Microbial diversity and soil functions

P. Nannipieri, J. Ascher, M. T. Ceccherini, L. Landi, G. Pietramellara & G. Renella

Dipartimento della Scienza del Suolo e Nutrizione della Pianta, Università degli Studi di Firenze, 50144 Firenze, Italy

Summary

Soil is a complex and dynamic biological system, and still in 2003 it is difficult to determine the composition of microbial communities in soil. We are also limited in the determination of microbially mediated reactions because present assays for determining the overall rate of entire metabolic processes (such as respiration) or specific enzyme activities (such as urease, protease and phosphomonoesterase activity) do not allow any identification of the microbial species directly involved in the measured processes. The central problem posed by the link between microbial diversity and soil function is to understand the relations between genetic diversity and community structure and between community structure and function. A better understanding of the relations between microbial diversity and soil functions requires not only the use of more accurate assays for taxonomically and functionally characterizing DNA and RNA extracted from soil, but also high-resolution techniques with which to detect inactive and active microbial cells in the soil matrix.

Soil seems to be characterized by a redundancy of functions; for example, no relationship has been shown to exist between microbial diversity and decomposition of organic matter. Generally, a reduction in any group of species has little effect on overall processes in soil because other microorganisms can take on its function.

The determination of the composition of microbial communities in soil is not necessary for a better quantification of nutrient transformations. The holistic approach, based on the division of the systems in pools and the measurement of fluxes linking these pools, is the most efficient. The determination of microbial C, N, P and S contents by fumigation techniques has allowed a better quantification of nutrient dynamics in soil. However, further advances require determining new pools, such as active microbial biomass, also with molecular techniques. Recently investigators have separated ^{13}C- and ^{12}C-DNA, both extracted from soil treated with a ^{13}C source, by density-gradient centrifugation. This technique should allow us to calculate the active microbial C pool by multiplying the ratio between labelled and total DNA by the microbial biomass C content of soil. In addition, the taxonomic and functional characterization of ^{13}C-DNA allows us to understand more precisely the changes in the composition of microbial communities affected by the C-substrate added to soil.

Introduction

At present there is a particular interest in the relation between biodiversity, simply defined as the number of species present in the system, and function in the soil. This is part of a more general concern to conserve biodiversity and its role in maintaining a functional biosphere. The tacit assumptions in many current studies are that (i) by characterizing diversity one will be able to understand and manipulate the working of ecosystems and (ii) the ability of an ecosystem to withstand serious disturbances may depend in part on the diversity of the system. The importance of biodiversity in the functionality of ecosys-

tems was stressed by Agenda 21, a document from the United Nations Conference on Environment and Development, prepared in Rio de Janeiro in 1992. The document promoted scientific and international cooperation for a better understanding of the importance of biodiversity and its functions in ecosystems. There is now a growing body of experimental evidence that most organisms are functionally redundant and that the functional characteristics of component species are at least as important as the number of species *per se* for maintaining essential processes (Andren & Balandreau, 1999; Bardgett & Shine, 1999). We believe that at least some minimum number of species is essential for ecosystem functioning under steady conditions and that a large number of species is probably essential for maintaining stable processes in changing

environments, the so-called 'insurance hypothesis' (Loreau *et al.*, 2001). However, our theories on terrestrial ecosystems have been developed from above-ground observations, whereas comparatively few studies have been made in soil (Wardle & Giller, 1996; Ohtonen *et al.*, 1997; Griffiths *et al.*, 2000). The links between biodiversity and soil functioning are therefore poorly understood.

Soil is fundamental and irreplaceable; it governs plant productivity of terrestrial ecosystems and it maintains biogeochemical cycles because microorganisms in the soil degrade, sooner or later, virtually all organic compounds including persistent xenobiotics and naturally occurring polyphenolic compounds. The living population inhabiting soil includes macrofauna, mesofauna, microfauna and microflora. In this brief review we focus on the relationship between microbial diversity and soil functionality, by considering that 80–90% of the processes in soil are reactions mediated by microbes (Coleman & Crossley, 1996; Nannipieri & Badalucco, 2003). Indeed, bacteria and fungi are highly versatile; they can carry out almost all known biological reactions. To provide a comprehensive view of the complex relations between microbial diversity and soil functionality we consider:

1 the complexity of soil as a biological system;

2 the problems in measuring microbial diversity and microbial functions in soil and the meaning of these measurements;

3 current ideas concerning the link between microbial diversity and soil functions;

4 instances when measurements of microbial diversity are unnecessary for a better understanding of soil functionality; and

5 the research needed for a better evaluation and manipulation of microbial diversity and soil functionality.

Soil as a microhabitat

Progress in testing contemporary ecological hypotheses in soil has been limited by the difficulty of accurately measuring species richness and evenness and by the lack of information about the species really involved in the measured microbial activities. Soil is a complex microhabitat (Figure 1) for the following distinctive properties (Nannipieri & Badalucco, 2003).

1 The microbial population in soil is very diverse. Torsvik *et al.* (1996) calculated the presence of about 6000 different bacterial genomes per gram of soil by taking the genome size of *Escherichia coli* as a unit. Microbial biomass is large: in a temperate grassland soil the bacterial and fungal biomass amounted to 1–2 and 2–5 t ha^{-1}, respectively (Killham, 1994).

2 Soil is a structured, heterogeneous and discontinuous system, generally poor in nutrients and energy sources (in comparison with the concentrations optimal for nutrient microbial growth *in vitro*), with microorganisms living in discrete microhabitats (Stotzky, 1997). The chemical, physical and biological characteristics of these microhabitats differ in both time and space. Scales of the habitats depend mainly on

the size of the organism: a few μm for bacteria; less than 100 μm for fungi; between 100 μm and 2 mm for Acari and Collembola; between 2 and 20 mm for Isopoda (Coleman & Crossley, 1996). Even if the available space is extensive in soil, the biological space, that is, the space occupied by living microorganisms, represents a small proportion, generally less than 5% of the overall available space (Ingham *et al.*, 1985). Another peculiarity is the presence of 'hot spots', zones of increased biological activity, such as aggregates with different physicochemical properties from the bulk of the soil (Sexstone *et al.*, 1985), zones with accumulated particulate organic matter (Parkin, 1987) or animal manures (Petersen *et al.*, 1996), and the rhizosphere (Lynch, 1990; Pinton *et al.*, 2001). Indeed, only a few microhabitats have the right set of conditions to allow microbial life. Several environmental factors, such as carbon and energy sources, mineral nutrients, growth factors, ionic composition, available water, temperature, pressure, air composition, electromagnetic radiation, pH, oxidation–reduction potential, surfaces, spatial relationships, genetics of the microorganisms and interaction between microorganisms, can affect the ecology, activity and population dynamics of microorganisms in soil. These environmental factors can change markedly, and so microhabitats in soil are dynamic systems.

According to Hattori (1973), almost 80–90% of the microorganisms inhabiting soil are on solid surfaces. Mechanisms by which these microorganisms interact with soil surfaces have been much studied. Chen (1998) and Huang & Bollag (1998) showed that some bacterial cells produce extracellular polysaccharides interacting with clay particles and that these clay–polysaccharide complexes can persist even after the death of the microbes. The use of traditional and more recent (e.g. confocal laser scanning, use of microorganisms with reporter genes) electron microscopy techniques with staining procedures has allowed us to locate the microbial groups, and inorganic and organic colloids in the soil matrix (Forster, 1994; Assmus *et al.*, 1995, 1997; Bakken, 1997). All microorganisms are aquatic and they live free or attached to surfaces, in water films surrounding solid particles, and inside aggregates (Stotzky, 1997).

