4 Role of Stabilised Enzymes in Microbial Ecology and Enzyme Extraction from Soil with Potential Applications in Soil Proteomics

Paolo Nannipieri

4.1 Introduction

The same enzyme can have different locations in the soil matrix being cytoplasmic, periplasmic (in Gram-negative bacteria) or attached (ectoenzymes) to the outer surface of active cells, intracellular in dead cells or cell debris, intracellular in resting cells, such as bacterial spores or protozoan cysts, or released as extracellular enzymes, which can be free in the soil solution, adsorbed on a substrate or on soil colloids (Burns 1982; Nannipieri 1994; Chap. 12). Some of these enzyme activities are not quantitatively important; e.g. "free" extracellular enzymes are supposed to be short-lived unless they are protected from proteolysis upon adsorption by soil minerals or entrapment by humic colloids (Burns 1982; Nannipieri et al. 2002). It is well established that enzyme activities of resting cells, such as bacterial spores, are very low and probably undetectable in soil enzyme assays (Atlas and Bartha 1981). A better interpretation of measurements of enzyme activities should be possible by distinguishing the extracellular activity due to enzymes adsorbed by soil minerals or entrapped by humic colloids from the activity of enzymes associated with active microbial cells (the cytoplasmic and periplasmic enzymes and the ectoenzymes; Nannipieri et al. 2002).

Of the different locations that an enzyme can have in the soil matrix, those associated with soil colloids have been extensively studied because they are markedly resistant to thermal denaturation and generally to proteolysis (Nannipieri et al. 2002). Thus, they can be active even under conditions limiting microbial activity and are not regulated by factors repressing or derepressing their synthesis. For this reason they are also called "stabilised" enzyme activities in soil. Research aimed at understanding the state of these enzymes in soil has been carried by two different approaches. The first concerns the study of properties of enzyme complexes prepared in the laboratory. For example, various enzymes have been adsorbed on pure clay minerals made homoionic to different cations (Stotzky 1986; Nannipieri et

Paolo Nannipieri: Dipartimento della Scienza del Suolo e Nutrizione della Pianta, P.le delle Cascine 28, 50144 Firenze, Italy, E-mail: paolo.nannipieri@unifi.it

Soil Biology, Volume 8
Nucleic Acids and Proteins in Soil
P. Nannipieri, K. Smalla (Eds.)

al. 1996; Chap. 7). Model humus–enzyme complexes have been prepared by entrapping enzymes during oxidative coupling or condensation reactions of phenolic compounds (Burns 1986; Nannipieri et al. 1996). The second approach concerns the extraction of enzyme complexes from soil with successive studies of the properties of the extracted complex. Only organic enzyme complexes or free enzymes have been extracted from soil (Nannipieri et al. 1996). Clay–enzyme complexes have not been extracted from soil and, as discussed below, their presence has not been detected by electron microscopy; however, there is indirect evidence that they can exist in soil.

The present knowledge on the extraction of enzymes from soil can also be used in studies about soil proteomics, which involves protein extraction from soil. There is the need to combine studies on soil functionality with genome characterisation, i.e. to relate the presence of genes with their expression, and this can be carried out not only by characterising DNA and mRNA extracted from soil, but also by monitoring protein syntheses and specific soil functions (Nannipieri et al. 2003; Smalla 2004). It is well established that the statement "one gene one protein" is no longer valid because several events can occur between gene expression and protein functions (Graves and Haystead 2002; Fig. 4.1). The mRNA molecule can undergo post-transcriptional control due to alternative splicing, polyadenylation and mRNA editing (Sarkar 1977; Newman 1998). Thus different protein isoforms can be generated from a single gene at this stage. Some stable mRNA-like transcripts (spliced and polyadenylate) are non-coding and function as RNA riboregulators (Erdmann et al. 1999). In addition, mRNA can be subjected to regulation and then the formed protein can be subjected to post-translational modifications and regulated by proteolysis (Kirschner 1999) and compartmentalisation (Colledge and Scott 1999). According to Krishna and Wold (1993), there are 200 different types of post-translational modifications.

