Biol Fertil Soils (2004) 40: 363–385
DOI 10.1007/s00374-004-0784-9

REVIEW ARTICLE

J. M. Lynch · A. Benedetti · H. Insam · M. P. Nuti · K. Smalla · V. Torsvik · P. Nannipieri

Microbial diversity in soil: ecological theories, the contribution of molecular techniques and the impact of transgenic plants and transgenic microorganisms

Received: 8 January 2003 / Revised: 12 July 2004 / Accepted: 19 July 2004 / Published online: 7 October 2004

Abstract This review mainly discusses three related topics: the application of ecological theories to soil, the measurement of microbial diversity by molecular techniques and the impact of transgenic plants and microorganisms on genetic diversity of soil. These topics were debated at the Meeting on Soil Emergency held in Erice (Trapani, Italy) in 2001 for the celebration of the 50th anniversary of the Italian Society of Soil Science. Ecological theories have been developed by studying aboveground ecosystems but have neglected the belowground systems, despite the importance of the latter to the global nutrient cycling and to the presence of life on the Earth. Microbial diversity within the soil is crucial to many functions but it has been difficult in the past to determine the major components. Traditional methods of analysis are useful but with the use of molecular methods it is now possible to detect both culturable and unculturable microbial species. Despite these advances, the link between microbial diversity and soil functions is still a major challenge. Generally studies on genetically modified bacteria have not addressed directly the issue of microbial diversity, being mainly focused on their persistence in the environment, colonization ability in the rhizosphere, and survival. Concerns have been raised that transgenic plants might affect microbial communities in addition to environmental factors related to agricultural practice, season, field site and year. Transgenic plant DNA originating from senescent or degraded plant material or pollen has been shown to persist in soil. Horizontal transfer of transgenic plant DNA to bacteria has been shown by the restoration of deleted antibiotic resistance genes under laboratory in filter transformations, in sterile soil or in planta. However, the transformation frequencies under field conditions are supposed to be very low. It is important to underline that the public debate about antibiotic resistant genes in transgenic plants should not divert the attention from the real causes of bacterial resistance to antibiotics, such as the continued abuse and overuse of antibiotics prescribed by physicians and in animal husbandry.

Keywords Biodiversity · DNA · Genetic diversity · Molecular methods · Polymerase chain reaction

J. M. Lynch (✉)
Forest Research,
Alice Holt Lodge,
Farnham, Surrey, GU10 4LH, UK
e-mail: jim.lynch@forestry.gsi.gov.uk
Fax: +44-1420-520417

A. Benedetti
Istituto Sperimentale per la Nutrizione delle Piante,
Via della Navicella 2–4,
1-00184 Rome, Italy

H. Insam
Institut für Mikrobiologie, Universität Innsbruck,
Technikerstr. 25,
6020 Innsbruck, Austria

M. P. Nuti
Department of Agarian Chemistry and Biotechnology,
University of Pisa,
via del Borghetto,
Pisa, Italy

K. Smalla
Institute of Plant Virology, Microbiology and Biosafety, Federal Biological Research Centre for Agriculture and Forestry,
Messeweg 11–12,
38104 Braunschweig, Germany

V. Torsvik
Department of Microbiology, University of Bergen,
P.O. Box 7800, Jahnebakken 5,
5020 Bergen, Norway

P. Nannipieri (✉)
Dipartimento della Scienza del Suolo e Nutrizione della Pianta,
P.le delle Cascine 28,
50144 Firenze, Italy
e-mail: paolo.nannipieri@unifi.it
Fax: +39-55-333373

Introduction

Biological diversity (or biodiversity) can be defined as the set of animal and vegetable species, their genetic material and the ecosystems they belong to, and it includes ecosystem, species and gene diversity (Ohtonen et al. 1997). It is a function of time (evolution) and space (geographic distribution). Ecosystem diversity defines the number and abundance of habitats, of biotic communities and of ecosystems within which the diverse organisms live and evolve (Ohtonen et al. 1997). Ecosystems are made up of interdependent communities of species related to their physical environment and vary in size. Species diversity encompasses both the number and abundance of species present in a given area and takes into account that the term "species" generally indicates a group of individuals able to (effectively or potentially) breed within the group and issue fecund offspring, where the progeny are morphologically similar (Ohtonen et al. 1997). The individuals of a species may resemble, or not resemble, each other, but within each species, all individuals should maintain the particular characteristics of that species. However, this standard definition cannot be applied to organisms that reproduce asexually (for example, bacteria and viruses). Genetic diversity designates gene and genotype variation within a species. It consists of the whole set of genetic information contained in the genes of all the animals, plants and microorganisms populating the Earth. Species are composed of individuals having different hereditary (genetic) characteristics. According to modern theories of evolution, the variability and adaptive capacity in the genetic code enable single species to evolve progressively and to survive in changing environments (Ohtonen et al. 1997).

Soil problems such as, soil loss, soil degradation, soil contamination are some of the 15 emergencies that mankind must resolve in the third millennium to safeguard the planet and ensure survival of mankind (Zichichi 1993). However, the issue does not yet command the attention it deserves, even though several millions of hectares of soil are lost every year, due to the spread of urban development, erosion, deforestation and pollution. Soil is a fascinating biological system with the microorganisms inhabiting soil responsible for much of its broad metabolic capacity (Nannipieri et al. 2003). Therefore, microbiological properties are considered to be more sensitive than chemical and physical properties to changes in management and environmental conditions. Changes in the composition of soil microflora can be crucial for the functional integrity of soil (Insam 2001). A recent paper by Nannipieri et al. (2003) has reviewed the interrelationship between the microflora, its diversity and function in soil. Effects of stresses, such as low pH and pollutants, on microbial diversity and soil functioning were discussed but no mention was made of the impact of genetically modified organisms (GMOs). This is an issue on which there has been some public scientific debate, some public concern and involvement of (inter)national agencies, as well as the establishment of (trans)national legislation.

This review combines the contributions of several speakers at the Meeting on Soil Emergency, held in Erice (Trapani, Italy) in 2001 for the celebration of the 50th anniversary of the Italian Society of Soil Science. A review has been already published covering papers dealing with the contribution of mycorrhiza to plant health and soil fertility (Jeffries et al. 2002). A common aspect of these contributions was that microbial diversity is one of the key issues when the theme of soil emergency is discussed due to the role of structural and functional diversity of soil microorganisms in soil fertility, productivity and ecological stability (Nannipieri et al. 2003). This review discusses the various methods for determining microbial diversity in soil with particular attention paid to molecular tools and their important contribution to a better measurement and understanding of microbial diversity in soil. We are experiencing rapid advances in molecular ecology, and it is only a question of time before molecular tools targeting functional genes will close the gap between diversity–structure information and microbial activities. This review will also discuss the application of ecological theories to soil for their importance in developing concepts in microbial ecology even though these theories have only generally been applied to aboveground systems. Other aspects discussed in this review are the effects of the exposure of soil microorganisms to GMOs because concern is growing on the use of GMOs in open environments, such as agricultural soils. In this regard studies on the persistence of transgenic DNA in soil are considered from the perspective of the possible transformation of plant-associated bacteria by transgenic DNA.

Microbial and functional diversity in soil and the role of ecological theories

To date ecological theories have been based on the study of aboveground ecosystems. Despite the fact that the soil biota plays a fundamental role in ecosystem functioning, through nutrient cycling, decomposition and energy flow, soil organisms have had a negligible influence on the development of contemporary ecological theories (Wardle and Giller 1996). Here we discuss how the study of soil microorganisms might contribute to the development of ecological theories, and how such theories might contribute to a better understanding of the belowground system.

Microbial diversity is usually taken as the number of individuals assigned to different taxa and their distribution among taxa (Atlas and Bartha 1998). This view could be enlarged nowadays to embrace the diversity of individuals assessed up to below the rank of biovar with high throughput analytical or global tools (Johnsen et al. 2001). These include the study of individual cells at the genomic and proteomic levels to obtain in vivo informational imaging. The global tools also apply to the study of functional biodiversity of single microbial cells and

communities with respect to the environment. The enlarged view seems to adequately fit the complexity of the soil environment.

The Shannon–Weaver (1969) index (H') is often used in the form of:

$$H' = -\sum (n_i/N)\log(n_i/N) = -\sum p_i(\log p_i)$$

where according to Odum (1969), n_i is the importance value for each species, N is the total of importance values and p_i is the importance probability for each species (n_i/N) (Odum 1969). Thus, the H' takes into account both the "richness" and the "evenness" component of diversity. In the case of community-level physiological profiling (CLPP) the operational unit species is replaced by the operational unit ability to degrade a certain compound (Zak et al. 1994).

On a landscape level, diversity may be viewed at different levels of resolution. Whittaker (1972) proposed to distinguish between diversity of species within a community of a habitat (α-diversity), rate and extent in change of species along a gradient of habitats (β-diversity) and richness of species over a range of habitats (γ-diversity). This concept, plausible for traditional habitat diversity, may also be used to describe soil microbial diversity concepts. However, soil biota are characterised by a spatial diversity with possible differences between rhizosphere and bulk soil, macroaggregates and micro-aggergates, macropores and micropores, different horizons, etc. (Fig. 1). Indeed within a soil, there are several microhabitats, e.g. the rhizoplane, the rhizosphere, aggregates, decaying organic matter, or the bulk soil. Typically, soils are also largely stratified habitats, with distinct horizons; each of them may be regarded as a separate entity. How the diversity of these microhabitats can be incorporated in a general soil microbial diversity concept is not known.

Numerous factors are known to affect diversity, or at least they do in theory. Among these are trophic interactions, spatial and temporal habitat heterogeneity, disturbance and eutrophication (Torsvik et al. 2002). There are supposedly negative effects such as stress, or positive effects like resource diversity or biological interactions (Fig. 2). Positive effects on diversity may be related to increased stability, resilience, resistance to stress, and even productivity (Griffiths et al. 1997; Nannipieri et al. 2003).

The theory that biological diversity and ecosystem stability are linked is nearly 50 years old, and may be regarded as a core dogma of early ecosystem theory. In his 1955 paper on "fluctuations of animal populations and a measure of stability", MacArthur (1955) hypothesised that community stability would arise from food web structures, rather than from the autecological properties of certain species. The premise was that in an ecosystem with numerous energy pathways, changing the number of one single species would have less effect on the other species than an equal change in an ecosystem with few energy

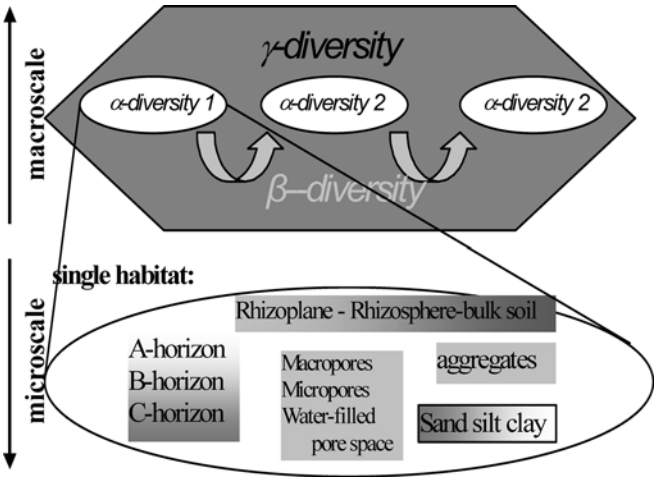

Fig. 1 According to Whittaker (1972) ecosystem diversity must be viewed at different levels of resolution: diversity of species within a community of a habitat (*α-diversity*), rate and extent in change of species along a gradient of habitats (*β-diversity*) and richness of species over a range of habitats (*γ-diversity*). In addition, in soil, spatial variability in biodiversity is also important with differences between rhizosphere and bulk soil, macroaggregates and micro-aggregates, macropores and micropores, different horizons, etc.

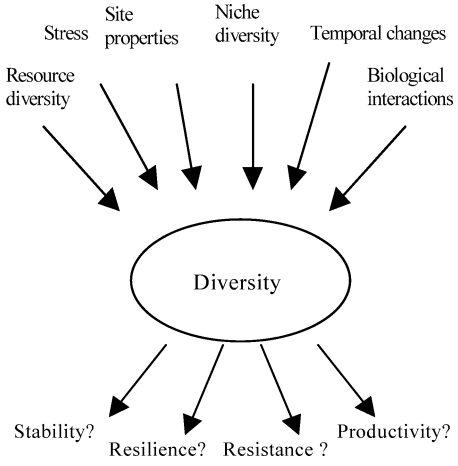

Fig. 2 External factors determine diversity, which in turn affects ecosystem properties like stability, resilience, redundancy and productivity

pathways. MacArthur used a simple foodweb model to show the stabilising effect of diversity and considered a sudden alteration of the abundance of one community member as a perturbation. His definition of stability was the degree to which the community resisted a given perturbation (Fig. 3). May (1973) challenged MacArthur's hypothesis and stated that disturbance was in absolute figures and as such rendered both communities equally susceptible to disturbance (Fig. 3). After 30 years, this dispute has still not been resolved since experimental evidence supporting the competing hypothesis remains controversial.

Since the introduction of MacArthur's food web model, several ecological theories, or models, have been proposed to explain the diversity–stability or diversity–productivity

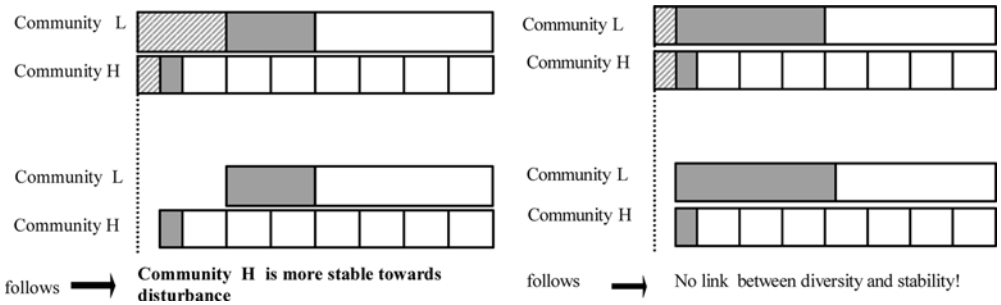

Fig. 3 Food web model of MacArthur (1955) (*left*), challenged by May (1973) (*right*). MacArthur hypothesised that when a low-diversity (*L*) community (here, with two members) is disturbed in a way that one community member is impaired by 50%, the disturbance has a higher impact than when a high-diversity community (*H*, 20 species) member is impaired. Thus, the high-diversity community would be more stable. According to May (1976), however, disturbance has to be put in absolute figures, which renders both communities equally susceptible to disturbance

relationships (McNaughton 1988; Griffiths et al. 2000; Hall et al. 2000). Among these ecological theories here we shall discuss the resource heterogeneity hypothesis (RHH) and insurance hypothesis.

Resource heterogeneity hypothesis

The RHH states that when an area is uniformly barren with respect to resource availability, it will not sustain many species and its productivity will be very low (Tilman 1982) (Fig. 4). As the mean quality of the habitat increases it is assumed that spatial variability and diversity of resources also increases, thereby allowing both productivity and diversity to increase. Past a certain point, however, the opposite occurs and there is a reduction of resource heterogeneity and hence diversity. This reduction occurs because the species that are competitively superior under such conditions are favoured when there are favourable niches everywhere (Hall et al. 2000).

As an example, Hall et al. (2000) found a hump-shaped relationship of resource heterogeneity and plant diversity. However, this humpback relationship holds only at the local and regional scales, while on a global scale the energy-richness relationship increases monotonically with energy. The relationship between microbial diversity and energy resource is not clear but the specific microflora of many plants (Westover et al. 1997) would suggest that with increasing plant diversity, which corresponds to an increase in resource heterogeneity for the microorganisms, microbial diversity also increases.