3 Another distinctive characteristic of soil as a microhabitat is the property of the solid phase to adsorb important biological molecules such as proteins and nucleic acids. In this way some extracellular enzymes adsorbed by clay minerals or entrapped by humic molecules can maintain their activity, being protected against proteolysis, and thermal and pH denaturation (Nannipieri *et al.*, 1990, 2002). Deoxyribonucleic acid (DNA) molecules adsorbed or bound to humic molecules, clay and sand particles are protected against degradation by nucleases, but they can still transform competent bacterial cells, a process through which one of the two strands of extracellular DNA is taken up by the bacterial cell and inserted into its chromosomal DNA (Lorenz & Wackernagel, 1987; Khanna & Stotzky, 1992; Paget *et al.*, 1992; Pietramellara *et al.*, 1997). In such a way the competent bacterial cell acquires all or part of the genes associated with the extracellular DNA.
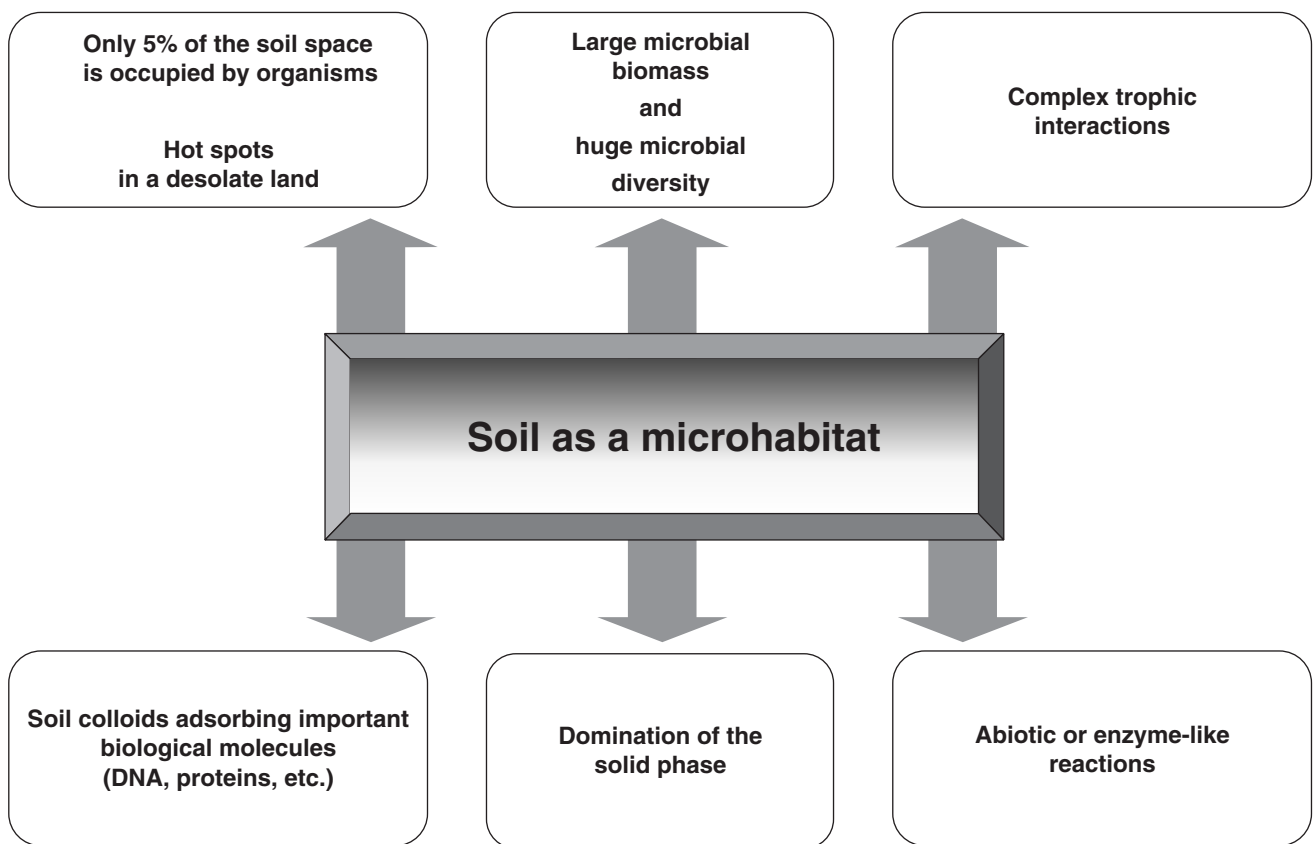
Figure 1 Schematic representation of the main characteristics of soil as a microhabitat.

The adsorption of organic compounds by soil colloids retards their microbial degradation; the location of potential substrates inside pores or microaggregates reduces their accessibility to soil microorganisms (Ladd *et al.*, 1996).

4 The surfaces of soil mineral components can themselves catalyse many reactions. Clay minerals and Mn(III and IV) and Fe(III) oxides catalyse electron transfer reactions, such as the oxidation of phenols and polyphenols with formation of humic substances (Huang, 1990; Ruggiero *et al.*, 1996). Other abiotic reactions catalysed by soil minerals include deamination, polymerization, polycondensation and ring cleavage. We think that microbe-mediated reactions prevail under natural conditions, whereas abiotic reactions prevail under harsh conditions and diminish microbial activity. These hypotheses cannot be verified because we have no accurate methods for determining the contribution of abiotic reactions. Huang (1990) and Ruggiero *et al.* (1996) suggested that abiotic transformations prevail under conditions hostile to microbial activity in soil.

Definition and measurement of microbial diversity

Microbial diversity is a general term used to include genetic diversity, that is, the amount and distribution of genetic information, within microbial species; diversity of bacterial and fungal species in microbial communities; and ecological diversity, that is, variation in community structure, complexity of interactions, number of trophic levels, and number of guilds. Here we consider microbial diversity simply to include the number of different fungal and bacterial species (richness) and their relative abundance (evenness) in soil microflora. Equations used to calculate species richness and evenness and diversity indices, which combine both richness and evenness, have been discussed by Kennedy & Smith (1998).

Microbial diversity is measured by various techniques such as traditional plate counting and direct counts as well as the newer molecular-based procedures and fatty acid analysis.

Plate and direct counts

In the past microbial diversity has been quantified by counting techniques such as the plate count technique or the Most Probable Number (MPN) technique (Bakken, 1997; Johnsen *et al.*, 2001). Thus investigators have followed changes in the number of specific taxonomic or functional groups by plating on agar media, and have assessed precisely the culturable diversity by isolating colonies from these media followed by identification by a variety of typing methods (Vandamme *et al.*, 1996). They have assessed the gross diversity of culturable microorganisms by plotting the different colonies identified on

the medium against the incubation time (Ishikuri & Hattori, 1985). However, the approach of determining microbial diversity from the number of isolates has become less popular, not only because a limited number of microorganisms can be cultured but also because the procedures are laborious. As a consequence, an increasing number of molecular techniques are now popular because they do not rely on isolation–cultivation microorganisms (Johnsen *et al.*, 2001). It is well established that plate counts estimate only 1–10% of the overall soil microflora. The discrepancy is essentially due to the interdependency of different organisms upon each other (for example, the endosymbiotic bacteria in specific worms and molluscs), to the inability to create in pure culture the environmental conditions faced by microorganisms in the soil environment, and to the fact that some microbial species are cultivable only under certain physiological conditions (Bakken, 1997; Muyzer & Smalla, 1998; Heuer *et al.*, 2001). Generally the choice of the growing medium markedly affects the colony formed (Sørheim *et al.*, 1989; Johnsen & Nielsen, 1999). Small bacteria cells (dwarf cells or ultramicrobacteria) are not culturable, that is, they cannot form colonies in agar. By considering that larger cells are supposed to account for about 80% of the bacterial volume, Bakken (1997) hypothesized that the culturable bacteria have an ecological significance in soil more important than that which appears from their small numbers.