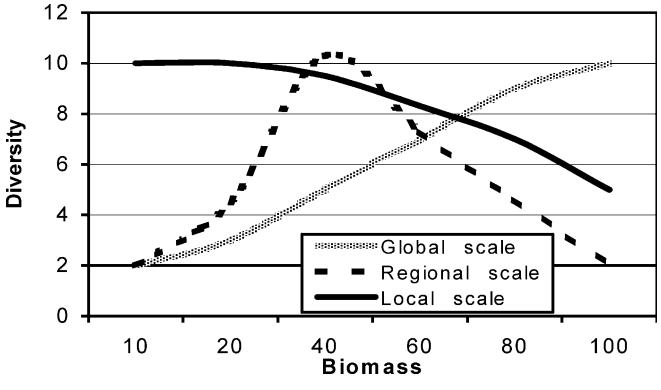

Fig. 4 Resource heterogeneity hypothesis (Tilman 1982) seen from a local-, regional- and global-scale perspective

Insurance hypothesis

According to the insurance hypothesis (Yachi and Loreau 1999), species richness has a positive effect on ecosystem productivity through a buffering effect against disturbances (reduction in the temporal variance of productivity) and thus a performance-enhancing effect (increase in temporal mean of productivity). The strength of this effect is determined by the individual species' responses to fluctuations, the degree of asynchronicity of responses and the detailed form of responses. This infers that especially in climatic regions with strong fluctuations in external factors, like seasonal drought or temperature cycles, diversity gains importance. Species that are functionally redundant at a given time are no longer redundant through time (Yachi and Loreau 1999). Griffiths et al. (2000) demonstrated recovery of microbial functions to transient stress (brief heating to 40°C) and this recovery was negatively affected when microbial diversity in soil was low. On the other hand, no recovery to persistent stress ($CuSO_4$ addition) was found; however, soils with higher microbial diversity were more resistant than soil with lower microbial diversity to persistent stress.

It is both structure and function that characterise ecosystems. Structures on the plant and animal level are much more evident than on the level of the soil microbiota. Is it, with respect to organic matter decomposition, important to know who is doing the job? Or may it be sufficient to ascertain that the various biochemical pathways are working properly? If so, functional diversity would be much more important than structural diversity. However there is a consensus that "some minimum number of species is essential for ecosystem functioning under constant conditions and that a larger number of species is probably essential for maintaining the stability of ecosystem processes in changing environments" (Loreau et al. 2001). According to Giller et al. (1997)

the extent of functional redundancy in soil microorganisms may result in there being no effect on the function of the microbial communities if microbial diversity is changed. If a large number of species conducts similar functions, a loss of any species has little effect on soil processes since other organisms fill the functional gap. Of course this is not valid for specialistic functions such as the antagonism to a pathogenic fungus like *Gaeumannomyces graminis* var. *tritici*. This function was found to reside in a single species (*Pseudomonas fluorescens*) and depends on a single function, the production of 2,4-diacetylphloroglucinol (DAPG) (Keel et al. 1992; Thomashow and Weller 1995).

Soil functionality

Several methods can be used to measure rates of microbial processes or enzyme reactions in soil. Most of these methods give potential rather than real activities because assays are carried out in the presence of saturating substrate concentrations, at optimal pH and temperature values and usually in soil slurries (Nannipieri et al. 2003). In situ rarely are the pH and the substrate concentration optimal, and temperature and moisture conditions can fluctuate. The monitoring of C oxidation to CO_2 when ^{14}C-labelled compounds are used or the monitoring of distribution of ^{15}N among the various soil N pools when ^{15}N-enriched compounds are used, represent two examples which can provide quantitative estimations of soil biological processes. The advantages and disadvantages of classic methods such as soil respiration, enzyme activities, nitrification, etc., in measuring soil functions have been discussed by Nannipieri et al. (2003).

One of the functional approaches that have gained increasing importance in soil ecology is CLPP (Garland and Mills 1991). This method employs microplates that contain up to 95 different C sources. The pattern of their utilization by a microbial community (e.g. the community contained in a sample of soil extract) can potentially provide functional information on the community, and be used to derive diversity parameters such as the H' or "catabolic versatility" (Burkhardt et al. 1993). However, the methodology has several drawbacks in that it is culture-dependent and changes in the composition of microbial communities can occur during incubation. Furthermore the contribution of fungi to soil functions cannot be determined using this approach (Nannipieri et al. 2003). These limitations were addressed by Degens and Harris (1997) who measured the utilization patterns of these compounds by soil microbial communities using short-term responses of the soils to the addition of amino acids, carboxylic acids, carbohydrates and organic polymers (substrate-induced respiration) [(Fig. 5)].

A long-disputed issue is the question of whether diversity is related to productivity and in particular to plant productivity. Data from the CLPP approach are frequently used to express functional diversity of soil (Yan et al. 2000; Dunfield and Germida 2001; Bending et al. 2002). Yan et al. (2000) found a broken-stick relationship between organic C and functional diversity, and they also recalculated the figures given by Sharma et al. (1997) who stated to have found a linear relationship between H' and microbial biomass. Also in that case, a broken-stick model gave the best fit (Fig. 6). From these data it may be concluded that up to a certain threshold (i.e. 1.7% organic C) functional diversity increases monotonically with soil microbial biomass.

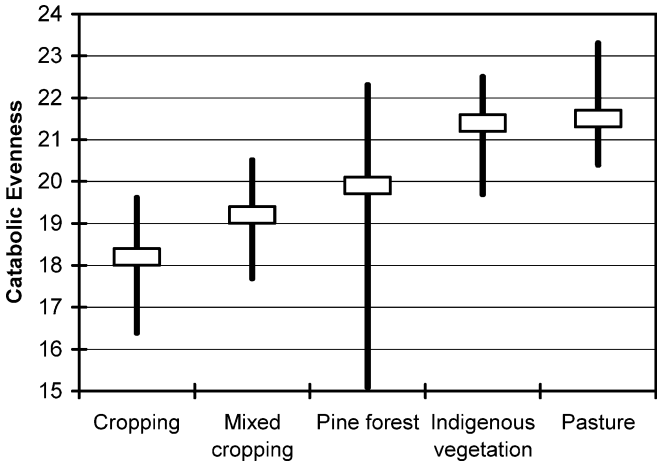

Fig. 5 The effect of cropping on the catabolic evenness of the soil microbiota

Molecular methods for measuring microbial diversity

Usually microbial diversity in soil has been analysed by methods such as viable and culturable methods, CLPP and flow cytometry. The limits of these methods have been frequently discussed (e.g. Torsvik et al. 1996). There is strong evidence that most of the soil bacteria observed under the microscope are viable and active, but are unable to form visible colonies on agar plates (Staley and Konopka 1985; Torsvik et al. 1990; Amann et al. 1995). Both culturable and unculturable microorganisms can be determined by using molecular techniques based on the

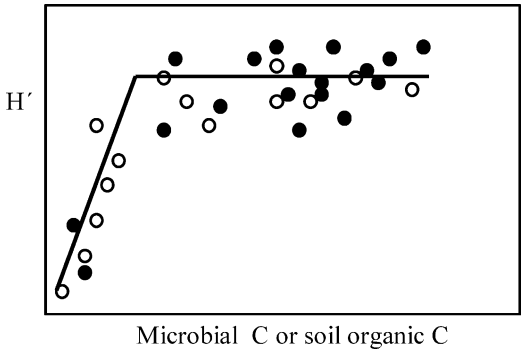

Fig. 6 Up to a certain threshold, functional diversity (H') increases monotonically with microbial and/or organic C in a soil. Data from Yan et al. (2000) (*open symbols*) and Sharma et al. (1997) (*closed symbols*)

extraction, purification and characterization of nucleic acids from environmental samples (Torsvik 1980). These techniques provide a more accurate measure of the extent of microbial diversity in soil. The information in nucleic acids of microorganisms can be used to investigate and compare diversity at different organization levels, ranging from variability within species to diversity of communities (Johnsen et al. 2001). This section gives an overview of the potentials and limitations of some molecular methods currently used to study microbial communities in soil. To demonstrate the power and feasibility of the molecular approaches, examples are given of their application to the study of indigenous soil microbial communities in relation to pollution and perturbation (Fig. 7).

Total genomic DNA (metagenome) derived from microbial communities can provide complementary information about the overall community structure ("species" composition) and the total genetic diversity (Torsvik et al. 1990; Ritz et al. 1997; Johnsen et al. 2001). For example, bacterial artificial chromosome (BAC) vectors can be used to clone large segments of DNA directly derived from soil microbial communities (Rondon et al. 2000). The BAC library can be used to study soil microbial diversity at a phylogenetic and functional level with both high and low resolution methods.

Intermediate resolution methods include the application of phylogenetic probes, either in slot/dot-blot hybridisations of community DNA or RNA or by fluorescence in situ hybridisation (FISH) of intact cells (Amann et al. 1990; Stahl and Amann 1991; Hahn et al. 1992; Johnsen et al. 2001). Such methods can be used to quantify target microorganisms and to determine the overall taxonomic composition of the microbial communities.

Other analyses provide resolution at intermediate level and are based on sequence differences of conserved genes such as those coding for ribosomal RNA (rRNA), the so-called rDNA (Johnsen et al. 2001). Common to most of these fingerprinting methods is the specific amplification of the target nucleic acids using polymerase chain reaction (PCR) (Saiki et al. 1985). The resulting amplicons are either subjected to restriction analysis or separated on the basis of denaturing or conformational properties (Johnsen et al. 2001). Restriction analyses of rRNA or rDNA [(amplified ribosomal DNA restriction analysis (ARDRA); terminal restriction fragment electrophoresis (T-RFLP)] can discriminate at higher levels of taxonomic rank and may even differentiate between species (Avaniss-Aghajani et al. 1994; Massol-Deya et al. 1995; Smit et al. 1997; Osborn et al. 2000). Osborn et al. (2000) have shown that the T-RFLP profiles for rRNA genes can be used to discriminate between *Sphingomonas* species. They argue that T-RFLP profiles generated from whole communities and/or from isolates or clones, when compared with databases of in silico predicted T-RFs, may provide the possibility of immediate phylogenetic assignment of some fragments in a profile. T-RFLP and community fingerprinting techniques [(denaturing/thermal gradient gel electrophoresis (DGGE/TGGE); single-strand conformational polymorphism (SSCP)], based on separation of PCR-amplified rRNA and rDNA molecules, have been used successfully to analyse the structure and dynamics of microbial communities (Muyzer et al. 1993; Heuer and Smalla 1997a; Schwieger and Tebbe 1998).

High resolution analyses include the fingerprinting of non-coding DNA regions (for example, rep-PCR-amplification of sequences between repetitive elements) and the

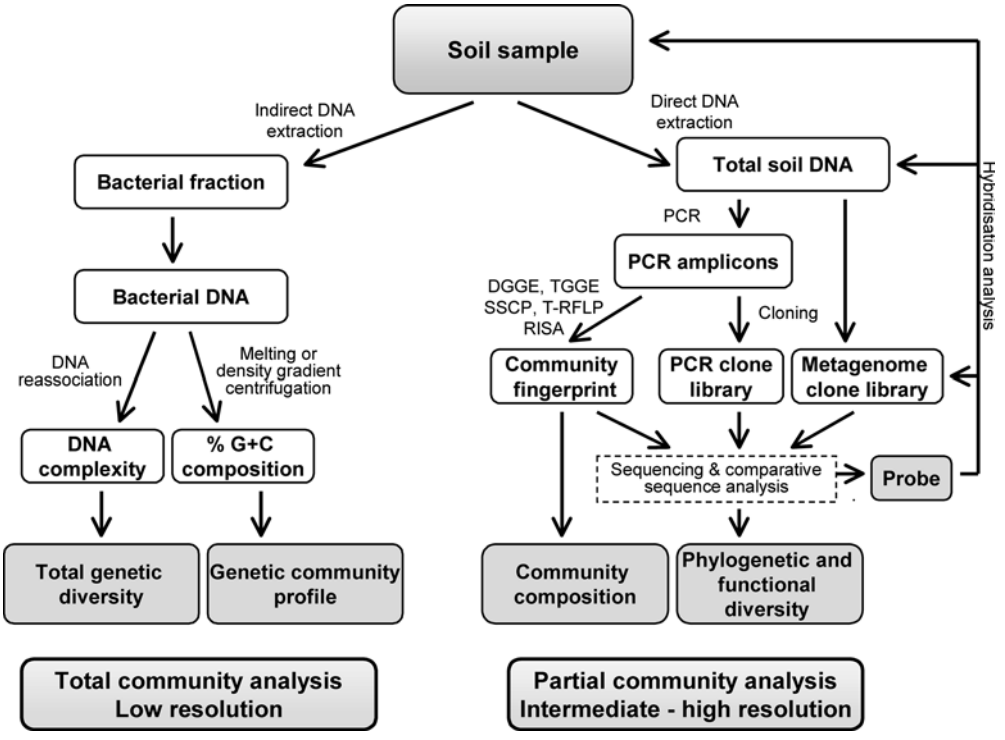

Fig. 7 An overview of culture-independent molecular methods for the analyses of microbial communities. *PCR* Polymerase chain reaction, *DGGE* denaturing gradient gel electrophoresis, *TGGE* temperature gradient gel electrophoresis, *SSCP* single-strand conformation polymorphism, *T-RFLP* terminal restriction fragment electrophoresis, *RISA* intergenic spacer analysis, *% G+C* mole % guanine +cytosine

sequencing of both coding and non-coding regions (Johnsen et al. 2001). Such methods are suitable for identification and classification of microbial strains at the species and subspecies level. High resolution fingerprinting methods have also been used to monitor specific populations in microbial communities and to assess the diversity of bacterial isolates and cloned genes (de Bruijn 1992; Hadrys et al. 1992; Borneman and Triplett 1997).

The total genomic DNA approach to biodiversity analysis

Information about the microbial community composition of soil can be obtained by measuring the base distribution [mole % guanine+cytosine (% G+C)] in community DNA. The base distribution of DNA can be determined by thermal denaturation because single-stranded DNA has approximately 35% higher absorbency than double-stranded DNA at 260 nm (Torsvik et al. 1996). DNA melting is measured at 260 nm in a UV-spectrophotometer with a thermostated cuvette holder by slowly increasing the temperature. The melting curves are converted to % G+C profiles (Torsvik et al. 1995, 1996; Ritz et al. 1997) and provide microbial community profiles indicative of the overall genetic diversity. Even if this analysis is considered to be low resolution, it can be used to indicate overall changes in microbial community structure, especially when the diversity is low. DNA base composition profiles can also be obtained by isopycnic centrifugation of bisbenzimide-DNA complexes in a CsCl gradient (Harris 1994; Holben and Harris 1995). Bisbenzimide binds preferentially to adenine-thymine pairs and will decrease the buoyant density of DNA. Thus, community DNA can be separated according to the base composition in a density gradient with bisbenzimide. The analyses have the limitation that two communities having similar base distributions do not necessarily have similar species composition, since different species often have the same base composition. On the other hand, if communities have different base distributions this provides strong evidence that they have different species composition. Therefore, the method can be used as a general indication of changes in microbial community composition following perturbation. The community DNA fractionation by bisbenzimide-CsCl gradient ultracentrifugation can be combined with further analyses of the different G+C% fractions by other molecular methods like PCR and DGGE (Nüsslein and Tiedje 1998). The advantage of this approach is that some of the less dominant microorganisms in the community that might not be detected by PCR without fractionation can be detected and analysed.

The complexity of DNA isolated from microbial communities is an estimate of the total genetic diversity. The rate at which denatured, single-stranded DNA re-anneals (reassociates) when the temperature is lowered to approximately 25°C below its melting point, is indicative of this genetic complexity (Torsvik et al. 1995). The rate decreases with increased DNA complexity, or number of different DNA types in solution (Torsvik et al. 1995). The fraction of reassociated DNA is plotted against the C_0t values, where C_0 is mole nucleotides per litre and t is time in seconds. The $C_0t_{1/2}$, at 50% DNA reassociation, is used to estimate DNA complexity. Figure 8 shows an example of C_0t curves for community DNA from microcosms of arable soil (Øvreås et al. 1998). The soil microcosms were either incubated with CH_4 gas as the major C source (CH) or under anaerobic conditions (N_2). Untreated microcosms were used as the control (K). The reassociation rate of *Escherichia coli* B genomic DNA with known complexity [4.1×10^6 base pairs (bp)] was used as a standard measure (Table 1). To estimate the size of soil community DNA, the $C_0t_{1/2}$ of community DNA was divided by $C_0t_{1/2}$ of the *E. coli* B genomic DNA, multiplied with the size of the *E. coli* B genome.

$$\frac{C_0t_{1/2}\ \text{soil community DNA}}{C_0t_{1/2}\ E.coli\ \text{genome}} \times E.coli\ \text{genome size (bp)}$$

The reassociation results suggest that perturbations caused a reduction in the microbial diversity (Fig. 8, Table 2). DNA from the unperturbed control soil had a $C_0t_{1/2}$ of 6,300 mol s^{-1} l^{-1}, which corresponds to approximately 8,000 different *E. coli* genomes. For DNA from the N_2-perturbed and CH_4-perturbed soils, the $C_0t_{1/2}$ values were reduced to 2,500 mol s^{-1} l^{-1} and 300 mol s^{-1} l^{-1}, respectively. This corresponds to a diversity of 3,200 and 340 different *E. coli* genomes.

When applied to microbial community DNA, the $C_0t_{1/2}$ is used as an index (Torsvik et al. 1995) of genetic diversity and encompasses both the total range of genetic information in the community (richness component) and

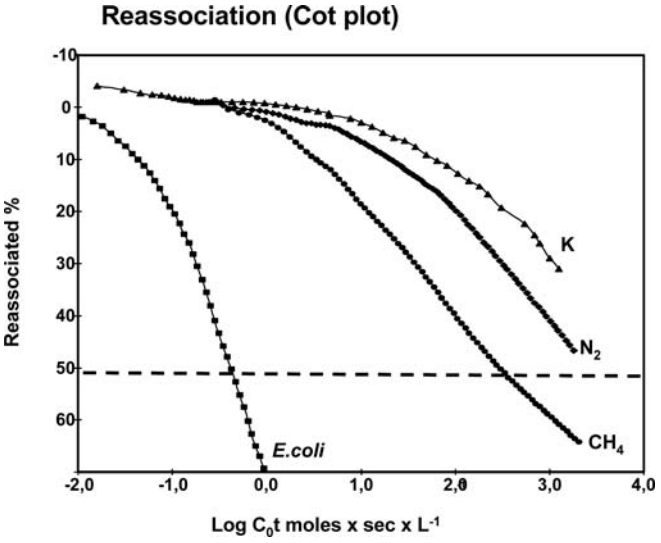

Fig. 8 Reassociation (C_0t plot, where C_0 is mole nucleotides per litre and t is time in seconds) of DNA from *Escherichia coli*, or bacterial fractions of untreated soil (*K*), soil incubated under anaerobic conditions (N_2) and soil incubated with CH_4 as major C source (CH) (Øvreås et al. 1998)

Table 1 Molecular methods for soil microbial diversity studies. *G+C* Guanine+cytosine, *PCR* polymerase chain reaction, *DGGE* denaturing gradient gel electrophoresis, *TGGE* temperature gradient gel electrophoresis, *SSCP* single-strand conformation polymorphism, *T-RFLP* terminal restriction fragment electrophoresis, *ARDRA* amplified ribosomal DNA restriction analysis, *RISA* intergenic spacer analysis, *FISH* fluorescence in situ hybridisation

Method	Type of information and resolution	Application in soil microbial analysis
DNA reassociation rate	Total genetic diversity, theoretical "species" number. Community "genome size". Low resolution	Global analysis of the genetic potential of communities. Comparative analysis of the overall biodiversity
Mole % G+C composition	Genetic community profile, overall community composition. Low resolution	Comparative analysis of overall changes in community composition
PCR—DGGE/TGGE sequencing of individual bands	Genetic fingerprinting of communities, affiliation of predominant community members. Intermediate resolution	Comparative analysis of community structure, spatial and temporal changes in community composition
PCR—SSCP sequencing of individual bands	Genetic fingerprinting of communities, affiliation of predominant community members. Intermediate resolution	Comparative analysis of community structure, spatial and temporal changes in community composition
PCR—T-RFLP	Community composition, relative abundance of numerically dominant community members. Intermediate resolution	Comparative analysis of distribution of microbial populations, monitoring changes in community composition
PCR—ARDRA	Genetic fingerprinting of simple communities, populations or phylogenetic groups. Discrimination at lower taxonomic (species) levels. High resolution	Comparative analysis of microbial population dynamics. Diversity within phylogenetic or functional groups of microorganisms
PCR—RISA	Genetic fingerprinting of populations or phylogenetic groups. Simultaneously analysis of different microbial groups. Discrimination at species or group level. High resolution	Comparative analysis of microbial population dynamics. Diversity within phylogenetic or functional groups of microorganisms
PCR of rDNA—cloning and sequencing	Phylogenetic diversity, identification of community members. High resolution	Phylogenetic diversity of community members
PCR of functional genes—cloning and sequencing	Functional diversity. High resolution	Comparative analysis of the functional potential of communities
RNA dot/slot blot hybridisation	Phylogenetic identification of metabolic active community members. Intermediate resolution	Qualitative and quantitative analysis of metabolic active populations in communities. Phylogenetic information on active community members
FISH	Detection and specific counting of metabolic active microorganisms. Intermediate resolution	Comparative analysis of community structure. Detection and identification of active cells. Direct phylogenetic information on community members

Table 2 Genetic diversity in soil microbial communities calculated from the reassociation kinetics in 6× standard saline citrate, 30% dimethylsulphoxide. Soil community DNA size in base pairs (bp), *Escherichia coli* genome equivalents (*E. coli* genome size: 4.1×10^6 bp) (Brønstad et al. 1996; Drønen et al. 1998). C_0 Mole nucleotides per litre, t time in seconds

DNA source	$C_0t_{1/2}$	Soil community DNA size base pairs	*E. coli* genome equivalents
E. coli	0.79	4.1×10^6	1
Pasture soil (StendO)	6,300	3.3×10^{10}	8,000
Arable soil (StendS)	270	1.4×10^9	340
Sewage sludge amended soil; uncontaminated	7,800	4.0×10^{10}	9,800
Sewage sludge amended soil; low metal	3,700	1.9×10^{10}	4,600
Sewage sludge amended soil; high metal	1,200	6.2×10^9	1,500

the distribution of this information among the different genetic types (evenness component). This makes it possible for two communities with different structures to have identical $C_0t_{1/2}$ values (Torsvik et al. 1995).

To estimate prokaryotic diversity, the DNA must be highly purified and free from eukaryotic DNA. Therefore, an indirect DNA extraction protocol has to be used where the bacteria are separated from the soil by fractionated centrifugation (Fægri et al. 1977; Torsvik 1995) prior to cell lysis. In addition, for an accurate estimation DNA of high purification, uniform fragment size should be used (Torsvik et al. 1990). The DNA derived from soil microbial communities is often very complex and the reassociation rate is low but it can be increased if the reassociation is measured in a saline citrate solution and 30% DMSO (Escara and Hutton 1980). However, good estimates of $C_0t_{1/2}$ require long reassociation times to reach 50% reassociation.

Large (100 kb) segments of soil community DNA can be cloned directly into the BAC vector without the need

for PCR amplification (Methagenome analysis, Rondon et al. 1999, 2000). The BAC vector, which is derived from the F-plasmid, can maintain large inserts stably in *E. coli* host. The BAC libraries can, in principle, accommodate the total microbial community genome, and can be used for genomic mapping, screening for specific genes and assessing phylogenetic and functional diversity. This can be done by hybridisation with specific probes, genetic fingerprinting and sequencing. An advantage of the method is that no PCR amplification is required, thereby circumventing the bias introduced by this step in many other fingerprinting and sequencing techniques. Each BAC clone represents a fragment of the metagenome and may contain genes and operons with their own promoters allowing them to be phenotypically expressed in the *E. coli* host. Information linking phylogeny and function of microorganisms in soil can therefore be gained by combining studies of clones harbouring rRNA and functional genes by clone walking and hybridisation (Gillespie et al. 2002).

Ribosomal RNA genes (rDNA) and their transcripts, the rRNA molecules, are the most widely used markers for classification and measuring phylogenetic diversity of microorganisms (Madigan et al. 2003). The rRNA-based methods have revealed highly diverging phylogenetic lineages within the prokaryotic microorganisms that represent the most extensive genetic diversity on Earth (Woese et al. 1990; Woese 1998). The culture-independent rRNA approaches have revealed novel lineages, phylogenetically different from cultured and characterized soil microbes. They have shown that microorganisms from similar habitats in separated geographical regions can be related, but also that microorganisms within the same functional group or guild may belong to different phylogenetic groups (phylotypes) reflecting niche differences (Thorseth et al. 2001). The hypothesis that microorganisms are cosmopolitan has been challenged by Tiedje et al. (2001), who found that microbial genotypes were peculiar to each sample site of the same continental region.

Genetic fingerprinting of soil microbial communities and the identification of the community members by comparison with fragment sizes or sequences in databases

Genetic fingerprinting methods based on PCR amplification differ in the various methods used to separate amplicons. New methodologies such as terminal restriction fragment length polymorphisms use fluorescent labelling of PCR amplicons and separate these in automated sequencing systems with laser detection of fluorescing DNA fragments (Liu et al. 1997). The PCR-based community fingerprinting techniques have several advantages (Wintzingerode et al. 1997). They are: (1) rapid, and allow parallel analyses of multiple samples, (2) reliable and highly reproducible, (3) provide both qualitative and quantitative information on populations within a community, and (4) allow the assessment of the phylogenetic affiliation of community members by comparison with fragment sizes or sequences in databases. However, PCR-based techniques also present several drawbacks such as: (1) bias in PCR-amplification due to preferential amplification of target DNAs from some bacteria, (2) formation of chimeric molecules, (3) derivation of several different PCR amplicons from a single bacterial strain due to the presence of several operons, and (4) the numbers of amplicons from complex communities can be too high to be readily separated and resolved (Wintzingerode et al. 1997).

DNA fingerprinting of PCR-amplified rDNA using methods such as DGGE and TGGE, provide information about the community composition. In DGGE/TGGE analysis, DNA fragments with the same length but different nucleotide sequences are separated (Muyzer et al. 1993; Heuer and Smalla 1997a). This separation is based on the differences in mobility of PCR-amplified DNA molecules in polyacrylamide gels in a linear denaturing gradient. In DGGE, a concentration gradient of denaturing chemicals (urea and formamide) is used whereas TGGE is based on a temperature gradient. DNA molecules with different sequences will differ in their melting behaviour. As DNA molecules migrate in the denaturing gel, they start to melt at particular melting domains and thus they become partially single-stranded. Partly denatured or fully denatured molecules stop migrating in the gel and DNA fragments occupy different positions in the gel according to their base composition and sequence variation (Muyzer et al. 1993; Heuer and Smalla 1997a). Variable regions (230–500 bp) of rDNA from microbial community DNA are PCR amplified using primers annealing to conserved regions that flank the variable regions. The forward primer is covalently linked to G+C-rich sequence (GC-clamp, usually up to 40 bases long) to prevent the complete melting of the double-stranded DNA (Muyzer et al. 1993; Heuer and Smalla 1997a). A limit of the technique is that the 16S rDNA of different organisms can contribute to a specific band in the denaturing gel. However, when applied to communities with low to moderate complexity, it is assumed that discernible bands obtained by DGGE/TGGE represent numerically predominant microbial populations in the community (Øvreås et al. 1997). Information about the taxon composition of the community can be obtained by blotting the gel and hybridising with phylogenetic probes, targeting the main phylogenetic subclasses of bacteria and Archaea (Raskin et al. 1994; Amann et al. 1995; Heuer et al. 1999). Well-separated DGGE bands can be excised from the gel and sequenced. By comparing the sequences of DGGE bands with those in a database, the phylogenetic affiliation of the original microorganisms can be obtained (Øvreås et al. 1998; Smalla et al. 2001; Heuer et al. 2002b).

DGGE/TGGE analysis of PCR amplicons derived from rRNA molecules by reverse transcriptase PCR might give fingerprints of the metabolically active microbial populations (Heuer and Smalla 1997a) and DGGE/TGGE methods are useful in the rapid screening of multiple

samples for distinguishing soil microbial communities (Nakatsu et al. 2000). They are convenient for investigating spatial and temporal differences in microbial communities and provide information about changes in dominating populations (Øvreås et al. 1998; Smalla et al. 2001).

SSCP, like DGGE/TGGE, detects sequence variations between different PCR amplicons normally derived from variable regions of the rDNA (Lee et al. 1996; Stach et al. 2001). In SSCP one primer is phosphorylated at the 5′ end, and the phosphorylated strand of the PCR amplicons is selectively digested with lambda exonuclease. The intact strands are separated by electrophoresis under non-denaturing conditions (low temperature) in a polyacrylamide gel optimal for SSCP. This optimal gel restricts duplex formation but allows intra-molecular folding of the DNA strands. The method is based on the differential intra-molecular folding of single-stranded DNA that is itself dependent upon DNA sequence variations. Thus, DNA secondary structure alters the electrophoretic mobility of the single-stranded PCR amplicons enabling them to be resolved. The reproducibility and discriminatory ability of the method is dependent on the fragment size and the position of the sequence variation within the fragment (Lee et al. 1996) and normally gives best results with fragments smaller than 400 bp. SSCP has been used to differentiate between pure cultures of soil microorganisms and to distinguish community fingerprints of non-cultivated rhizosphere microbial communities from different plants (Schwieger and Tebbe 1998; Schmalenberger and Tebbe 2002). SSCP analysis should in principle be easier to carry out than DGGE/TGGE, as no primers with GC-clamp or specific apparatus for gradient gels are required. A limitation of the method, in addition to potential PCR bias, is that a single bacterial species may yield several bands due to the presence of several operons or more than one conformation of the single-stranded PCR amplicons.

T-RFLP analysis is based on the restriction endonuclease digestion of fluorescent end-labelled PCR amplicons (Avaniss-Aghajani 1994; Liu et al. 1997; Marsh et al. 2000; Osborn et al. 2000). These PCR amplicons are derived from microbial community DNA using primers that anneal to consensus sequences flanking the variable regions in rRNA genes. Both primers are normally labelled at the 5′ end with fluorescent phosphoramidite dyes. The amplicons are restriction enzyme digested and separated either by gel or capillary gel electrophoresis. The fluorescently labelled fragments are detected with a laser detector in an automated analyser and thus this technique only detects the "terminal" end labelled restriction fragments. Thus, T-RFLP combines the three techniques PCR, RFLP and gel electrophoresis. By identifying the appropriate restriction site positions on 16S rDNA sequences in databases using a computer program, T-restriction fragment (T-RF) size distribution for rRNA with different restriction enzymes can be predicted and experimentally obtained T-RF profiles for PCR amplicons or clone libraries of 16S rDNA derived from community DNA can be compared to the predicted T-RF profiles for different phylogenetic groups. Such analyses have been used to distinguish communities and to study community structure and dynamics in soils (Dunbar et al. 2000). In addition to analyses based on housekeeping genes (e.g. rDNA), T-RFLP has be used to analyse functional genes such as mercury resistance genes, and particulate methane monooxygenase genes (Bruce 1997; Horz et al. 2001).