Direct counting by fluorescence microscopy can give 100–1000 times more than the numbers obtained by plate counting (Johnsen *et al.*, 2001). Several stains specific to proteins or nucleic acids have been used; they include fluorescein isothiocyanate (FITC), acridine orange (AO), ethidium bromide (EB) and europium chelate with a fluorescent brightener differential stain (DFS) for bacteria. Phenol aniline blue (PAB) has been used to stain hyphae in agar films and on membrane filters, whereas metabolically active hyphae have been counted after their staining with fluorescent diacetate (FDA) (Bloem *et al.*, 1995). Stains for active bacterial cells include FDA or redox probes such as 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) or 5-cyano-2,3-ditoyl tetrazolium chloride (CTC). Bloem *et al.* (1995) improved the direct counting method with a video camera on an epifluorescence microscope. However, this procedure does not allow counting specific microbial species, and some of the stains used do not discriminate between living and dead microbial cells.

Molecular techniques

The molecular techniques generally involve extraction of nucleic acid, directly or indirectly, from soil. They are independent of culture, and according to their sensitivity can detect species, genera, families or even higher taxonomic groups.

Low-resolution methods include the analysis of base distribution (mole percentage guanine + cytosine) of DNA and the determination of rates at which denatured single-stranded DNA reassociates when temperature is lowered to approximately 25°C below the DNA melting point (Torsvik *et al.*, 1994, 1996). These techniques give an overall indication of microbial diversity and can be used to monitor overall changes in the composition of a microbial community's composition after stress or changes in management.

High-resolution analyses allow the detection of microbial strains at the species and subspecies level. They usually give 'fingerprints' of non-coding DNA regions or involve the sequencing of both coding and non-coding regions. These techniques include rep-polymerase chain reaction (PCR) amplification of sequences between repetitive elements, ribosomal inter space analysis (RISA), which is based on the length polymorphism of the spacer region between 16S and 23S rRNA genes (Borneman & Triplett, 1997), and random amplified polymorphic DNA (RAPD), which does not require a preliminary knowledge of the genome (Nei & Li, 1985; Harry *et al.*, 2001). In RISA, PCR products are separated by gel electrophoresis, and the separated bands can be sequenced. A limit of this technique is the number of spacer sequences in the database. Recently, Borneman (1999) monitored microorganisms responding to nutrient addition to soil with the thymidine analogue bromodeoxyuridine. Bromodeoxyuridine-labelled DNA extracted from soil DNA by immunocapture was subjected to RISA analysis.

The latest developments include the creation of bacterial artificial chromosomes (BAC) and the application of DNA microarrays (Rondon *et al.*, 2000; Tiedje *et al.*, 2001). The former technique clones high-molecular-weight soil DNA (metagenome) which can be analysed at a phylogenetic and functional level. Microarrays of DNA are powerful for rapidly characterizing the composition and functions of microbial communities because a single array can contain 500–1000 different DNA spots with the possibility of a very broad hybridization with a broad identification capacity (Tiedje *et al.*, 2001).

Most of the molecular methods have intermediate resolution, because they allow the detection of microbial groups rather than microbial species. Usually these techniques give fingerprints that allow multiple sample analysis to study spatial and temporal variation in the composition of soil microflora (Muyzer & Smalla, 1998). Profiles of multiple replicates can be run next to each other, and thus allow easy detection of differences in band profiles. These techniques are characterized by 16S-rRNA PCR. Bacterial ribosomal DNA contains both conserved and variable regions; as sequencing of these genes has been carried out frequently in microbial ecology, there is a considerable database of known sequences. A successful analysis depends on the efficient DNA extraction from soil followed by a purification step. The DNA can be extracted from soil by direct methods of *in situ* lysis or by indirect methods of initial cell extraction before lysis. In both cases, the methods used often include various combinations of bead beating, detergents, enzymatic lysis, and solvent extraction to

obtain crude preparation of nucleic acids (Frostegård *et al.*, 1999; Krsek & Wellington, 1999). Care is needed in interpreting the composition of microbial communities by molecular techniques because the method of extraction can influence patterns obtained by amplified ribosomal DNA restriction analysis (ARDRA) or RISA (Martin-Laurent *et al.*, 2001). Usually, an efficient extracting solution also solubilizes humic molecules, which inhibit the PCR, and thus it is followed by long procedures of purification (Krsek & Wellington, 1999; Martin-Laurent *et al.*, 2001). Another problem when extracting DNA from soil is the lysis of bacterial cells. Many Gram-positive bacteria require strong procedures to be lysed (Head *et al.*, 1998). However, strong extractants should be avoided because they degrade DNA molecules to fragments below 1 kb, and short DNA fragments may lead to the generation of chimeric 16S rRNA after amplification (Liesack *et al.*, 1991).

Among the intermediate-resolution techniques, denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE) are the most used for characterizing bacterial communities in environments such as hydrothermal vents, hot springs, activated sludge, phyllosphere, biodegraded wall paintings, and soil (Heuer *et al.*, 2001). They are rapid and simple (Muyzer & Smalla, 1998; Heuer *et al.*, 2001). The electrophoretic mobility of DNA fragments (obtained after PCR amplification) in polyacrylamide gels within a linear denaturant gradient (due to chemicals, DGGE, or temperature, TGGE) depends on the base composition of the DNA (Muyzer & Smalla, 1998; Heuer *et al.*, 2001). These techniques allow us to differentiate two molecules differing at the level of a single base (Muyzer & Smalla, 1998). In addition to the simplicity and rapidity, another advantage of DGGE or TGGE is that the identity of bands can be investigated by hybridization with specific probes or by extraction and sequencing (Muyzer & Smalla, 1998).

However, we emphasize that all techniques based on PCR, such as DGGE and TGGE, have several drawbacks (von Wintzingerode *et al.*, 1997). Amplification of PCR can be inhibited if contaminants are not removed by the purification process, and the preferential or selective amplification in the presence of DNA from mixed communities can occur. Another bias of these techniques is the production of chimeric or heteroduplex DNA molecules. Many of these problems can be avoided if (i) all DNA molecules are equally accessible to primer hybridization and form primer–template hybrids with equal efficiencies, (ii) in all templates there is the same extension efficiency of DNA polymerase, and (iii) all templates are equally affected by the exhaustion of substrates (von Wintzingerode *et al.*, 1997).

In addition to the drawbacks of each PCR-based technique, DGGE and TGGE fingerprints present other biases (Table 1). Only the dominant populations are revealed, and bands from more than one species may be hidden behind a single band, resulting in an underestimation of the bacterial diversity (Heuer *et al.*, 2001). Finally, the same isolate can have different bands because multiple copies of operon in a single species are there (Heuer *et al.*, 2001). In addition, we must keep in mind that the fingerprinting depends on the primer used. Heuer *et al.* (2001) reported that 14 different regions (A, B, C, D, E, F, V1–V3 and V5–V9) of the 16S rDNA have been used to generate fingerprints of bacterial communities. Usually V6 is the best one for optimal top-to-bottom analysis of bacterial communities of soil. According to Gomes *et al.* (2001), one can distinguish the different phylogenetic groups of bacteria with the following primers: F984GC, F27, R1378 and R1494 (bacteria), F243HGC (Actinomycetales), F203α (α-proteobacteria) and F948β (β-proteobacteria).