ARDRA is a powerful tool for bacterial identification and classification at species level (Massol-Deya et al. 1995) and it has been used to group and classify large sets of isolates and clones (Smit et al. 1997; Øvreås and Torsvik 1998; Cheneby et al. 2000). Automated ARDRA has been performed with fluorescent PCR amplicons obtained by incorporating fluorescently labelled dUTP during PCR. After restriction enzyme digestion, the fragments are separated on an automated DNA sequencing gel (Pukall et al. 1998). The restriction patterns data can then be compared with restriction analysis of rDNA sequences of known bacteria obtained using database sequences.

RISA is based on the length polymorphism of the ribosomal intergenic spacer region between the 16S and 23S rRNA genes (Borneman and Triplett 1997; Ranjard et al. 2000b); the length of this spacer region has been shown to be strain or species specific, and varies from 50 bp to over 1,500 bp. PCR-amplified products are separated by agarose gel electrophoresis. The non-coding ribosomal internal spacer region is variable in both size and nucleotide sequence even within closely related strains, and the method has been successfully used to characterise, classify and type strains, and to fingerprint simple communities and mixed populations (Nagpal et al. 1998; Ranjard et al. 2000a). An automated version of RISA, called ARISA, has been developed using PCR amplification of the intergenic spacer with a fluorescence-labelled forward primer. The fragments are separated according to their size on an automated capillary electrophoresis system, and their sizes are compared to those in DNA databases (Fisher and Triplett 1999; Ranjard et al. 2001). Several primers targeting different phylogenetic groups in the same sample can be used to evaluate simultaneously the population dynamics of different microbial phylotypes within a community (Ranjard et al. 2000a,b).

Genetic fingerprinting methods are limited in their ability to discriminate between communities with high diversity where the number of PCR amplicons are too high to be readily resolved (Tiedje et al. 2001). In complex communities, rRNA-based fingerprinting techniques can be used to partially analyse the community, focusing on a subset of the community by applying primers targeting specific phylogenetic (e.g. Archaea) or functional (methanogenic) groups of microorganisms. According to Gomes et al. (2001) it is possible to distinguish different phylogenetic groups of bacteria by using the following primers F984GC, F27, R1378 and R1494 (bacteria), F243HGC (Actinomycetales), F203α (α-Proteobacteria) and F948β (β-Proteobacteria). Specific primers are also available to detect specific genes such as those of *Paenibacillus* (Da Silva et al. 2003), *Burkholderia* (Salles

et al. 2002) and *Bacillus* (Garbeva et al. 2003). The assessment of fungal diversity has been problematic because the primers used can co-amplify DNA from other eukaryotic organisms such as plants, algae and nematodes (Kowalchuk et al. 1997). The fungal primers developed by Vainio and Hantula (2000) have been successfully applied to study the diversity of fungal communities in bulk and rhizosphere soil of maize grown in tropical soils (Gomes et al. 2003).

Another approach to identify community members is to apply specific enrichments to enhance the growth of the microorganism of interest. This strategy is particularly useful in studies of functional groups or guilds.

Hybridisation techniques

Phylogenetic oligonucleotide probes homologous to sequences of 16S or 23S rRNA are designed by aligning and comparing sequences in rRNA databases. The probe specificity depends on the variability of the target sequence. By choosing sequences in conserved, variable and hypervariable regions of the rRNA, probes can target phylogenetic groups at different taxonomic levels, ranging from domain to subspecies. The relative abundance of major phylotypes in soil microbial communities can be assessed by quantitative slot dot blot hybridisation of community DNA or by FISH with group-specific phylogenetic probes. In the FISH method, phylogenetic probes are labelled with fluorescent dyes and used for in situ detection of single cells in environmental samples by whole cell hybridisation (Amann et al. 1990). Excellent reviews of the procedures and methodologies are provided by Amann et al. (1995) and Wagner et al. (2003). Suitable FISH probes can be found at the probeBase website (Loy et al. 2003; URL: http://www.microbial-ecology.net/probebase/).

Hybridisation methods can help to resolve the species composition within specific parts (organism groups) of the community. Slot/dot blot and Southern blot hybridisation of community fingerprints (e.g. DGGE profiles) with phylogenetic probes has proved particularly useful in studying changes in communities and in identifying the numerically dominant community members (Øvreås et al. 1997). A combination of slot blot hybridisation and FISH was used to distinguish the community structure of low and high metal-contaminated soils (Chatzinotas et al. 1998; Sandaa et al. 1999b).

In the FISH technique, the target microorganisms are visualized using epifluorescence microscopy. There are some limitations to the standard FISH method with respect to sensitivity that prevents detection of cells with low ribosome content. Low physiological activity is often correlated with low ribosome content per cell, therefore slow-growing or starving cells may not be detected (Amann et al. 1995). To overcome this limitation, a tyramide signal amplification technique has been adopted for FISH, which allows the analysis of slow-growing microorganisms (Pernthaler et al. 2002).

FISH has been used to identify uncultured microorganisms, studying the distribution of, and quantifying microbial populations. By counting under the microscope after hybridisation with a set of different phylogenetic probes, the number of phylogenetic groups and the relative distribution of individuals among taxa can be determined.

Analysis of cloned rRNA genes

Cloning techniques provide an alternative to fingerprinting methods for analysing PCR amplicons and have been widely used to analyse microbial communities. Clone libraries of PCR-amplified rRNA genes in DNA from environmental samples are made in cloning vectors (Borneman et al. 1996). Bacterial, fungal and archaeal rRNA genes have been amplified in separate PCR reactions using domain-specific and universal primers (Lane et al. 1985; Lane 1991; Raskin et al. 1994; Suzuki and Giovannoni 1996; Gomes et al. 2003). The cloned amplicons can be compared by fingerprinting methods such as ARDRA (Sandaa et al. 2001). Clones can then be classified by dot/slot blot hybridisation with phylogenetic probes (Manz et al. 1992; Amann et al. 1995). Sequencing of the cloned rRNA genes and comparing the sequences with those obtained from databases provides information about affiliation of the cloned sequences (Liesack and Stackebrandt 1992; Sandaa et al. 1999a,b; Alfreider et al. 2002).

Assessing the impact of agricultural management and pollution on microbial diversity and community structure in soil using molecular methods with different levels of resolution

The use of diversity and changes in community structure as ecological indicators of perturbations and pollution have been investigated in soils subjected to different agricultural management and to heavy metal-contaminated sewage sludge (Nannipieri et al. 2003).

Microbial diversity, as determined by the reassociation technique discussed above, of an arable (StendS) soil cropped to cereals, vegetables and potatoes was compared with that of an organic soil (StendO), used solely as a pasture field during the last decades; the two soils have similar texture (sandy loam) and are situated in western Norway 400 m apart (Brønstad et al. 1996; Drønen et al. 1998). The reassociation technique showed that the complexity of microbial community DNA, as calculated from the $C_0t_{1/2}$ values, was approximately 1.4×10^9 bp in the StendS soil and 3.3×10^{10} bp in the StendO soil (Table 2). This corresponds to a genetic diversity of approximately 340 and 8,000 completely different genomes with *E. coli* genome size (4.1×10^6 bp) in the StendS and StendO series, respectively. It was suggested that the total genetic diversity in the arable soil was approximately 24 times lower than in the relatively undisturbed pasture soil and that the overall genetic diversity provides a good

indicator of disturbance caused by agricultural management. PCR combined DGGE indicated that the number of different bacterial types in both soils was high, meaning that there was a high "species" richness component of diversity in both soils (Øvreås et al. 1998). The bacterial populations in the arable soil were probably less evenly distributed than in the pasture soil, containing a few numerically dominant bacterial populations and many with low abundance. This would cause a low evenness and hence a lower genetic diversity in the arable soil as compared to the pasture soil.

The microbial diversity, determined by the reassociation technique, of control field soils amended with "uncontaminated" sewage sludge was compared with that of contaminated field soils treated with metal-amended sewage sludge at two rates of application (low and high metal contamination) for several years (McGrath et al. 1995; Sandaa et al. 1999b). On the one hand, the base composition (mole % G+C) profiles for the three treatments were very similar. On the other hand, the total genetic diversity, as measured by the DNA:DNA reassociation was different. The DNA complexity of the uncontaminated soil community was 4.0×10^{10} bp (Table 2). This corresponds to a genetic diversity of approximately 9,800 different bacterial genomes with *E. coli* genome size. The diversity in the metal-polluted soils was reduced and depended on the level of pollution. The complexity of DNA from the low and high metal-polluted soils was 1.9×10^{10} bp and 6.2×10^{9} bp corresponding to approximately 4,600 and 1,500 *E. coli*-like genomes, respectively.

The community structure of the low and high metal-contaminated soils was investigated by hybridisation with group-specific phylogenetic probes [α-Proteobacteria (ALF1b); β-Proteobacteria (BET42a); γ-Proteobacteria (GAM42a); δ-Proteobacteria (SRB385); cytophaga-flexibacter-bacteroides (CF319a); Gram positives with a low mole% G+C (LGCb); Gram positive with a high mole % G+C (HGC69a)] (Sandaa et al. 1999a,b, 2001). The probes were applied to the total bacterial community (FISH), bacterial isolates and PCR-based clone libraries of 16S rRNA (slot blot hybridisation). Approximately 300 clones and 300 bacterial isolates were analysed altogether. The culture-independent methods (FISH and clone analyses) gave similar results and showed greater differences in community composition than the culture-dependent methods (isolate analysis). The most abundant group of clones in the low metal-contaminated soil was the cytophaga-flexibacter-bacteroides group. This group was twice as abundant in the low as in the high metal-contaminated soil. In the high metal-contaminated soil, clones belonging to the α-Proteobacteria were numerically dominant (Fig. 9). With respect to the isolates, 30–37% of them belonged to Gram-positive bacteria with low mole% G+C. Accordingly, this was the largest group of isolates in both soils. Interestingly this group made up <1% of the clones. In the high metal-contaminated soil the abundance of isolates and clones belonging to the α-Proteobacteria subclass differed markedly, as the percentage of clones was 38% and that of isolates was only 14%.

These investigations revealed that the total microbial diversity in relatively undisturbed and unpolluted soil was high, and that upon perturbation and pollution the total soil microbial diversity was dramatically reduced. Microcosm investigations indicate that some population types become numerically dominant under stress. Pollution may induce profound changes in the community structure (Tiedje et al. 2001). The results suggest that quantitative measures of microbial diversity and qualitative analysis of community structure can discriminate between soil samples subjected to different levels of pollution and be useful indicators of stress and perturbation.

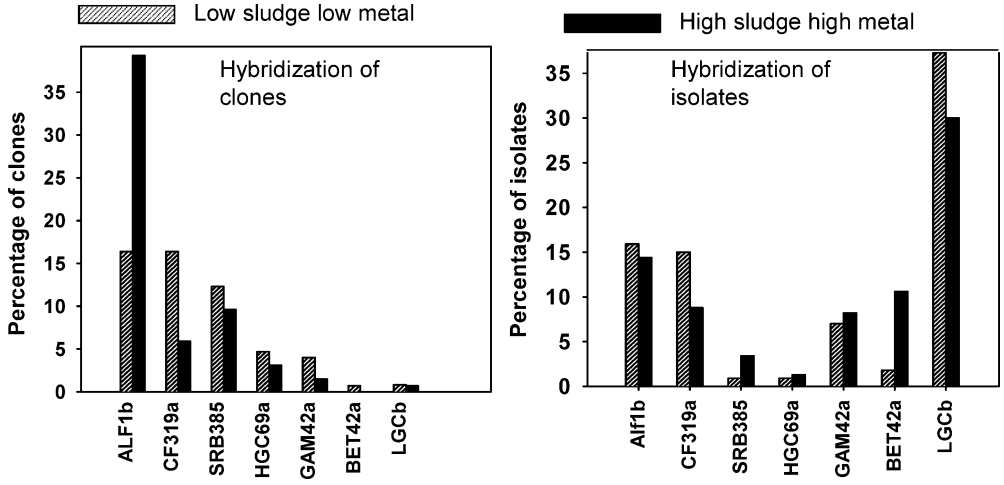

Fig. 9 Changes in community structure in sewage sludge-amended soil with low and high concentrations of heavy metals. Group-specific 16S and 23S rRNA phylogenetic probes were used for whole cell FISH and for slot blot hybridisation of 300 clones of PCR amplified total 16S and 23S rDNA from the soils. For FISH a control soil without contamination was also investigated. Probes against the following phylogenetic groups of bacteria were applied: α-Proteobacteria (*ALF1b*); β-Proteobacteria (*BET42a*); γ-Proteobacteria (*GAM42a*); δ-Proteobacteria (*SRB385*); cytophaga-flexibacter-bacteroides (*CF319a*); Gram positives with a low mole % G+C (*LGCb*); Gram positive with a high mole % G+C (*HGC69a*) (Torsvik et al. 2000). For abbreviations, see Fig. 7

Microbial communities affected by transgenic microorganisms

Microbial inoculants, some of which have an historical record of safe use since 1896 (the well-known rhizobia, for the inoculation of legumes) or the 1930s (e.g. *Bacillus thuringiensis*, for the biological control of invertebrate pests) are being applied in modern agriculture over a surface of >30 million ha each year (Nuti 1994). Most of these inoculants consist of rhizobial inoculants (12–24×10^6 ha) and other uses are *Azolla-Anabaena* for the inoculation of rice, azospirilli as inoculants for cereal crops (mainly sorghum, corn, wheat) or grasses, mycorrhizal fungi for a variety of trees, bushes and crops (e.g. *Citrus*, *Quercus*, *Salix*, *Tilia*, *Corylus*, *Populus*, *Cystus*, and corn, onions, etc.), and bacilli, *Pseudomonas* and *Trichoderma* species as biological control agents (i.e. plant protection agents against plant pathogens, mainly fungi). Other potential applications for microbial inoculants currently include bioremediation and phytoremediation, phosphate solubilization, soil aggregation, sewage treatment, bioleaching, oil recovery, coal scrubbing and biogas production (Latour et al. 1996; Van Veen et al. 1997). The most studied genetically modified (GM) microbial inoculants have so far been *Rhizobium* sp., *Pseudomonas* sp., and *Azospirillum* sp. (Corich et al. 1995, 1996; Resca et al. 2001) and (GM) bacteria have been used as seed inoculants at field-scale level to test their effectiveness and to gain deeper insight into the biosafety-related aspects of their use, i.e. their fate in the soil environment (Basaglia et al. 2003; Dighton et al. 1997; Lynch 2002a, 2002b; Van Veen et al. 1997).

Pseudomonas fluorescens SBW 25, isolated from the phylloplane of sugar beet, has been shown to readily colonise the rhizosphere of sugar beet, as well as the phylloplane and rhizosphere of wheat (De Leij et al. 1998a). The marker genes (*lacZY* and *kan*^{r}*xylE*) were chosen to facilitate identification and detection of the GM organisms by simple culture methods and were positioned 1 Mb apart on the 6.5-Mb chromosome to ensure genotypic and phenotypic stability and to facilitate any gene exchange between microbial populations associated with the two crops (De Leij et al. 1998a). There was no movement of the marker genes and only very small and transient effects on indigenous communities. Subsequent studies carried out by De Leij et al. (1998b) investigated the potential metabolic burden of the inserted genes on the ecological competence of a variety of constructs that were modified with the marker genes used in the release study strain of the bacterium. Whereas kanamycin resistance did not affect the fitness of the organism, both of the other marker inserts did reduce ecological competence. Hence, even though the modified bacterium was competent in the field, the wild-type is even more competent.