The assessment of fungal diversity in soil by molecular techniques has not been as successful as the characterization of bacterial diversity because the concentration of fungal DNA is much less than that of bacterial DNA (Borneman & Hartin, 2000). Another problem has been the use of specific primers without co-amplification of DNA from other eukaryotic organisms such as plants, algae and nematodes (Kowalchuk

Table 1 Disadvantages of denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE) (from Muyzer & Smalla, 1998 and Heuer *et al.*, 2001)

Problem	Cause
Different fingerprints can be generated from the same DNA mixture	The available primers cover different regions of the 16S rDNA.
One single band might not correspond to a single bacterial species	**1** Bands from more than one species might be hidden behind one band. **2** DNA fragments of different species might have similar electrophoretic mobility. **3** A single species might have different operons coding for 16S rDNA with sequence heterogeneity.
Dominant population representing at least 1% of the total community can be detected	This is the sensitivity limit of the technique in the fingerprint.

et al., 1997). We can now use DGGE and TGGE to generate fingerprints for the fungal community of soil because specific primers are available for fungal 18S rRNA (Smit *et al.*, 1999; Borneman & Hartin, 2000; van Elsas *et al.*, 2000).

Phospholipid fatty acid (PLFA) analysis

Another approach used to overcome the problem of selective culturing for assessing the composition of soil microflora is phospholipid fatty acid (PLFA) analysis (Tunlid & White, 1992; Frostegård & Bååth, 1996; Bossio & Scow, 1998; Zelles, 1999; Pankhurst *et al.*, 2001). This technique is based on the extraction, fractionation, methylation and chromatography of the phospholipid component of soil lipids. Phospholipids are thought to be related to the viable component of soil microflora because they are present as important components of membranes of living cells and break down rapidly when the cells die, and they cannot survive long enough to interact with soil colloids (Zelles, 1999). In addition, they have another prerequisite of a biomarker: they make up a fairly constant proportion of the biomass of organisms. Changes in the phospholipid profiles are generally related to variation in the abundance of microbial groups and can be interpreted by reference to a database of pure cultures and known biosynthetic pathways (Zelles, 1999). Direct extraction of PLFAs or whole fatty acids (WSFAMEs) from soil does not permit detection at the species level and can be used to estimate only gross changes in community structure. However, identification of species by fatty acid analysis is possible with standard cultural-based media and databases (MIDI, Newark, Delaware). Lechevalier (1989) and Zelles (1999) have listed many fatty acids isolated from specific microbial groups. Bacterial biomass can be calculated by summing up several fatty acids (15:0, a15:0, i15:0, i16:0, i17:0, cy17:0, cy19:0 and 16:1ω7c), whereas fungal biomass can be obtained from the 18:2ω6c fatty acid (Frostegård & Bååth, 1996).

Microbial diversity in soil

Communities of plants and animals generally compete because the majority of species are rare and a few species are abundant (Loreau *et al.*, 2001). In these communities the driving factor is the competitive interactions among species. By considering the result of a small subunit rRNA gene-based approach carried out by Zhou *et al.* (1997), Tiedje *et al.* (2001) suggested that competition in microbial communities of surface soils with prevalence of any microbial species was absent because the various microbial species inhabiting soil are spatially separated for most of the time. They assumed that the contact among microhabitats lasts for a very short time immediately after rain, when water bridges are formed between the various soil particles and aggregates. Rapid drainage maintains the spatial isolation among the various microhabitats of soil. This hypothesis is confirmed by the fact that the saturation of soil with water resulted in a predominance of one or a few species (Tiedje *et al.*, 2001). However, it does not take into account the mixing and transport by soil fauna and the stability of communities in biofilms at the interface between roots and soil which are not so strongly affected by wetting and drying. An alternative hypothesis to explain the large microbial diversity of surface soil is based on the presence of a greater variety and content of organic compounds there than deeper (Tiedje *et al.*, 2001). This presence would be responsible for the diverse heterotroph-dominated microbial community in surface soil. However, microbial diversity of soil from preferential flow paths (cracks, fissures, biopores such as earthworm burrows or root channels) was similar to that of the bulk soil (Bundt *et al.*, 2001), in spite of the fact that the former sample showed a greater concentration of organic C and organic N and greater microbial biomass values than the latter sample.

Plants exert a strong influence on the composition of microbial communities in soil through rhizodeposition and the decay of litter and roots. The link between plant species and microbial communities in the rhizosphere soil is strict, being the result of co-evolution (Brimecombe *et al.*, 2001).

An interesting question about microbial diversity in soil is: are microbial species inhabiting soil ubiquitous or native to particular places only? If microorganisms are cosmopolitan and no differences exist among different soil types all over the Earth then we may assume that microorganisms can be dispersed everywhere by wind, water currents, birds and human activity. On the other hand, if microbial species have a restricted distribution, that is, are geographically unique, the microbial diversity in soil must be very large globally (Tiedje *et al.*, 2001). To find out Tiedje *et al.* (2001) took samples from the 5–10 cm soil depth (and not from the surface layer so as to avoid recent additions to the surface) from many sites worldwide. They included soil from parks and nature reserves, so as to minimize human effects, from Mediterranean and boreal ecosystems in southwest Australia, southwest South Africa, central Chile and central California, northern Saskatchewan and northern Russia. The DNA of the fluorescent *Pseudomonas*, isolated from all soil samples, was analysed by ARDRA, intergenic transcribed spacer fragment length polymorphism (ITS-RFLP) and rep-PCR genomic fingerprinting (Tiedje *et al.*, 2001). The third analysis, having the finest resolution, showed that genotypes were peculiar to each sampled site of the same continent region (Tiedje *et al.*, 2001). The same genotypes were found in a 200-m transect, and a positive and significant relationship occurred between genetic distance and geographic distance.

Microbial and biochemical functions in soil

Microbial and biochemical characteristics are used as potential indicators of soil quality, even if soil quality depends on

a complex of physical, chemical and biological properties (Kennedy & Papendick, 1995). The rationale for the use of microbial and biochemical characteristics as soil quality indicators is their central role in cycling of C and N and their sensitivity to change (Nannipieri *et al.*, 1990).

Microbial activity is a term used to indicate the vast range of activities carried out by microorganisms in soil, whereas biological activity reflects not only microbial activities but also the activities of other organisms in the soil, including plant roots (Nannipieri *et al.*, 1990). Although the two terms are conceptually different they are confused. Various methods have been used to determine microbiological activity (Table 2). Some of them measure the rate of entire metabolic processes; for example, the evolution of CO_2 reflects the catabolic degradation under aerobic conditions; nitrification activity represents the rate of ammonia oxidation to nitrate; thymidine incorporation represents the rate of DNA synthesis in bacteria; and dehydrogenase activity represents the intracellular flux of electrons to O_2 and is due to the activity of several intracellular enzymes catalysing the transfer of hydrogen and electrons from one compound to another (Nannipieri *et al.*, 1990). As in other assays in soil biochemistry, dehydrogenase assays are based on the use of synthetic electron acceptors, which are less effective than O_2 (Nannipieri *et al.*, 1990). Another established method, the hydrolysis of fluorescein diacetate (FDA), a colourless compound, to fluorescein which is coloured (Schnurer & Rosswall, 1982; Adam & Duncan, 2001), is also a measurement of the contribution of several enzymes such as non-specific esterases, proteases and lipases, all of which are involved in the decomposition of organic matter in soil. Since more than 90% of the energy flow in a soil system passes through microbial decomposers, and since heterotrophic microorganisms are predominant in soil, FDA hydrolysis is thought to reflect overall soil microbiological activity. However, we must interpret the FDA data cautiously because the measured enzyme activities depend on the contribution of both extracellular and intracellular enzyme activities. As mentioned above, stable extracellular enzyme activities are associated with soil colloids and persist even in harsh environments that would limit intracellular microbiological activity (Nannipieri *et al.*, 2002). Thus, only strictly intracellular

Table 2 Some parameters used to determine microbiological activity (Alef & Nannipieri, 1995)

Basal respiration	Dehydrogenase activity
Substrate induced respiration	Fluorescein diacetate hydrolysis
Nitrogen mineralization	Heat output
Nitrification rate	Thymidine incorporation
Potential denitrification activity	Leucine incorporation
Nitrogen fixation	Specific enzyme activities
Adenylate energy charge	Arginine ammonification
ATP content	Dimethyl sulphoxide reduction

enzyme activities can truly reflect microbial activity because the contribution of free extracellular enzyme released by active microbial cells is negligible; indeed, these enzymes are short-lived because they are degraded by proteases unless they are adsorbed by clays or immobilized by humic molecules (Burns, 1982). Unfortunately, the present enzyme assays do not distinguish the contribution of intracellular from extracellular and stabilized enzyme activities, and thus they do not give valid information on the distribution and comparative importance of reactions mediated by microbes (Nannipieri *et al.*, 2002). In the case of enzymes maintaining their activity in the extracellular environment, Landi *et al.* (2000) have suggested that calculating the ratio between the measured enzyme activity and the microbial biomass would provide more meaningful information on the location of the measured enzyme activities. However, any change in the ratio does not depend exclusively on variations in the stabilized extracellular enzyme activity because the intracellular enzyme activity can also increase or decrease without any change in microbial biomass (Nannipieri *et al.*, 2002).