In terms of functional genes, *P. fluorescens* F113 strain was isolated from sugar beet and found to produce the antibiotic DAPG (Shanahan et al. 1992). Besides being active against *Pythium* damping-off disease, it was also active against the potato soft-rot pathogen *Erwinia carotovora* subsp. *atroseptica* and the potato cyst nematode *Globodera rostochiensis*. For comparative purposes, strain F113 G22 was engineered to produce a Tn*5*::lacZY DAPG-negative derivative of F113 that does not have the ability to inhibit the growth of plant pathogenic fungi. This construct and the indigenous bacterial populations were used to study the impact of *Pseudomonas* strains on the rhizosphere microflora. One of the main approaches used was to determine the effect of the inoculated construct and indigenous bacterial population on the rhizosphere/soil enzymes *N*-acetyl glucosaminidase, chitobiosidase, acid and alkaline phosphatase, phosphodiesterase, aryl sulfatase and urease, whose activities play important roles in the C, N, P and S cycles in soil (Naseby and Lynch 2002). None of the soil enzyme activities was affected by the GM marker (GMM) under any of the soil amendments (addition of lactose to kanamycin).

However, studies on the effects of inoculation with GMM have not directly addressed the issue of the microbial diversity, being mainly focused on other essential aspects of risk assessment profile, e.g. their persistence in the environment and colonization ability in the rhizosphere. A shift in the fungal population of wheat roots, as indicated by cluster analysis of replicate ARDRA-generated profiles, occurred when both *P. putida* WCS358r and the respective GMM WCS358r::phr producing phenazine-1-carboxylic acid (antimicrobial compound) were applied to seeds (Viebahn et al. 2003). The most prominent effect occurred at the beginning of the field experiments, when the numbers of introduced bacteria were relatively high; the GMM impact on the composition of fungal population lasted longer than the WCS358r impact.

International quality standards for microbial inoculants are scattered throughout different countries and are highly diverse (Nuti and Sirsi 2003). It would be beneficial, for the protection of farmers and for an easier control or monitoring by (inter)national agencies or competent authorities, to have an established and consistent legislation at international level; including the European Union where such a legislative framework does not exist yet.

Scientific knowledge has progressed considerably over the last two decades, allowing more accurate tracking of organisms in the environment and this identification at species and strain level. This can be achieved via a combination of conventional tools and innovative molecular techniques, as discussed in other paragraphs of this review.

Microbial communities affected by transgenic plants

Several thousand field releases of transgenic crop plants have been performed during the last decade and several transgenic crop plants have been commercialised (James 2003). However, there are actually very few studies (Kowalchuk et al. 2003) published which have tried to analyse the potential effects of transgenic crops on soil microbial communities. Does this reflect the perception

that the effects of transgenic crops on soil microbial populations are expected to be negligible or at least less important compared to other biosafety issues of transgenic crops such as out-crossing to weedy species, effects on non-target organisms or the appearance of new viruses. Or is it that the effects on the soil microbiota are just more difficult to analyse? What kind of effects of transgenic crops might be expected? Presently, two kinds of scenarios are recognised in which the large-scale use of transgenic crops could have an effect on microbial communities in rhizosphere and bulk soils:

I. When the structural and functional composition of the soil microbial community in the vicinity of the roots is changed as a result of an altered root exudation or released transgene product with antifungal, antibacterial activity, or others.
2. When bacterial rhizosphere populations would be able to capture and stably integrate transgenic plant DNA, in particular antibiotic resistance genes used as markers in transgenic crops.

Whereas the first scenario might impact plant health or soil functions, the impact of the second following horizontal gene transfer (HGT) of marker genes would be more indirect and primarily seen as the extent to which such a HGT event would contribute to an additional spread of these genes to other bacterial populations.

Plant breeding by traditional techniques, as well as transgenic modification, might affect the structural and functional diversity of the rhizosphere microbial community, through for example an altered root morphology and physiology, and plant exudation, and thus might affect the balance of plant-beneficial and deleterious microbes. In particular, if the transgenic modification was made to improve the resistance of the plant towards bacterial or fungal pathogens by releasing transgene products such as cell wall-attacking enzymes or compounds like T4-lysozyme, chitinases or cecropine, unintended changes in the microbial community cannot be excluded and should therefore be assessed (Kowalchuk et al. 2003). However, to evaluate any potential shifts in microbial rhizosphere communities as a result of transgene expression it is of utmost importance that baseline data are available to relate potential changes to natural fluctuations. As mentioned, nucleic acid-based analysis of bacterial communities allows us to overcome biases of cultivation-dependent methods (Van Elsas et al. 2000). To study spatial and temporal variation in rhizosphere and soil bacterial communities, multiple sample analysis with the use of molecular techniques is essential (Muyzer et al. 1993; Van Elsas and Smalla 1996; Muyzer and Smalla 1998; Schwieger and Tebbe 1998; Gelsomino et al. 1999).

Recently, several studies were published in which molecular fingerprints have been used to analyse the dynamics in the rhizosphere during plant growth development, and the effect of the plant species on the relative abundance of bacterial populations in the rhizosphere (Normander and Prosser 2000; Schmalenberger and Tebbe 2000; Schwieger and Tebbe 2000; Duineveld et al. 2001, Gomes et al. 2001, 2003; Smalla et al. 2001). Strong shifts in the relative abundance of the microbial rhizosphere community could be observed during plant development (Gomes et al. 2001, 2003; Heuer et al. 2002a,b; Smalla et al. 2001).

Effects of transgenic crops on the structural and functional composition of microbial rhizosphere communities are studied best at the stage of small-scale field releases, since greenhouse observations have been shown to deviate considerably from what is observed under field conditions (Kowalchuk et al. 2003). To test the performance of transgenic lines, field tests are done in which the transgenic plants are grown in completely randomised field designs together with the unmodified parental crop on different sites. Such field tests are also ideal for studying potential differences between the parental crop variety and different transformant lines since they allow a sufficient number of replicates to be analysed and samples to be taken at different stages of plant growth development and from different field sites (Kowalchuk et al. 2003). Usually, different transformation events are tested because they can show considerable variation in the level and stability of gene expression.

To date only a few studies have been sought to analyse the potential effects of transgenic crop plants on the composition of bacterial communities in the rhizosphere under field conditions (Lottmann et al. 1999, 2000; Dunfield and Germida 2001; Heuer et al. 2002a; Schmalenberger and Tebbe 2002; Kowalchuk et al. 2003). In the study by Dunfield and Germida (2001), four herbicide-tolerant and four conventional oilseed varieties were grown at four different locations across Canada and the rhizosphere microbial communities were characterised over two field seasons using fatty acid methyl ester (FAME) analysis and Biolog CLPP. The transgenic glyphosate-tolerant oilseed variety Quest seemed to be unique and the microbial rhizosphere communities associated with Quest were different from those of the three other glufosinate ammonium-tolerant and the four conventional oilseed varieties. Differences found for the other varieties seemed to be significantly influenced by the soil type. However, since the transgenic variety Quest was not compared with the parental non-transgenic line it remains unclear whether the changes observed were caused by the genetic modification. The objective of the study by Heuer et al. (2002a) was to characterise the structure and dynamics of bacterial communities in the rhizosphere of potato under field conditions, and to compare these to those of the transgenic T4-lysozyme-expressing potato plants. In contrast to many other transgenic plants, this genetic modification was targeted at bacteria, and it was shown by Düring and Mahn (1999) that plant-associated bacteria were indeed affected, in so far as the susceptibility of the transgenic potato plants to infections by *Erwinia carotovora* was significantly reduced. Furthermore, it was demonstrated that a detectable amount of T4 lysozyme was released from the roots (De Vries et al. 1999) resulting in bactericidal activity at the root surface (Ahrenholtz et al. 2000). Two

T4 lysozyme-producing lines, a transgenic control without the T4 lysozyme gene and the parental line, were investigated over 3 years at two distant field sites with different soil types. The bacterial communities were analysed by three different approaches which were intended to complement each other. In the first approach, the relative abundance of bacterial species in the rhizosphere was determined based on the cultivation and characterisation of isolates by fatty acid analysis. The second approach was the catabolic profiling of the communities as functional units using Biolog GN microplates (Garland and Mills 1991; Heuer and Smalla 1997b). The third approach was based on the analysis of 16S rRNA genes fragments amplified from total rhizosphere DNA by DGGE (Heuer et al. 1999; Muyzer and Smalla 1998) or by cloning and sequencing. This allowed workers to monitor changes of the bacterial rhizosphere consortia, including bacteria not readily culturable or those in a non-culturable state. In this study, all methods revealed that environmental factors related to plant developmental stage, field site or year, but not the T4 lysozyme expression of the transgenic plants, influenced the rhizosphere communities. In some cases, differences in the rhizosphere community structure to one or all other cultivars were detected at some of the samplings which were not attributable to the T4 lysozyme production but most likely to differences observed in growth characteristics of the cultivar. Thus, the risk associated with transgene effects on the bacterial communities seems to be below accepted background levels. The methods applied in the study by Heuer et al. (2002a) seemed ideally suited to the detection of changes in microbial rhizosphere communities since seasonal shifts in bacterial rhizosphere communities were easily detectable. Although only a small proportion of the bacterial community contributes to the Biolog patterns (Smalla et al. 1998), differences in the catabolic potential of the bacterial communities were sensitively and quantitatively detected. The isolation and characterisation of dominant rhizobacteria by FAME analysis provided information on the taxonomic composition of the dominant culturable members of the rhizosphere bacterial community. However, the characterisation of many isolates would have been required to statistically confirm differences between the potato lines. Most suitable for this kind of study seemed to be the D/TGGE approach, which allowed the cultivation-independent analysis of large numbers of samples with the option as for the characterisation of differentiating bands.

In other studies performed under greenhouse conditions, transient effects of transgenic crops on soil microorganisms and processes have been reported (Di Giovanni et al. 1999; Donegan et al. 1999; Siciliano and Germida 1999; Oger et al. 2000; Gyamfi et al. 2002; Sessitsch et al. 2003). However, due to the experimental design used these effects could often not be related to natural fluctuations. A relevant effect of the transgenic plants on soil microbial communities should cause more profound changes than those due to environmental factors related to season and field site (Heuer et al. 2002a,b; Kowalchuk et al. 2003).

Persistence of DNA in soil and horizontal transfer of transgenic plant DNA to bacteria

Natural transformation is the most likely mechanism for horizontal transfer of genes from transgenic crops to bacteria (Nielsen et al. 1998; Bertolla and Simonet 1999). Natural transformation is defined as a DNase-sensitive process by which competent bacteria can take up free DNA (Stuart and Carlson 1986). The single-stranded DNA taken up by the bacteria can either be integrated into the bacterial genome by homologous recombination, or form an autonomous replicating element. Natural transformation provides a mechanism of gene transfer that enables competent bacteria to generate genetic variability by "sampling" the DNA present in their surroundings (Nielsen et al. 2000a). From laboratory experiments, >40 bacterial species from different environments are known to be naturally transformable (Lorenz and Wackernagel 1994; Nielsen et al. 1998). Recent findings by Demanèche et al. (2001b) indicated that the number of naturally transformable bacteria could be higher than previously thought. Prerequisites for natural transformation are: the availability of free DNA; the development of competence; and the take-up and stable integration of the captured DNA. However, there is very limited knowledge of how important natural transformation by bacterial or plant DNA is in different environmental settings such as soil, compost, manure, sewage, etc. Two aspects have been, or are presently being, studied, by several groups to address natural transformation in the environment:

(1) How long does free DNA persist, e.g. in soil, and is the DNA still available for natural transformation?
(2) How frequently do different bacterial species under environmental conditions reach the state of competence?

Lightning-mediated gene transfer has been demonstrated recently under laboratory-scale conditions (Demanèche et al. 2001a) providing another potential route for uptake of transgenic plant DNA to bacteria.

Investigations made by different groups have shown that in spite of the ubiquitous occurrence of DNases, high-molecular free DNA can be detected in different environments (Nielsen et al. in press; De Vries et al. 2003). It is supposed that free DNA released from microorganisms or decaying plant material can serve as a nutrient source or as a reservoir of genetic information for autochthonous bacteria. Different abiotic and biotic factors seem to affect the persistence of free DNA in soil (Gallori et al. 1994; Paget and Simonet 1994) and reports on the persistence of DNA in non-sterile soil have only recently been published (Blum et al. 1997; Nielsen et al. 1997a; Gebhard and Smalla 1999; De Vries et al. 2003). Microbial activity was pinpointed as an important biotic factor affecting the persistence of free DNA in soil, where

stimulating microbial activity often coincided with an increase in DNase activity (Blum et al. 1997). Nielsen et al. (2000a) showed that cell lysates of *P. fluorescens*, *Burkholderia cepacia* and *Acinetobacter* spp. were available as sources of transforming DNA to *Acinetobacter* sp. BD 413 cell populations in sterile and non-sterile soil for a few days, and that cell debris protects DNA from inactivation in soil, with the cell walls possibly playing an important role in protecting DNA after cell death (Paget and Simonet 1997). Long-term persistence of transgenic plant DNA has also been observed in different studies (Widmer et al. 1996, 1997; Paget and Simonet 1997; Gebhard and Smalla 1999; De Vries et al. 2003) under microcosm and field conditions. A more rapid decrease in transgenic DNA was observed at higher soil humidity and temperature. Both factors are thought to contribute to a higher microbial activity in soil (Widmer et al. 1996; Blum et al. 1997). Field releases of transgenic rhizomania-resistant sugar beet plants were accompanied by studies on the persistence of transgenic DNA from sugar beet litter in soil (Gebhard and Smalla 1999). To detect transgenic DNA independently of cultivation, total community DNA was extracted directly from soil and amplified with three different primer sets specific for the transgenic DNA. Parts of the construct were detectable for up to 2 years. Similar studies in soil microcosms amended with free transgenic DNA, also showed long-term persistence (Gebhard and Smalla 1999).

Transgenic DNA can be degraded during plant senescence and during microbial degradation of the plant residue in soil (Ceccherini et al. 2003). However, measured amounts of transgenic plant DNA can escape these degradation processes (Ceccherini et al. 2003) and the long-term persistence, even of a small percentage of released plant DNA, is assumed to enhance the likelihood of bacterial transformation.