The need to measure the activities of a large number of enzymes and to combine these measured activities in a single index has been emphasized to provide information on microbial activity in soil (Nannipieri *et al.*, 2002). Note that it is conceptually wrong to assume a simple relationship between a single enzyme activity and microbiological activity in soil.

Most of the assays used to determine microbiological activities in soil present the same problem: measuring potential rather than real activities (Burns, 1982; Nannipieri *et al.*, 1990). Indeed, assays are generally made at optimal pH and temperature and at saturating concentration of substrate. Furthermore, synthetic rather than natural substrates are often used, and soil is incubated as a slurry (Nannipieri *et al.*, 1990).

Recent measurements, such as the community-level physiological profile (CLPP), otherwise known as the BIOLOG® approach, and the mineralization kinetics of compounds added to soil have the advantage of combining both functional diversity and degradation rates.

Investigators often measure microbiological activity in soil by determining soil respiration in the absence (basal respiration) or in the presence of specific organic substrates or organic residues (Nannipieri *et al.*, 1990). The CO_2 produced by the oxidation of the added compound or residue can be determined only with ^{14}C-labelled material. Indeed, it can be erroneous to subtract the CO_2 evolved from the control soil (basal respiration) from that of the treated soil because of the priming effect (acceleration or inhibition of the mineralization of native organic matter in the treated soil) (Shen & Bartha, 1996). Soil respiration can be insensitive to pollution (Brookes, 1995; Landi *et al.*, 2000), whereas the lag time prior to mineralization of amino acid, as determined by evolution of CO_2, can increase in the presence of heavy metals (Nordgren *et al.*, 1988). Hopkins *et al.* (1997) suggested that the L:D respiration

ratio of several amino acids can be a sensitive measure of microbial stress in soil because the microorganisms are less discriminative between the stereoisomeric forms of amino acids. Indeed, acid pH (Hopkins *et al.*, 1997) and Cd pollution decreased the L:D glutamic acid respiration ratio of soil (Landi *et al.*, 2000). Respiration has also been measured to determine nutrient limitations in soil. In glucose-treated soil, there is an initial increase (2–6 hours) in respiration followed by a second increase at 6–10 hours. The first increase is proportional to soil microbial biomass (Anderson & Domsch, 1973). The second increase reflects assimilation and microbial growth, and it is larger in the presence than in the absence of the nutrient (nitrogen, phosphorus or sulphur) when microbial growth is limited (Stotzky & Norman, 1961; Alden *et al.*, 2001). Also, the rates of ^{14}C-leucine or ^{3}H-thymidine incorporation increased in the presence of the nutrient limiting bacterial growth in soil (Alden *et al.*, 2001). Pennanen *et al.* (1998) and Alden *et al.* (2001) have suggested that by monitoring acetate incorporation in ergosterol, one could determine nutrients that limit fungal growth in soil. Another approach for determining nutrient-limited bacterial growth uses bacteria of a reporter gene (van Overbeek *et al.*, 1997; Jensen & Nybroe, 1999). The disadvantage of this method is that one has to use one strain for each limiting nutrient.

BIOLOG® is nowadays much in favour to measure microbial functional diversity in soil because the utilization of available carbon is the key factor governing microbial growth in soil (Garland & Mills, 1991; Insam & Rangger, 1997). In addition, the technique is rapid and simple. However, there are several drawbacks: it is culture dependent, and reproducible results can be obtained only if replicates contain identical community profiles and are of similar inoculation density (Insam, 1997); changes in the microbial community can occur during the incubation (Smalla *et al.*, 1998); and the contribution of fungi is not measured because of their slow growth (Haack *et al.*, 1995).

Degens & Harris (1997) proposed the measurement of the patterns of *in situ* catabolic potential of microbial communities for overcoming the problems with BIOLOG®. They used differences in the individual short-term respiration responses (or substrate induced respiration, SIR) of soils to the addition amino acids, carboxylic acids, carbohydrates and organic polymers to assess patterns of microbial communities. Although the approach of Degens & Harris (1997) does not present the problems of the BIOLOG® technique (see above), it has not been widely used since it was first published in 1997. This method permits the determination of catabolic richness, which is a measure of the number of substrates oxidized to CO_2, and catabolic evenness, E, given by

$$E = 1 / \sum_{i=1}^{N} p_i^2,$$

where p_i is the proportion of the total respiration response (i.e. is equal to $r_i / \Sigma r_i$), r_i is the response for substrate i and N is number of substrates (Degens *et al.*, 2001).

The application of molecular techniques can improve the accuracy of determining microbial activities in soil. The traditional assays determine the rates of metabolic processes or activities of specific reactions that can be carried out by several microbial species. By using molecular techniques one might be able to determine the actual species involved in the processes being measured. This can be obtained by the direct targeting of 16S rRNA (Felske *et al.*, 1998). Although methods for extracting DNA from soil are now well established, only a few extraction methods of RNA from soil have been reported (Moran *et al.*, 1993; Felske *et al.*, 1996; Duarte *et al.*, 1998). These are less than ideal because they involve multiple steps for purification and are impractical for processing large numbers of samples. In addition, recovery of intact mRNA molecules from soil is difficult because RNA is not stable. Recent protocols have been developed for the co-extraction of DNA and RNA from soil, making possible the characterization of bacterial diversity with the differentiation of the active bacteria (Griffiths *et al.*, 2000; Hurt *et al.*, 2001). Glassware and utensils used for RNA extraction are treated (such as by heating at 500°C for 4 hours) to inactivate RNases (Hurt *et al.*, 2001). In addition, the efficient co-extraction of RNA and DNA makes it possible to calculate the RNA/DNA ratio, which can be an important indicator of the metabolic status of microbial communities in soil.

Microbial diversity and soil functions

As mentioned above, the links between biodiversity and the functions of terrestrial ecosystems have been studied mainly in above-ground systems. A well-known relation between biodiversity and function is that described by the hump-shaped curve, in which there is an increase in plant production (i.e. the function) concurrent with increasing biodiversity until a certain point is reached; then a further increase in biodiversity results in a decrease in plant production (Loreau *et al.*, 2001). Other concepts proposed by current macroecological theories include stability defined as the property of an ecosystem to withstand perturbations. Stability includes both resilience (i.e. the property of the system to recover after disturbance) and resistance (i.e. the inherent capacity of the system to withstand disturbance) (McNaughton, 1994). Tilman (1996) found that variations in plant populations in grassland caused by interspecific competition produced compensating effects over the total community and increased stability as measured by primary production. In microbial systems, functional stability is not necessarily related to community stability. Fernandez *et al.* (1999) studied the behaviour of a well-mixed and functionally stable (i.e. constant pH and oxygen demand) metanogenic reactor fed with glucose. They discovered that the structure of the community was dynamic, as revealed by ARDRA.