Recombinant DNA of T4-lysozyme-expressing potatoes was frequently detected in rhizosphere extracts of field-grown transgenic potato plants (De Vries et al. 2003). The authors showed that two sources of DNA seem to contribute to the spread of transgenic plant DNA: the growing root system and pollen. In contrast to previous studies, De Vries et al. (2003) were able to show long-term persistence of transforming DNA in field soil for 4 years using a novel and specific biomonitoring technique. Since plant DNA can persist adsorbed to soil particles or be protected in plant cells this DNA could be captured by competent bacteria colonising in the close vicinity of the residue (Saxena and Stotzky 2000). Until recently, it was unclear whether bacteria could be transformed by plant DNA at all. The high content of non-bacterial DNA and the much higher methylation rate of plant DNA were supposed to prevent a transfer of antibiotic resistance genes from transgenic plants to bacteria (Nielsen et al. 1998). Several groups failed to detect HGT from transgenic plants to bacteria, perhaps because of a scarcity of homologous sequences in the bacteria (Nielsen et al. 1997c; De Vries et al. 2001) or the use of poorly transformable bacteria (Schlüter et al. 1995). However, the ability of *Acinetobacter* sp. BD413 pFG4Δ*npt*II to capture and integrate transgenic plant DNA based on homologous recombination under optimised laboratory conditions has been recently demonstrated (Gebhard and Smalla 1998). Restoration of the 317-bp deletion, resulting in the emergence of kanamycin-resistant *Acinetobacter* sp. BD413, was observed not only with transgenic plant DNA but also with transgenic plant homogenates (Gebhard and Smalla 1998). Also for *Acinetobacter* sp. BD413 cells containing an *npt*II-gene with a 10-bp deletion, transformation with DNA from various transgenic plants (*Solanum tuberosum*, *Nicotiana tabacum*, *Beta vulgaris*, *Brassica napus*, *Lycopersicon esculentum*) carrying *npt*II as the marker gene resulted in the incorporation of the deleted *npt*II gene on the bacterial genome (De Vries and Wackernagel 1998; De Vries and Wackernagel 2002). Both studies had in common that *Acinetobacter* sp. BD413 was transformed and that an *npt*II gene carrying an internal deletion served for sequence homology and as a detection system (incorporation of the *npt*II gene resulted in kanamycin resistance). *Ralstonia solanacearum*, the causal agent of bacterial wilt, was reported to develop competence and to exchange genetic information in planta (Bertolla et al. 1997, 1999). Gene exchange was demonstrated when tomato plants infected with *R. solanacearum* were inoculated with plasmid DNA or during co-infection with different *R. solanacearum* carrying different genetic markers; however, transformation was not observed during colonisation of transgenic plants (Bertolla et al. 1999). A new finding concerning horizontal transfer of antibiotic resistance genes from plants to bacteria is the capture of DNA from transgenic plants by bacteria by homologous recombination (Nielsen et al. 2000b). However, how does one assess the significance of such a finding? First of all, it is important to look at the frequencies of such transfer events. Compared to transformation with chromosomal or plasmid DNA, transformation frequencies with plant DNA or plant homogenates were drastically reduced when the experiments done initially by filter transformation of *Acinetobacter* sp. BD413 pFG4 were taken a step further and performed in sterile and non-sterile soil (Nielsen et al. 2000a,b). Transformation of *Acinetobacter* sp. BD413 pFG4 by transgenic sugar beet DNA could be detected in sterile but not in non-sterile soil (Nielsen et al. 2000b) and the authors estimated that numbers of transformants in non-sterile soil would be between 10^{-10} and 10^{-11} and thus below the level of detection. Experimental studies have confirmed the low probability of integration of transgenes into the bacterial genome of the recipient in the absence of significant DNA homology (Nielsen et al. 1997c). Where homologous DNA is present, studies on gene transfer by natural transformation have revealed that additive integration of non-homologous genetic material can occur when flanking homology is present (Gebhard and Smalla 1998; Nielsen et al. 1998). However, the possible existence of hot spots for gene transfer by transformation, such as the digestive tract of soil mesofauna, cannot be ruled out.

Among the different steps in the process of natural transformation, the release of DNA from cells, its persistence and its availability to be taken up by competent bacteria have been well documented (Paget and Simonet 1997). The major limiting factor for natural transformation remains the presence of competent bacteria and the development of competence. In most studies on transformation, competent bacteria have been inoculated in the soil system studied (Gallori et al. 1994; Nielsen et al. 1997a; Sikorski et al. 1998). Nielsen et al. (1997b, 2000b) have, however, shown that non-competent *Acinetobacter* sp. strain BD413 cells residing in soil can become competent after addition of nutrients. The nutrient solutions used to stimulate competence development in *Acinetobacter* sp. BD413 populations contained inorganic salts and simple compounds corresponding to rhizosphere exudates (Nielsen et al. 2000b).

Presently, we still know very little about the importance of transformation processes in environmental habitats. In contrast, HGT by conjugation or mobilisation under different environmental conditions is much better documented (Thomas and Smalla 2000). It cannot be excluded that HGT from plants to bacteria takes place in different environmental niches, but the ecological impact of such rare events depends upon the selection and dissemination of the acquired trait. The emergence of bacterial antibiotic resistances as a consequence of the wide-scale use of antibiotics by humans has resulted in a rapid evolution of antibiotic resistance. Mobile genetic elements such as transferable plasmids, transposons and integrons have played a key role in the dissemination of antibiotic resistance genes amongst bacterial populations, and have contributed to the acquisition and assembly of multiple antibiotic resistance in bacterial pathogens (Tschäpe 1994; Levy 1997; Witte 1998). Since bacteria circulate between different environments and different geographic areas, the global nature of the problem of bacterial antibiotic resistances requires that data on their prevalence, selection and spread are obtained in a more comprehensive way than before. Few studies have provided data on the prevalence of antibiotic resistance genes used as markers in transgenic plants. Studies on the dissemination of the most widely used marker gene, *npt*II, in bacteria from sewage, manure, river water and soils demonstrated that in a high proportion of kanamycin-resistant enteric bacteria, the resistance is encoded by the *npt*II-gene (Smalla et al. 1993).

Bacteria resistant to multiple antibiotics are not restricted to clinical environments but can easily be isolated from environmental samples and food (Perreten et al. 1997; Dröge et al. 2000; Smalla et al. 2000; Heuer et al. 2002b). There is substantial movement of antibiotic resistance genes and antibiotic resistant bacteria between different environments. In assessing the antibiotic resistance problem, the nature of the antibiotic itself and the antibiotic resistance trait have been identified as important factors (Levy 1997). The genetic plasticity of bacteria has largely contributed to the efficiency by which antibiotic resistance has emerged. However, HGT events have no *a priori* consequence unless there is antibiotic selective pressure (Levy 1997). Given the facts that: (1) antibiotic resistance genes, often located on mobile genetic elements, are already widespread in bacterial populations, and (2) that HGT events from transgenic plants to bacteria are supposed to occur at extremely low frequencies and have not yet been detected under field conditions, it is unlikely that antibiotic resistance genes used as markers in transgenic crops will contribute significantly to the spread of antibiotic resistance in bacterial populations. There is no doubt that the present problems in human and veterinary medicine were created by the unrestricted use of antibiotics in medicine and animal husbandry, and not by transgenic crops carrying antibiotic resistance markers. Thus, the public debate about antibiotic resistance genes in transgenic plants should not divert the attention from the real causes of bacterial resistance to antibiotics such as the continued abuse and overuse of antibiotics prescribed by physicians and in animal husbandry (Salyers 1996).

Conclusions

The key issue is whether a reduction in biodiversity is of any significance to life on this planet. Using mathematical modelling, May (1976) demonstrated that as the number of interacting species increases, the stability of the whole system is likely to decrease, unless special conditions are met. This highlights the unexpected vulnerability of complex ecosystems such as tropical rainforests and coral reefs. May (1976) did not apply his ideas to microbial communities, but there is no reason to think that the concepts would not apply. Indeed ecological theories have been generally applied to aboveground systems.

Despite the better determination of microbial diversity in soil by the use of molecular techniques, the relationships between microbial diversity and soil functionality is still largely unclear. In soil there is a redundancy of certain functions and for this reason a reduction in microbial diversity generally does not alter the rate of processes such as C and N mineralization (Griffiths et al. 1997; Nannipieri et al. 2003). Of course this is not valid for specialistic functions. The determination of all microbial species inhabiting soil is not only a problematic task but also it may not improve our knowledge of the links between microbial diversity and soil functions. It may be more rewarding to measure changes in the composition of keystone species, such as nitrifiers, only responsible for a process occurring in soil, like autotrophic nitrification. On the other hand it is problematic to carry out the determination of all microbial activities occurring in soil and to integrate the obtained results in a single index so as to assess quantitatively the soil metabolic activity (Nannipieri et al. 2003). The determination of the reaction rate limiting the overall rate of the main process can reduce the amount of experimental work without compromising the assessment of the metabolic activity.

As an example of anthropogenic influence on soil microbial diversity we have focussed on the impact of GM plants and GM microorganisms on soil microflora. Transgenic plants do not always affect bacterial composition and when these effects have been observed, they have not been related to natural fluctuations. Indeed, relevant effects of transgenic plants on microbial communities should cause deeper changes than the commonly accepted changes due to environmental factors related to season, field site and year.

Transgenic DNA can be degraded during plant senescence by plant nuclease and during microbial degradation of the plant remains in soil. However, parts of the construct of the transgenic rhizomania-resistant sugar beet were detectable for up to 2 years in soil (Gebhard and Smalla 1999). Contradictory results have been observed on the transfer of antibiotic resistant genes from transgenic plants to bacteria. However, where this process occurs, the observed transformation frequencies are very low. It is important to underline that the public debate about antibiotic resistance genes in transgenic plants should not divert the attention from the real causes of bacterial resistance to antibiotics, such as the continued abuse and overuse of antibiotics prescribed by physicians and in animal husbandry.

Since the statement "one gene one protein" is an oversimplification, because modifications can occur both at post-transcriptional and post-translational level and regulation can occur at the protein translational level, DNA and mRNA measurements should be combined with the application of the proteomic approach to soil so as to have measurements of protein expression in soil. This combined approach could give better insights into the links between microbial diversity and soil functionality.

Bearing in mind that a complex food web enhances the soil's resilience to disturbances, we should actively promote actions that ensure high microbial diversity, the measurement of which is still problematic in soil.

Acknowledgements We gratefully acknowledge the support of COST Action 831 for supporting the attendance of authors to the Erice (Trapani, Italy) meeting in 2001. Some of the data presented and discussed in this review by A. Benedetti and P. Nannipieri have been obtained in the research MISA project financed by the Ministero delle Politiche Agericole e Forestali, Italy.

References

Ahrenholtz I, Harms K, de Vries J, Wackernagel W (2000) Increased killing of *Bacillus subtilis* on hair roots of transgenic T4 lysozyme-producing potatoes. Appl Environ Microbiol 66:1862–1865

Alfreider A, Peters S, Tebbe CC, Rangger A, Insam H (2002) Microbial community dynamics during composting of organic matter as determined by 16S ribosomal DNA analysis. Compost Sci Util 10:303–312

Amann RI, Krumholz L, Stahl DA (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J Bacteriol 172:762–770

Amann RI, Ludwig W, Schleifer K-H (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169

Atlas RM, Bartha R (1998) Microbial ecology. Fundamentals and applications, 4th edn. Addison-Wesley, Reading

Avaniss-Aghajani E, Jones K, Chapman D, Brunk C (1994) A molecular technique for identification of bacteria using small subunit ribosomal RNA sequences. Biotechniques 17:144–146

Basaglia M, Casella S, Peruch U, Poggiolini S, Vamerali T, Mosca G, Vanderleyden J, De Troch P, Nuti MP (2003) Field release of genetically marked *Azospirillum brasiliense* in association with *Sorghum dicolor* L. Plant Soil 256:281–290

Bending GT, Turner MK, Jones JE (2002) Interactions between crop residues and soil organic matter quality and the functional diversity of soil microbial. Soil Biol Biochem 34:1037–1082

Bertolla F, Simonet P (1999) Horizontal gene transfer in the environment: natural transformation as a putative process for gene transfer between transgenic plants and microorganisms. Res Microbiol 150:375–384

Bertolla F, van Gijsegem F, Nesmer X, Simonet P (1997) Conditions for natural transformation of *Ralstonia solanacearum*. Appl Environ Microbiol 63:4965–4968

Bertolla F, Frostegard A, Brito B, Nesmer X, Simonet P (1999) During infection of its host, the plant pathogen *Ralstonia solanacearum* naturally develops a state of competence and exchanges genetic material. Mol Plant Microbe Interact 12:467–472

Blum SAG, Lorenz MG, Wackernagel W (1997) Mechanisms of retarded DNA degradation and prokaryotic origin of DNases in nonsterile soils. Syst Appl Microbiol 20:513–521

Borneman J, Triplett EW (1997) Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. Appl Environ Microbiol 63:2647–2653

Borneman J, Skroch PW, O'Sullivan KM, Palus JA, Rumjanek NG, Jansen JL, Nienhuis J, Triplett EW (1996) Molecular microbial diversity of an agricultural soil in Wisconsin. Appl Environ Microbiol 62:1935–1943

Brønstad K, Drønen K, Øvreås L, Torsvik V (1996) Phenotypic diversity and antibiotic resistance in soil bacterial communities. J Ind Microbiol Biotechnol 17:253–259

Bruce KD (1997) Analysis of mer gene subclasses within bacterial communities in soils and sediments resolved by fluorescent-PCR-restriction fragment length polymorphism profiling. Appl Environ Microbiol 63:4914–4919

de Bruijn FJ (1992) Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl Environ Microbiol 58:2180–2187

Burkhardt C, Insam H, Hutchinson TC, Reber HH (1993) Impact of heavy metals on the degradative capabilities of soil bacterial communities. Biol Fertil Soils 16:154–156

Ceccherini MT, Potè J, Kay E, Van VT, Marechal J, Pietramellara G, Nannipieri P, Vogel TM, Simonet P (2003) Degradation and transformability of DNA from transgenic leaves. Appl Environ Microbiol 69:673–678

Chatzinotas A, Sandaa R-A, Hahn D, Schönhuber W, Daae FL, Torsvik V, Zeyer J, Amann R (1998) Studies on bacterial diversity in bulk soils using different molecular techniques. Syst Appl Microbiol 21:588–592

Cheneby D, Philippot L, Hartmann A, Henault C, Germon JC (2000) 16S rDNA analysis for characterization of denitrifying bacteria isolated from three agricultural soils. FEMS Microbiol Ecol 34:121–128

Corich V, Giacomini A, Concheri G, Tritzerfeld B, Vendramin E, Struffi P, Basaglia M, Squartini A, Casella S, Nuti M.P, Peruch U, Poggiolini S, De Troch P, Vanderleyden J, Fedi S, Fention A, Moenne-Loccoz Y, Dowling DN, O'Gara F (1995) Environmental impact of genetically modified *Azospirillum brasilense*, *Pseudomonas fluorescens* and *Rhizobium leguminosarum* released as soil/seed inoculants. In: Jones D (ed) Biosafety results of field tests with genetically modified plants and microorganisms. USDA-UCLA Publ., Monterey, CA. USA pp 371–378

Corich V, Bosco F, Giacomini A, Basaglia M, Squartini A, Nuti MP (1996) Fate of genetically modified *Rhizobium leguminosarum* biovar *viciae* during prolonged storage of commercial inoculants. J Appl Bacteriol 81:319–328

Da Silva KRA, Salles JF, Seldin L, van Elsas JD (2003) Application of a novel *Paenibacillus*-specific PCR-DGGE method and sequence analysis to assess the diversity of *Paenibacillus* spp. in the maize rhizosphere. J Microbiol Methods 54:213–231

Degens BP, Harris JA (1997) Developments of a physiological approach to measuring the catabolic diversity of soil microbial communities. Soil Biol Biochem 29:1309–1320

De Leij FAAM, Bailey MJ, Whipps JM, Thompson IP, Bramwell PA, Lynch JM (1998a) Gene release and biomonitoring. In: Lynch J, Wiseman A (eds) Environmental biomonitoring. The biotechnology, ecotoxicology interface. Cambridge University Press, Cambridge, pp 70–100

De Leij FAAM, Thomas CE, Bailey MJ, Whipps JM, Lynch JM (1998b) Effect of insertion site and metobolic load on the environmental fitness of a genetically modified *Pseudomonas fluorescens*. Appl Environ Microbiol 64:2634–2638

Demanèche S, Bertolla F, Buret F, Nalin R, Sailand A, Auriol P, Vogel TM, Simonet P (2001a) Laboratory-scale evidence for lightning-mediated gene transfer in soil. Appl Environ Microbiol 67:3440–3444

Demanèche S, Kay E, Gourbière F, Simonet P (2001b) Natural transformation of *Pseudomonas fluorescens* and *Agrobacterium tumefaciens* in soil. Appl Environ Microbiol 67:2617–2621

De Vries J, Wackernagel W (1998) Detection of *npt*II (kanamycin resistance) genes in genomes of transgenic plants by marker-rescue transformation. Mol Gen Genet 257:606–613

De Vries J, Wackernagel W (2002) Integration of foreign DNA during natural transformation of *Acinetobacter* sp. by homology-facilitated illegitimate recombination. Proc Natl Acad Sci 99:2094–2099

De Vries J, Harms K, Broer I, Kriete G, Mahn A, Düring K, Wackernagel W (1999) The bacteriolytic activity in transgenic potatoes expressing a chimeric T4 lysozyme gene and the effect of T4 lysozyme on soil- and phytopathogenic bacteria. Syst Appl Microbiol 22:280–286

De Vries J, Meier P, Wackernagel W (2001) The natural transformation of the soil bacteria *Pseudomonas stutzeri* and *Acinetobacter* sp. by transgenic plant DNA strictly depends on homologous sequences in the recipient cells. FEMS Microbiol Lett 195:211–215

De Vries J, Heine M, Harms K, Wackernagel W (2003) Spread of recombinant DNA by roots and pollen of transgenic potato plants, identified by highly specific biomonitoring using natural transformation of an *Acinetobacter* sp. Appl Environ Microbiol 69:4455–4462

Dighton J, Jones HE, Robinson CH, Beckett J (1997) The role of abiotic factors, cultivation practices and soil fauna in the dispersal of genetically modified microorganisms in soils. Appl Soil Ecol 5:109–131

Di Giovanni GD, Watrud LS, Seidler RJ, Widmer F (1999) Comparison of parental and transgenic Alfalfa rhizosphere bacterial communities using Biolog GN metabolic fingerprinting and enterobacterial repetitive intergenic consensus sequence-PCR (ERIC-PCR). Microb Ecol 37:129–139

Donegan KK, Seidler RJ, Doyle JD, Porteus LA, Di Giovanni G, Widmer F, Watrud LS (1999) A field study with genetically engineered alfalfa inoculated with recombinant *Sinorhizobium meliloti*: effects on the soil ecosystem. J Appl Ecol 36:920–936

Dröge M, Pühler A, Selbitschka W (2000) Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. Mol Gen Genet 263:471–482

Drønen AK, Torsvik V, Goksøyr J, Top EM (1998) Effect of mercury addition on plasmid incidence and gene mobilizing capacity in bulk soil. FEMS Microbiol Ecol 27:381–394

Duineveld BM, Kowalchuk GA, Keijzer A, van Elsas JD, van Veen JA (2001) Analysis of bacterial communities in the rhizosphere of *Chrysanthemum* via denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA as well as DNA fragments coding for 16S rRNA. Appl Environ Microbiol 67:172–178

Dunbar J, Ticknor LO, Kuske CR (2000) Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. Appl Environ Microbiol 66:2943–2950

Dunfield KE, Germida JJ (2001) Diversity of bacterial communities in the rhizosphere and root interior of field-grown genetically modified *Brassica napus*. FEMS Microbiol Ecol 38:1–9

Düring K, Mahn A (1999) Freisetzung und Resistenzprüfung transgener Lysozym-Kartoffeln. In: Schiemann J (ed) Freisetzungsbegleitende Sicherheitsforschung mit gentechnisch veränderten Pflanzen und Mikroorganismen. Proceedings of the BMBF Workshop on Biological Safety, 25–26 May 1998. BEO, Braunschweig, pp 39–44

Escara JF, Hutton JR (1980) Thermal stability and renaturation of DNA in dimethyl sulfoxide solutions: acceleration of the renaturation rate. Biopolymers 19:1315–1327

Fægri A, Torsvik VL, Goksøyr J (1977) Bacterial and fungal activities in soil: separation of bacteria and fungi by a rapid fractionated centrifugation technique. Soil Biol Biochem 9:105–112

Fisher MM, Triplett EW (1999) Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. Appl Environ Microbiol 65:4630–4636

Gallori E, Bazzicalupo M, Dal Canto L, Fani R, Nannipieri P, Vettori C, Stotzky G (1994) Transformation of *Bacillus subtilis* by DNA bound on clay in non-sterile soil. FEMS Microbiol Ecol 15:119–126

Garbeva P, van Veen JA, van Elsas JD (2003) Predominant *Bacillus* spp. in agricultural soil under different management regimes via PCR-DGGE. Microb Ecol 45:302–316

Garland JL, Mills AL (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Appl Environ Microbiol 57:2351–2359

Gebhard F, Smalla K (1998) Transformation of *Acinetobacter* sp. strain BD413 by transgenic sugar beet DNA. Appl Environ Microbiol 64:1550–1554

Gebhard F, Smalla K (1999) Monitoring field releases of genetically modified sugar beets for persistence of transgenic plant DNA and horizontal gene transfer. FEMS Microbiol Ecol 28:261–272

Gelsomino A, Keijzer-Wolters A, Cacco G, van Elsas JD (1999) Assessment of bacterial community structure in soil by polymerase chain reaction and denaturing gradient gel electrophoresis. J Microbiol Methods 38:1–15

Giller KE, Beare MH, Lavelle P, Izac A-M, Swift MJ (1997) Agricultural intensification, soil biodiversity and agroecosystem function. Appl Soil Ecol 6:3–16

Gillespie DE, Brady SF, Bettermann AD, Cianciotto NP, Liles MR, Rondon MR, Clardy J, Goodman RM, Handelsman J (2002) Isolation of antibiotics Turbomycin A and B from a metagenomic library of soil microbial DNA. Appl Environ Microbiol 68:4301–4306

Gomes NCM, Heuer H, Schönfeld J, Costa R, Hagler-Mendonca L, Smalla K (2001) Bacterial diversity of the rhizosphere of maize (*Zea mays*) grown in tropical soil studied by temperature gradient gel electrophoresis. Plant Soil 232:167–180

Gomes NCM, Fagbola O, Costa R, Rumjanek NG, Buchner A, Mendonça-Hagler L, Smalla K (2003) Dynamics of fungal communities in bulk and maize rhizosphere soil in the tropics. Appl Environ Microbiol 69:3758–3766

Griffiths BS, Ritz K, Weatley RE (1997) Relationship between functional diversity and genetic diversity in complex microbial communities. In: Insam H, Rangger A (eds) Microbial communities, functional versus structural approaches. Springer, Berlin Heidelberg New York, pp 1–18

Griffiths BS, Ritz K, Bardgett RD, Cook R, Christensen S, Ekelund F, Sorensen SJ, Baath E, Bloem J, de Ruiter PC, Dolfing J, Nicolardot B (2000) Ecosystem response of pasture soil communities to fumigation-induced microbial diversity reductions: an examination of the biodiversity-ecosystem function relationship. Oikos 90:279–294

Gyamfi S, Pfeifer U, Stierschneider M, Sessitsch A (2002) Effects of transgenic glufosinate-tolerant oilseed rape (*Brassica napus*) and the associated herbicide application on eubacterial and *Pseudomonas* communities in the rhizosphere. FEMS Microbiol Ecol 41:181–190

Hadrys H, Balick M, Schierwater B (1992) Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. Mol Ecol 1:55–63

Hahn D, Amann RI, Ludwig W, Akkermans ADL, Schleifer KH (1992) Detection of microorganisms in soil after in situ hybridization with rRNA-targeted, fluorescently labelled oligonucleotides. J Gen Microbiol 138:879–887

Hall SJ, Gray SA, Hammett ZL (2000) Biodiversity-productivity relations: an experimental evaluation of mechanisms. Oecologia 122:545–555

Harris D (1994) Analyses of DNA extracted from microbial communities. In: Ritz K, Dighton J, Giller KE (eds) Beyond the biomass. British Society of Soil Science, Sayce, Wiley, Chichester, pp 111–118

Heuer H, Smalla K (1997a) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) for studying soil microbial communities. In: van Elsas JD, Wellington EMH, Trevors JT (eds) Modern soil microbiology. Dekker, New York, pp 353–373

Heuer H, Smalla K (1997b) Evaluation of community level catabolic profiling using BIOLOG GN microplates to study microbial community changes in potato phyllosphere. J Microbiol Methods 30:49–61

Heuer H, Hartung K, Wieland G, Kramer I, Smalla K (1999) Polynucleotide probes that target a hypervariable region of 16S rRNA genes to identify bacterial isolates corresponding to bands of community fingerprints. Appl Environ Microbiol 65:1045–1049

Heuer H, Kroppenstedt RM, Lottmann J, Berg G, Smalla K (2002a) Effects of T4-lysozyme release from transgenic potato roots on bacterial rhizosphere communities are negligible relative to natural factors. Appl Environ Microbiol 68:1325–1335

Heuer H, Krögerrecklenfort E, Egan S, van Overbeek LS, Guillaume G, Nikolakopoulou TL, Wellington EMH, van Elsas JD, Collard J-M, Karagouni AD, Smalla K (2002b) Gentamycin resistance genes in environmental bacteria: prevalence and transfer. FEMS Microbiol Ecol 42:289–302

Holben WE, Harris D (1995) DNA-based monitoring of total bacterial community structure in environmental samples. Mol Ecol 4:627–631

Horz HP, Yimga MT, Liesack W (2001) Detection of methanotroph diversity on roots of submerged rice plants by molecular retrieval of pmoA, mmoX, mxaF, and 16S rRNA and ribosomal DNA, including pmoA-based terminal restriction fragment length polymorphism profiling. Appl Environ Microbiol 67:4177–4185

Insam H (2001) Development in soil microbiology since mid 1960s. Geoderma 100:389–402

James C (2003) Global review of commercialized transgenic crops: 2002. ISAA Briefs 29:3–38

Jeffries P, Gianinazzi S, Perotto S, Turnau K, Barea J-M (2002) The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. Biol Fertil Soils

Johnsen K, Jacobsen CS, Torsvik V, Sørensen J (2001) Pesticide effects on bacterial diversity in agricultural soils—a review. Biol Fertil Soils 33:443–453

Keel C, Schnider U, Maurhofer M, Voisard C, Burger U, Haas D, Defago G (1992) Suppression of root diseases by *Pseudomonas fluorescens* CHA0: importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. Mol Plant Microbe Interact 5:4–13

Kowalchuk GA, Gerards S, Woldendorp JW (1997) Detection and characterization of fungal infections of *Ammophila arenaria* (Marran grass) roots by denaturing gradient gel electrophoresis of specifically amplified 18S rDNA. Appl Environ Microbiol 63:3858–3865

Kowalchuk GA, Bruinsma M, van Veen JA (2003) Assessing responses of soil microorganisms to GM plants. Trends Ecol Evol 18:403–410

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, Chichester, pp 115–175

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc Natl Acad Sci 82:6955–6959

Latour X, Philippot L, Corberand T, Lemanceau P (1996) The establishment of an introduced community of fluorescent pseudomonads in the soil and in the rhizosphere is affected by the soil type. FEMS Microbiol Ecol 30:163–170

Lee DH, Zo YG, Kim SJ (1996) Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single strand conformation polymorphism. Appl Environ Microbiol 62:3112–3120

Levy SB (1997) Antibiotic resistance: an ecological imbalance. In: Antibiotic resistance: origins, evolution, selection and spread, Ciba Foundation Symposium 207. Wiley, Chichester, pp 1–14

Liesack W, Stackebrandt E (1992) Occurrence of novel groups of the domain bacteria as revealed by analysis of genetic material isolated from an Australian terrestrial environment. J Bacteriol 174:5072–5078

Liu W-T, Marsh TL, Cheng H, Forney LJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl Environ Microbiol 63:4516–4522

Loreau M, Naeem S, Inchausti P, Bengtsson J, Grime JP, Hector A, Hooper DU, Huston DU, Raffaelli D, Schmid B, Tilman D, Wardle DA (2001) Biodiversity and ecosystem functioning: current knowledge and future challenges. Science 294:804–808

Lorenz MG, Wackernagel W (1994) Bacterial gene transfer by natural genetic transformation in the environment. Microbiol Rev 58:563–602

Lottmann J, Heuer H, Smalla K, Berg G (1999) Influence of transgenic T4-lysozyme-producing potato plants on potentially beneficial plant-associated bacteria. FEMS Microbiol Ecol 29:365–377

Lottmann, J, Heuer H, de Vries J, Mahn A, Düring K, Wackernagel W, Smalla K, Berg G (2000) Establishment of introduced antagonistic bacteria in the rhizosphere of transgenic potatoes and their effect on the bacterial community. FEMS Microbial Ecol 33:41–49

Loy A, Horn M, Wagner M (2003) probeBase—an online resource for rRNA-targeted oligonucleotide probes. Nucleic Acids Res 31:514–516

Lynch JM (2002a) Effect of living modified organisms on the soil. In: Roseland CR (ed) LMOs and the environment. United States Department of Agriculture, Md.

Lynch JM (2002b) Resilience of the rhizosphere to anthropogenic disturbance. Biodegradation 13:21–27

MacArthur RH (1955) Fluctuations of animal populations, and a measure of stability. Ecology 36:533–536

Madigan MT, Martinko JM, Parker J (2003) Brock biology of microorganisms, 10th edn. Pearson Education, Prentice Hall, Upper Saddle River, Toronto

Manz W, Amann R, Ludwig W, Wagner M, Schleifer K-H (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. Syst Appl Microbiol 15:593–600

Marsh TL, Saxman P, Cole J, Tiedje J (2000) Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. Appl Environ Microbiol 66:3616–3620

Massol-Deya AA, Odelson DA, Hickey RF, Tiedje JM (1995) Bacterial community fingerprinting of amplified 16S and 16-23S ribosomal DNA gene sequences and restriction endonuclease analysis (ARDRA). In: Akkermans ADL, van Elsas JD, deBruijn FJ (eds) Molecular microbial ecology manual. Kluwer, Dordrecht, pp 1–8

May RM (1973) Stability and complexity in model ecosystems. Princeton University Press, Princeton, N.J.