It is difficult to measure both resistance and resilience in soil. Generally microbe-mediated processes are the most sensitive to perturbations in the soil; for this reason the capacity of

soil to recover from perturbations can be assessed by monitoring microbial activities (Pankhurst *et al.*, 1997; Seybold *et al.*, 1999).

The links between microbial diversity and soil functioning, as well those between stability (resilience or resistance) and microbial diversity in soil, are unknown because, as stated above, it is difficult to measure microbial diversity. In addition, we generally measure soil functions by determining the rates of microbial processes, without knowing the microbial species effectively involved in the measured process. According to O'Donnell *et al.* (2001), the central problem of the link between microbial diversity and soil function is to understand the relations between genetic diversity and community structure and between community structure and function.

The links between microbial diversity and soil functions have been studied by approaches based on the use of
1 soils with the same texture but different microbial composition;
2 repeated CHCl₃ fumigations of soil to decrease microbial diversity;
3 specific biocides for killing specific soil microorganisms; and
4 sterile soils inoculated with soil microorganisms.
The second and third approaches are destructive, whereas the fourth is constructive (Griffiths *et al.*, 2000).

Degens *et al.* (2001) investigated the relation between microbial diversity and soil functioning. They measured catabolic evenness in two silty clay loam soils subjected to three different stresses (decline in pH, increase in electrical conductivity and increase in Cu concentration) and two cyclic disturbances (wetting and drying, and freezing and thawing). The two soils had different catabolic evennesses: 21.4 in the soil under permanent pasture for more than 20 years and 19.0 in the soil under arable crops (2 years potatoes, 3 years wheat, 4 years maize and 26 years barley). The arable soil was less resistant to cell stresses and all disturbances than the pasture soil (Degens *et al.*, 2001). Since the organic C content, stability of aggregates, cation exchange capacity and microbial biomass were also greater in the pasture soil, these factors might also have increased the resistance of soil microorganisms to stresses and disturbances. For example, the organic C content and microbial biomass are generally correlated in soil, and this shows that organic matter is a good habitat for microorganisms. Organic matter can also adsorb compounds toxic to microorganisms. The catabolic evenness showed the typical hump-shaped pattern, generally observed in plant communities (Tilman, 1996), with an increase with minimal stress and disturbance, followed by a decrease, more marked in the pasture soil at greater stress. Unfortunately, Degens *et al.* (2001) did not measure the composition of the soil microflora, and thus the relation between catabolic evenness and microbial evenness or richness could not be directly assessed.

Griffiths *et al.* (2001a) studied stability (resistance and resilience) of soils with different microbial diversities as a result of their different use that were otherwise similar. The soils were clay loam cropped with either a single annual species or six annual species; the B horizons of a petroleum polluted sandy loam soil (formerly a petrol station) under remediation and the relatively unpolluted control soil; and a horticultural loamy sand soil. Only small differences were detected by BIOLOG® analysis, whereas protozoan numbers were significantly different and were smaller in the polluted soil (about one third of the protozoan biomass of the uncontaminated soil). In addition, no fungal growth was recorded in the polluted soil (in contrast to the other) when treated with plant residues (Griffiths *et al.*, 2001a). Short-term (25 hours) decomposition of added shoots of *Lolium perenne* was monitored at 15°C under constant moist conditions after different perturbations: amendment with 500 μg Cu g⁻¹ soil; heating at 40°C for 18 hours; freezing at −20°C for 18 hours. Soil resistance was calculated by the following equation:

$$\% \text{ change from the control} = 100 \frac{(\text{Control CO}_2 - \text{Treated CO}_2)}{\text{Control CO}_2}.$$

The grassland soil was more resistant to both Cu and heat than the polluted soil, which was also less resistant to heat than the respective non-contaminated site. However, the polluted soil showed resilience after the Cu stress because soil respiration recovered after 15 days.

Griffiths *et al.* (2001b) created different degrees of microbial diversity (determined by DGGE and protozoan identification, respectively) artificially by inoculating a gamma irradiated agricultural clay loam with serially diluted soil suspensions prepared from the parent soil. The number of bacterial, fungal and protozoan taxa decreased with increasing dilution. There was no consistent relationship between microbial diversity and soil functions. Some functions increased with dilution (SIR), others were not affected (thymidine and leucine incorporation, NO₃⁻ accumulation, respiratory growth response), and some declined only at the greatest dilution (short-term respiration from added grass, nitrification rates, BIOLOG®). In addition, there was no difference in resilience since the decomposition of continuously labelled (¹⁴C) barley straw recovered after transient heat stress (40°C for 18 hours) to its pre-stress rate. Using sterile soil inoculated with different dilutions of the non-sterile soil has been criticized because only extractable microorganisms are used and the resulting community of the inoculated soil has an unrealistically small microbial diversity (Griffiths *et al.*, 2000).

Another destructive approach is to kill certain microbial groups with specific biocides so as to study their influence on soil functioning. The bacteriocide streptomycin and the fungicide cycloheximide have been used to inhibit selectively the respiratory response of bacteria and fungi to glucose with the aim of determining the bacteria:fungi ratio in soil (Anderson & Domsch, 1973). This method is based on two assumptions: the ratio of bacteria:fungi in the sensitive component of soil microflora is the same as that in the inhibitor-insensitive component

(West, 1986); and the fungal and bacterial components of soil microflora respond equally to addition of glucose (Wardle & Parkinson, 1990 a,b). Ingham & Coleman (1984) used amphotericin (a saprophytic fungicide), diazinon (an insecticide), carbofluran (a carbamate nematicide), cygon (an acaricide), and *p*-chloronitrobenzene (a fungicide specific for phycomycetous fungi), in addition to streptomycin and cycloheximide. However, biocides have the disadvantages that their effects are not restricted to their target group (Ingham & Coleman, 1984; Landi *et al.*, 1993). In addition, the biocides can be adsorbed by soil colloids or used as an energy and nutrient source by the surviving microorganisms (Badalucco *et al.*, 1994).

Chander *et al.* (2002) found that soil fumigated with chloroform, with a much smaller microbial biomass than the corresponding non-fumigated soil, respired about the same amount of ^{14}C-CO_2 from labelled straw as the non-fumigated soil. They also found that pollution with zinc affected respiration of non-fumigated and fumigated soils in the same way, indicating that the ratio between substrate C-to-microbial biomass C (larger in the fumigated than the non-fumigated soil) was not important. The ratio of CO_2 to ^{14}C-to-microbial biomass ^{14}C was linearly related to Zn pollution. Griffiths *et al.* (2000) found that soil fumigated with chloroform, with greater microbial diversity, was more resistant and resilient than soil with a less diverse community to perturbations such as heating at 40°C for 18 hours or treatment with 500 μg Cu g^{-1} soil. However, they suggested that the observed effects were due to the physiological influence of $CHCl_3$ fumigation on the microbial community rather than differences in microbial diversity.

The studies of Griffiths *et al.* (2000, 2001b) indicate that the effect of microbial diversity on microbial functions in soil depends on the measured function. According to Dighton (1997), only by functional assays, which integrate both microbial community and species composition, can the functional aspects of microbial community be assessed. In ecology, the negative correlation between variation of functional measurements and diversity has been generally found in fairly uniform systems (McGrady-Steed *et al.*, 1997; Naeem & Li, 1997). Redundancy of functions is believed to be typical in soil, and this could explain the reason for the lack of any observed relation between microbial diversity and soil functions. No relationship has been found between microbial diversity and decomposition of organic matter, and a reduction in any group of species has little effect on overall soil process since other microorganisms can carry out this function.

In conclusion, despite the recent progress in methods, the relations between genetic diversity and taxonomic diversity are not well understood, and even less is known about the manner in which genetic diversity and taxonomic diversity of microorganisms affect microbial functional diversity (Wardle & Giller, 1996; Griffiths *et al.*, 1997, 2001a,b; Ohtonen *et al.*, 1997).

The holistic approach and soil functioning

The extraction of large amounts of DNA and RNA from soil and the accurate taxonomic and functional characterization of these acids can increase our knowledge of the composition of soil microflora, of pathogenicity, of trophic interactions between soil microorganisms and of the production of bioactive molecules by soil microorganisms. These have important implications for agriculture, the environment, industry and pharmacology. However, the molecular approach does not seem sufficient for a better understanding of soil functioning (Figure 2). In this regard it is also important to understand the link between community structure and functions in microhabitats, that is, the spatial distribution of microbial species and their spatial and temporal links with the various microbe-mediated reactions *in situ*. For this reason, high-resolution techniques must be applied to soil preparations to be examined by electron microscopy. Ultracytochemical methods have been successfully applied to locate microbial cells in the soil matrix without giving any taxonomic characterization (Forster, 1994; Assmus *et al.*, 1995, 1997). It is also difficult to associate microbial activities with the locations of species within the soil matrix; the presence of electron-dense components in the soil has limited the location of active enzymes to microhabitats associated with microbial and root fragments (Forster, 1985). Thus, acid phosphomonoesterase activity has been detected in roots, soil microorganisms and small (7 nm × 20 nm) fragments of microbial membranes, but not in clays or humic particles (Ladd *et al.*, 1996).

However, it is not always rewarding to determine the composition of soil microflora and the various microbially mediated activities when assessing the functionality of the soil. Determining the composition of the soil microflora, the concentration of each metabolite, and the rate of each transformation reaction can be not only time-consuming, laborious and expensive, but also unnecessary for quantifying nutrient cycling or assessment of soil quality.

Information on transformation rates of nutrients in soil has been obtained with labelled compounds, such as ^{14}C-, ^{13}C-labelled or ^{15}N-enriched compounds. In the holistic approach, the system is partitioned into pools with a functional meaning, and fluxes between these pools represent physical (such as leaching and volatilization) or abiotic or biotic transformations (Nannipieri *et al.*, 1994). Then, the distribution of the isotope (reflecting the behaviour of the added compound) between the various pools can be followed, and the behaviour of the added compound can be discriminated with respect to that of the native C or N. Particular attention has been given to nutrients, such as N, present in a large organic reservoir. For example, the most popular models for describing N mineralization and immobilization consider microbial biomass as an undifferentiated whole, and a significant source and sink of nutrients, and consider microbial decomposition of organic matter and microbial synthesis occurring simultaneously in
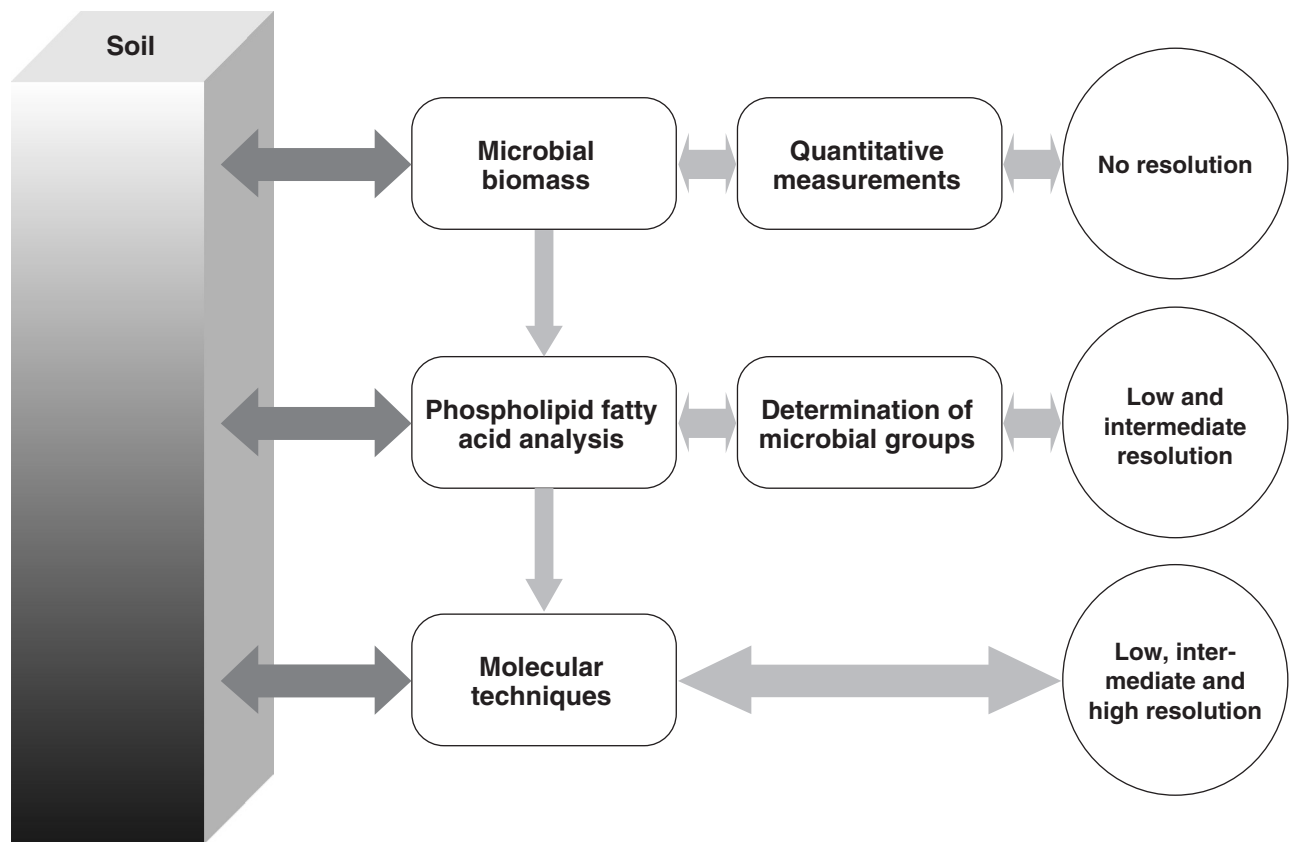
Figure 2 Scientific insights provided by determining the composition of microbial communities or by using the holistic approach in soil.

soil. When determining the mineralization and immobilization turnover of N (MIT) conventionally we assume that ammonium is the end product of the decomposition and that the ammonium may be immobilized by a proliferating microbial population or taken up by plant roots. Alternatively we may assume the so-called direct route, which involves the uptake of simple organic molecules (such as amino acids) directly through cell membranes. Once assimilated, amino acids are deaminated, and only the surplus N is released (i.e. mineralized) into the extracellular ammonium pool in the soil. Barraclough (1997) demonstrated that both MIT and the direct route can operate concurrently in the soil. The organic pool is also measured as an undifferentiated pool, even though we know that it is heterogeneous in terms of biological activity, because a proportion of it cycles rapidly whilst some components cycle very slowly (Nannipieri *et al.*, 1994). A better quantification of N transformations in soil can be obtained if we split the organic N pool into at least the more resistant and less resistant N organic pool, and the microbial biomass into the active and the inactive microbial pool. Current models represent both pools, but their N content is not measurable (Nannipieri & Badalucco, 2003). Nannipieri *et al.* (1994) proposed to set up methods based on the molecular techniques for determining new microbial pools in soil. Radajewski *et al.* (2000) have recently reported an example of this approach. They separated the ^{13}C-DNA extracted from

soil treated with a ^{13}C source by density-gradient centrifugation and characterized the ^{13}C-DNA taxonomically and functionally by gene probing and sequence analysis. This technique is very promising because we might be able to calculate the active microbial C pool by multiplying the ratio between labelled and total DNA fractions by the content of microbial C in the soil. In addition, the taxonomic and functional characterization allows us to understand more precisely the changes in the composition of microbial communities affected by the added C-substrate.