May RM (ed) (1976) Theoretical ecology, principles and applications. Blackwell, Oxford

McGrath SP, Chaudri AM, Giller KE (1995) Long-term effects of land application of sewage sludge: Soils, microorganisms, and plants. J Ind Microbiol 14:94–104

McNaughton SJ (1988) Diversity and stability. Nature 333:204–205

Muyzer G, Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek J Microbiol Serol 73:127–141

Muyzer G, de Waal ED, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700

Nagpal ML, Fox KF, Fox A (1998) Utility of 16S–23S rRNA spacer region methodology: how similar are interspace regions within a genome and between strains for closely related organisms? J Microbiol Methods 33:211–219

Nakatsu CH, Torsvik V, Øvreås L (2000) Soil community analysis using DGGE of 16S rDNA polymerase chain reaction products. Soil Sci Soc Am J 64:1382–1388

Nannipieri P, Ascher J, Ceccherini MT, Loretta L, Giacomo P, Giancarlo R (2003) Microbial diversity and soil functions. Eur J Soil Sci 54:655–670

Naseby DC, Lynch JM (2002) Enzymes and micro-organisms in the rhizosphere. In: Dick RP, Burns R (eds) Enzymes in the environment. Dekker, New York, pp 109–123

Nielsen KM, van Weerelt DM, Berg TN, Bones AM, Hagler AN, van Elsas JD (1997a) Natural transformation and availability of transforming chromosomal DNA to *Acinetobacter calcoaceticus* in soil microcosms. Appl Environ Microbiol 63:1945–1952

Nielsen KM, Bones AM, van Elsas JD (1997b) Induced natural transformation of *Acinetobacter calcoaceticus* in soil microcosms. Appl Environ Microbiol 63:3972–3977

Nielsen KM, Gebhard F, Smalla K, Bones AM, van Elsas JD (1997c) Evaluation of possible horizontal gene transfer from transgenic plants to the soil bacterium *Acinetobacter calcoaceticus* BD413. Theor Appl Genet 95:815–821

Nielsen KM, Bones AM, Smalla K, van Elsas JD (1998) Horizontal gene transfer from transgenic plants to terrestrial bacteria-a rare event? FEMS Microbiol Rev 22:79–103

Nielsen KM, Smalla K, van Elsas JD (2000a) Natural transformation of *Acinetobacter* sp. strain BD413 with cell lysates of *Acinetobacter* sp., *Pseudomonas fluorescens*, and *Burkholderia cepacia* in soil microcosms. Appl Environ Microbiol 66:206–212

Nielsen KM, van Elsas JD, Smalla K (2000b) Transformation of *Acinetobacter* sp. strain BD413 (pFG4Δ*nptII*) with transgenic plant DNA in soil microcosms and effects of kanamycin on selection of transformants. Appl Environ Microbiol 66:1237–1242

Nielsen KM, Choi M, Pietramellara G, Nannipieri P, Bensasson D (in press) Extracellular DNA: persistence in various environments and availability to bacteria. In: Schink B, Vincent W, Van Elsas JD (eds) Advances in microbial ecology. Kluwer, New York

Normander B, Prosser J (2000) Bacterial origin and community composition in the barley phytosphere as a function of habitat and presowing conditions. Appl Environ Microbiol 66:4372–4377

Nüsslein K, Tiedje JM (1998) Characterization of the dominant and rare members of a young Hawaian soil bacterial community with small-subunit ribosomal DNA amplified from DNA fractionated on the basis of its guanine and cytosine composition. Appl Environ Microbiol 64:1283–1289

Nuti MP (1994) Biosafety of genetically modified soil microbial inoculants. BRIDGE Programme, CEC ELWW/DGXII, Brussels, pp 1–11

Nuti MP, Sirsi E (2003) Science/legislation interface. In: Migheli Q, Ruiz Sanz JE (eds) Quality control and efficacy assessment of microbial inoculants, COST 830/E, Europlomas. European Commission, Brussels

Odum EP (1969) The strategy of ecosystem development. Science 164:262–270

Oger P, Mansouri H, Dessaux Y (2000) Effect of crop rotation and soil cover on the alteration of the soil microflora generated by the culture of transgenic plants producing opines. Mol Ecol 9:881–890

Ohtonen R, Aikio S, Vare H (1997) Ecological theories in soil biology. Soil Biol Biochem 29:1613–1619

Osborn AM, Moore ER, Timmis KN (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environ Microbiol 2:39–50

Øvreås L, Torsvik V (1998) Microbial diversity and community structure in two different agricultural soil communities. Microb Ecol 36:306–315

Øvreås L, Forney L, Daae FL, Torsvik V (1997) Distribution of bacterioplankton in meromictic Lake Sælenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl Environ Microbiol 63:3367–3373

Øvreås L, Jensen S, Daae FL, Torsvik V (1998) Microbial community changes in a perturbed agricultural soil investigated by molecular and physiological approaches. Appl Environ Microbiol 64:2739–2742

Paget E, Simonet P (1994) On the track of natural transformation in soil. FEMS Microbiol Ecol 15:109–118

Paget E, Simonet P (1997) Development of engineered genomic DNA to monitor the natural transformation of *Pseudomonas stutzeri* in soil-like microcosms. Can J Microbiol 43:78–84

Pernthaler A, Pernthaler J, Amann R (2002) Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. Appl Environ Microbiol 68:3094–3101

Perreten V, Schwarz F, Cresta L, Boeglin M, Dasen G, Teuber M (1997) Antibiotic resistance spread in food. Nature 389:801–802

Pukall R, Brambilla E, Stackebrandt E (1998) Automated fragment length analysis of fluorescently labeled 16S rDNA after digestion with 4-base cutting restriction enzymes. J Microbiol Methods 32:55–63

Ranjard L, Brothier E, Nazaret S (2000a) Sequencing bands of RISA fingerprints for the characterization and the microscale distribution of soil bacterial populations responding to mercury spiking. Appl Environ Microbiol 66:5334–5339

Ranjard L, Poly F, Nazaret S (2000b) Monitoring complex bacterial communities using culture-independent molecular techniques: application to soil environment. Res Microbiol 151:167–177

Ranjard L, Poly F, Lata J-C, Moguel C, Thioulouse J, Nazaret S (2001) Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: biological and methodological variability. Appl Environ Microbiol 67:4479–4487

Raskin L, Stromley JM, Rittmann BE, Stahl DA (1994) Group-specific 16S rRNA hybridization probes to describe natural communities of Methanogens. Appl Environ Microbiol 60:1232–1240

Resca R, Basaglia M, Poggiolini S, Vian P, Bardin S, Walsh UF, Enriquez Barreiros CM, O'Gara F, Nuti MP, Casella S, Peruch U (2001) An integrated approach for the evaluation of biological control of the complex *Polymyxa betae*/beet necrotic yellow vein virus, by means of seed inoculants. Plant Soil 232:215–226

Ritz K, Griffiths BS, Torsvik VL, Hendriksen NB (1997) Analysis of soil and bacterioplancton community DNA by melting profiles and reassociation kinetics. FEMS Microbiol Lett 149:151–156

Rondon MR, Raffel SJ, Goodman RM, Handelsman J (1999) Toward functional genomics in bacteria: analysis of gene expression in *Escherichia coli* from a bacterial artificial chromosome library of *Bacillus cereus*. Proc Natl Acad Sci USA 96:6451–6455

Rondon MR, August PR, Bettermann AD, Brady SF, Grossman TH, Liles MR, Loiacono KA, Lynch BA, MacNeil IA, Minor C, Tiong CL, Gilman M, Osburne MS, Clardy J, Handelsman J, Goodman RM (2000) Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Appl Environ Microbiol 66:2541–2547

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230:1350–1354

Salles JF, De Souza FA, van Elsas JD (2002). Molecular method to assess the diversity of *Burkholderia* species in environmental samples. Appl Environ Microbiol 68:1595–1603

Salyers A (1996) The real threat from antibiotics. Nature 384:304

Sandaa R-A, Enger Ø, Torsvik V (1999a) Abundance and diversity of Archaea in heavy-metal contaminated soils. Appl Environ Microbiol 65:3293–3297

Sandaa R-A, Torsvik V, Enger Ø, Daae FL, Castberg T, Hahn D (1999b) Analysis of bacterial communities in heavy metal-contaminated soils at different levels of resolution. FEMS Microbiol Ecol 30:237–251

Sandaa R-A, Torsvik V, Enger Ø (2001) Influence of long-term heavy-metal contamination on microbial communities in soil. Soil Biol Biochem 33:287–295

Saxena D, Stotzky G (2000) Insecticidal toxin from *Bacillus thuringensis* is released from roots of transgenic Bt corn in vitro and in situ. FEMS Microbial Ecol 33:35–39

Schlüter K, Fütter J, Potrykus I (1995) Horizontal gene transfer from a transgenic potato line to a bacterial pathogen (*Erwinia chrysanthemi*) occurs—if at all—at an extremely low frequency. Nat Biotechnol 13:94–98

Schmalenberger A, Tebbe CC (2002) Bacterial community composition in the rhizosphere of a transgenic, herbicide-resistant maize (*Zea mays*) and comparison to its non-transgenic cultivar *Bosphore*. FEMS Microbiol Ecol 40:29–37

Schwieger F, Tebbe CC (1998) A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. Appl Environ Microbiol 64:4870–4876

Schwieger F, Tebbe CC (2000) Effect of field inoculation with *Sinorhizobium meliloti* L33 on the composition of bacterial communities in rhizospheres of a target plant (*Medicago sativa*) and a non-target plant (*Chenopodium album*)-linking of 16S rRNA gene-based single-strand conformation polymorphism community profiles to the diversity of cultivated bacteria. Appl Environ Microbiol 66:3556–3565

Sessitsch A, Kan F-Y, Pfeifer U (2003) Diversity and community structure of culturable *Bacillus* spp. populations in the rhizosphere of transgenic potatoes expressing the lytic peptide cecropin B. Appl Soil Ecol 22:149–158

Shanahan P, O'Sullivan DJ, Simpson P, Gennor JD, O'Gara F (1992) Isolation of 2,^{4}-diocetylphloragtuinal from a fluourescent pseudomonal and investigation of physiological parameters influencing production. Appl Environ Microbiol 58:353–358

Shannon CE, Weaver W (1969) The mathematical theory of communication. University of Illinois Press, Urbana, Ill.

Sharma S, Piccolo A, Insam H. (1997) Different carbon source utilization profiles from four tropical soils of Ethiopia. In: Insam H, Rangger A (eds) Microbial communities. Functional versus structural approaches. Springer, Berlin Heidelberg New York, pp 132-139

Siciliano SD, Germida JJ (1999) Taxonomic diversity of bacteria associated with the roots of field-grown transgenic *Brassica napus* cv. Quest, compared to the non-transgenic *B. napus* cv. Excel and *B. rapa* cv. Parkland. FEMS Microbiol Ecol 29:263–272

Sikorski J, Graupner S, Lorenz MG, Wackernagel W (1998) Natural transformation of *Pseudomonas stutzeri* in a non-sterile soil. Microbiology 144:569–576

Smalla K, van Overbeek LS, Pukall R, van Elsas JD (1993) Prevalence of *npt*II and Tn*5* in kanamycin resistant bacteria from different environments. FEMS Microbiol Ecol 13:47–58

Smalla K, Wachtendorf U, Heuer H, Liu W-T, Forney L (1998) Analysis of BIOLOG GN substrate utilization patterns by microbial communities. Appl Environ Microbiol 64:1220–1225

Smalla K, Heuer H, Götz A, Niemeyer D, Krögerrecklenfort E, Tietze E (2000) Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of IncQ-like plasmids. Appl Environ Microbiol 66:4854–4864

Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heuer H, Berg G (2001) Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. Appl Environ Microbiol 67:4742–4751

Smit E, Leeflang P, Wernars K (1997) Detection of shifts in microbial community structure and diversity in soil caused by copper contamination using amplified ribosomal DNA restriction analysis. FEMS Microbiol Ecol 23:249–261

Stach JE, Bathe S, Clapp JP, Burns RG (2001) PCR-SSCP comparison of 16S rDNA sequence diversity in soil DNA obtained using different isolation and purification methods. FEMS Microbiol Ecol 36:139–151

Stahl DA, Amann R (1991) Development and application of nucleic acid probes. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, Chichester, pp 205–248

Staley JT, Konopka A (1985) Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu Rev Microbiol 39:321–346

Stuart GJ, Carlson CA (1986) The biology of natural transformation. Annu Rev Microbiol 40:211–235

Suzuki MT, Giovannoni SJ (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl Environ Microbiol 62:625–630

Thomas CM, Smalla K (2000) Trawling the horizontal gene pool. Microbiol Today 27:24–27

Thomashow LS, Weller DM (1995) Current concepts in the use of introduced bacteria for biological disease control: mechanisms and antifungal metabolites. In: Stacey G, Keen N (eds) Plant–microbe interactions, vol 1. Chapman & Hall, New York, pp 187–235

Thorseth IH, Torsvik T, Torsvik VL, Daae FL, Pedersen R-B, Keldysh -98 Scientific party (2001) Diversity of life in ocean floor basalt. Earth Planet Sci Lett 194:31–37

Tiedje JM, Cho JC, Murray A, Treves D, Xia B, Zhou J (2001) Soil teeming with life: new frontiers for soil science. In: Rees RM, Ball BC, Campbell CD, Watson CA (eds) Sustainable management of soil organic matter. CAB, Wallingford, pp 393–412

Tilman D (1982) Resource competition and community structure. Princeton University Press, Princeton, N.J.

Torsvik VL (1980) Isolation of bacterial DNA from soil. Soil Biol Biochem 12:15–22

Torsvik V (1995) Cell extraction method. In: Akkermans ADL, van Elsas JD, de Bruijn FJ (eds) Molecular microbial ecology manual. Kluwer, Dordrecht, pp 1–15

Torsvik V, Goksøyr J, Daae FL (1990) High diversity in DNA of soil bacteria. Appl Environ Microbiol 56:782–787

Torsvik V, Daae FL, Goksøyr J (1995) Extraction, purification, and analysis of DNA from soil bacteria. In: Trevors JT, van Elsas JD (eds) Nucleic acids in the environment: methods and applications. Springer, Berlin Heidelberg New York, pp 29–48

Torsvik V, Sørheim R, Goksoyr J (1996) Total bacterial diversity in soil and sediment communities—a review. J Ind Microbiol 17:170–178

Torsvik V, Daae FL, Sandaa R-A, Øvreås L (2000) Molecular biology and genetic diversity of microorganisms. In: Seckbach J (ed) Journey to diverse microbial worlds. Kluwer, Dordrecht, pp 43–57

Torsvik V, Øvreås L, Thingstad TF (2002) Prokaryotic diversity-magnitude, dynamics and controlling factors. Science 296:1064–1066

Tschäpe H (1994) The spread of plasmids as a function of bacterial adaptability. FEMS Microbiol Ecol 15:23–32

Vainio EJ, Hantula J (2000) Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. Mycol Res 104:927–936

Van Elsas JD, Smalla K (1996) Methods for sampling soil microbes. In: Hurst CJ, Knudsen GR, McInerney MJ, Stetzenbach LD, Walter MV (eds) Manual of environmental microbiology. ASM, Washington, D.C., pp 383–390

Van Elsas JD, Smalla K, Tebbe CC (2000) Extraction and analysis of microbial community nucleic acids from environmental matrices. In: Jansson JK, van Elsas JD, Bailey MJ (eds) Tracking genetically-engineered microorganisms. Eurekah, Austin, Tex., pp 29–51

Van Veen JA, Van Overbeck LS, Van Elsas JD (1997) Fate and activity of microorganisms into soil. Mol Biol Rev 61:121–133

Viebahn M, Glandorf DCM, Ouwens TWM, Smit E, Leeflang P, Wernars K, Thomashow LS, van Loon LC, Bakker PAHM (2003) Repeated introduction of genetically modified *Pseudomonas putida* WCS358r without intensified effects on the indigenous microflora of field-grown wheat. Appl Environ Microbiol 69:3110–3118

Wagner M, Horn M, Daims H (2003) Fluorescence in situ hybridisation for the identification and characterisation of prokaryotes. Curr Opin Microbiol 6:302–309

Wardle DA, Giller KE (1996) The quest for a contemporary ecological dimension to soil biology. Soil Biol Biochem 28:1549–1554

Westover KM, Kennedy AC, Kelleys SE (1997) Patterns of rhizosphere microbial community structure associated with co-occurring plant species. J Ecol 85:863–873

Whittaker RH (1972) Evolution and measurement of species diversity. Taxon 21:213–251

Widmer F, Seidler RJ, Watrud LS (1996) Sensitive detection of transgenic plant marker gene persistence in soil microcosms. Mol Ecol 5:603–613

Widmer F, Seidler RJ, Donegan KK, Reed GL (1997) Quantification of transgenic plant marker gene persistence in the field. Mol Ecol 6:1–7

Wintzingerode FV, Gobel UB, Stackebrandt E (1997) Determination of microbial diversity in environmental samples: pit falls of PCR-based rRNA analysis. FEMS Microbiol Rev 21:213–229

Witte W (1998) Medical consequences of antibiotic use in agriculture. Science 279:996–997

Woese CR (1998) Default taxonomy: Ernst Mayr's view of the microbial world. Proc Natl Acad Sci 95:11043–11046

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc Natl Acad Sci 87:4576–4579

Yachi S, Loreau M (1999) Biodiversity and ecosystem productivity in a fluctuating environment: the insurance hypothesis. Proc Natl Acad Sci 96:1463–1468

Yan A, McBratney B, Copeland L (2000) Functional substrate biodiversity of cultivated and uncultivated A horizons of vertisols in NW New South Wales. Geoderma 96:321–343

Zak JC, Willig MR, Moorhead DL, Wildman HG (1994) Functional diversity of microbial communities: a quantitative approach. Soil Biol Biochem 26:1101–1108

Zichichi A (1993) Scienza ed Emergenze Planetarie. Rizzoli, Rome