Instead of measuring the composition of soil microflora, it might be less laborious and time-consuming to monitor the behaviour of key species which can function as indicators of the status of the soil microflora. However, Kimball & Levin (1985) have been concerned about the use of single populations to monitor the response of a soil ecosystem to perturbations because this response might not predict what will happen throughout the community and cannot identify all the interactions that may be altered.

Conclusions

Our understanding of the links between microbial diversity and soil functions is poor because we cannot measure easily the microbial diversity, even if we can detect unculturable

microorganisms by molecular techniques. In addition, the present assays for measuring microbial functions determine the overall rate of entire metabolic processes, such as respiration, or specific enzyme activities, without our identifying the active microbial species involved. According to O'Donnell *et al.* (2001), the central problem with the link between microbial diversity and soil function is to understand the relations between genetic diversity and community structure and between community structure and function. The recent advances in RNA extraction from soil might permit us to determine active species in soil (Griffiths *et al.*, 2000; Hurt *et al.*, 2001). Further advances in understanding require us to determine the composition of microbial communities and microbial functions in microhabitats.

Griffiths *et al.* (2000, 2001b) showed that the effect of microbial diversity on microbial functions depends on the function measured. Some functions increased (substrate induced respiration, SIR) with decreasing microbial diversity in soil, others were not affected (thymidine and leucine incorporation, NO_3^- accumulation, respiratory growth response), and some declined when microbial diversity was small (short-term respiration from added grass, potential nitrification rates, BIOLOG®) (Griffiths *et al.*, 2001b). No relation exists between microbial diversity and decomposition of organic matter, and a reduction in any group of species has little effect on overall soil process because the surviving microorganisms can carry out the decomposition of organic matter (Andren *et al.*, 1995; Brookes, 1995; Giller *et al.*, 1998).

The use of molecular techniques has improved the determination of the composition of soil microflora (Figure 2). Recent advances include bacterial artificial chromosomes (BAC) cloning libraries, which can allow the functional and taxonomic analysis of large segments of soil DNA (metagenome) with further insights into pathogenicity, competitiveness, substrate range and bioactive molecule production by soil microorganisms (Rondon *et al.*, 2000).

However, the determination of the composition of microbial communities in soil can be unnecessary for a better understanding of soil functions. For example, the holistic approach with the use of labelled compounds allows us to determine the distribution of nutrients among the various pools. However, further advances in quantifying nutrient dynamics in soil require the determination of new pools, with new molecular techniques (Nannipieri *et al.*, 1994). With regard to this, Radajewski *et al.* (2000) separated ^{13}C- and ^{12}C-DNA, both
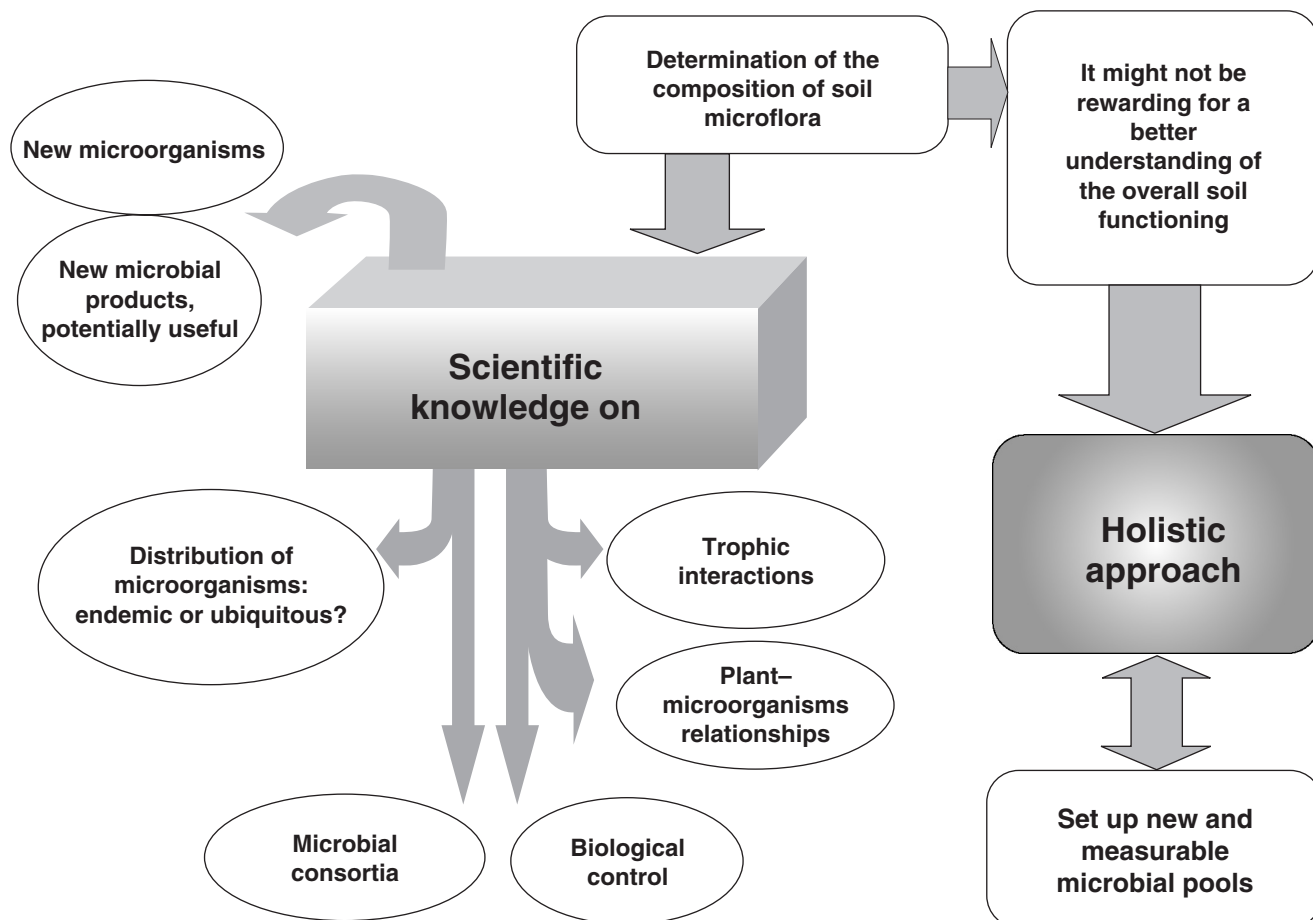
Figure 3 Taxonomic resolution of methods used to determine microbial biomass and microbial composition in soil.

extracted from soil treated with a ^{13}C source, by density-gradient centrifugation, and then ^{13}C-DNA was taxonomically and functionally characterized by gene probe and sequence analysis. This technique may also allow us to calculate the active microbial C pool if we multiply the ratio between labelled and total DNA by the microbial biomass C content of soil.

Soil microbiologists and biochemists have to consider carefully the meaning of determinations. The measurement of microbial biomass by the fumigation technique was an important step for a better quantification of nutrient cycling in soil because it allowed the determination of the microbial C, N, P and S content (Jenkinson, 1988; Nannipieri *et al.*, 1994). However, it is conceptually wrong to use this method for determining microbial activity. The C/N ratio of microbial biomass can give indications of the relative prevalence of fungi over bacteria and vice versa. However, microbial composition can be determined by phospholipid fatty acid (PLFA) analysis or molecular techniques (Figure 3), depending on the degree of resolution required (Zelles, 1999).

We should use these new techniques with caution. We sometimes tend to accept the new methods without critically considering their limits. With regard to this, we should note the behaviour of some soil microbiologists who use the community-level physiological profile (CLPP) as a new technique to determine microbial diversity although it is culture dependent, but criticize the use of plate counts and suggest using molecular techniques to determine microbial diversity.

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