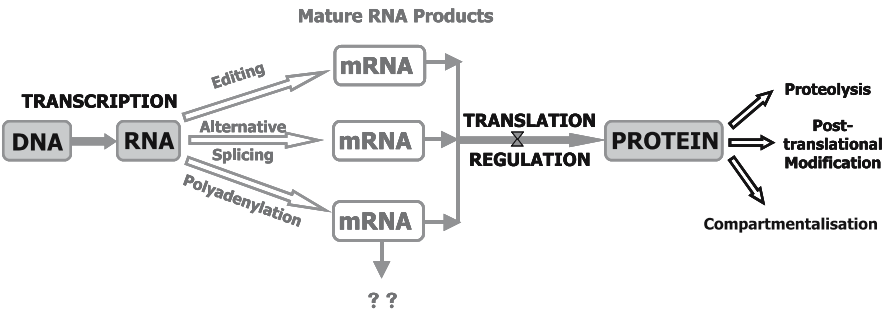

Fig. 4.1. Several processes occurring between gene expression and function of physiologically active proteins (partially modified from Graves and Haystead 2002)

The aims of this review are to discuss: (1) the indirect evidence that supports the presence of active enzymes adsorbed by clay minerals or entrapped by humic molecules in soil; (2) the various methods used to extract "stabilised" enzymes from soil and the characterisation of the extracted complexes; (3) the role of stabilised enzymes in microbial ecology; (4) the application of proteomics to soil with the manifold problems due to the complexity of the system; and (5) the contribution of the existing knowledge on the extraction of enzymes from soil to set up efficient and conceptually correct methods of protein extractions from soil. As for the application of any innovative technique and approach to soil, a successful application of proteomics to soil requires careful consideration of the complexity of the soil system. Of course, since soil proteomics is still in its infancy, the discussion on soil proteomics will be mostly speculative and it will partly overlap with what is discussed in Chap. 5.

The extraction of enzymes from soil has been carried out with the aim of understanding the mechanisms responsible for the formation of organo-mineral complexes in soil and the protection of enzymes by soil colloids against proteolysis. Thus, attention has been focused on extracellular hydrolases which could be active in the extracellular environment and whose activity could be determined in soil. Hydrolase activities were also selected for their importance in nutrient cycling. Thus the most studied enzymes were urease, phosphatases, proteases, β-glucosidases, etc. In contrast, the attention of proteomics might be focused on specific enzymes which respond to changes in environmental factors and soil pollution or can play important roles in biotic processes. Of course the two approaches are not exhaustive and other aspects, such as the origin of enzymes, still require further research.

4.2 Evidence for the Presence of Stabilised Enzymes in Soil

The most important piece of evidence of the presence of stabilised enzymes in soil derives from studies on the nitrogen (N) distribution in soil carried out in the 1960s and 1970s and based on the use of acid hydrolysis of soil (Bremner 1965; see also Chap. 2). Amino acids are the main identifiable organic N compounds in soil hydrolysates, where they can range from 30 to 45% of total soil N (Stevenson 1986; Schulten and Schnitzer 1998). However, net N mineralisation, i.e. the amount of N made available to plants, accounts for 2% of the total N, and it corresponds to 100–200 kg N per hectare, a normal rate of N fertilisation (Stevenson 1986). An intriguing question is how this organic N is slowly mineralised. A better understanding of the state of proteins and protein–colloid complexes in

soil can clarify mechanisms of the N mineralisation. By considering that the percentage of total N in microbial biomass averages to 4% (Jenkinson 1988) and that acid hydrolysis breaks peptide bonds, it is reasonable to hypothesise that most of the amino acid N in soil hydrolysates derives from extracellular protein N stabilised in soil by soil colloids. The percentage of total N present as amino acid N (85%) determined by ^{15}N NMR was even greater than that found after acid hydrolysis of soil (Knicker et al. 1993; Schulten and Schnitzer 1998). However, direct analysis by ^{15}N NMR spectrometry is difficult because natural ^{15}N abundance is very low (0.366%; see Chap. 2).

The visualisation by scanning electron microscopy of soil sections prepared by ultracytochemical tests has allowed the detection of enzymes such as acid phosphatase, succinic dehydrogenase, peroxidase, and catalase in root and microbial cells and acid phosphatase in fragments of microbial membranes as small as 7 × 20 nm (Foster and Martin 1981; Foster 1985; Ladd et al. 1996). Unfortunately, the presence of electron-dense soil components, such as minerals, or soil components, such as humic molecules, which aspecifically react with the counterstainer OsO_4, caused problems for the detection of enzymes adsorbed by clay minerals or englobated by humic complexes. These problems were partially overcome by using electron probe microanalysis and proper controls, where the enzyme was inhibited or the substrate not added (Ladd et al. 1996). By using this approach no phosphatase activity was found on quartz particles while enzyme activity associated to microbial cells was detectable, and there was also little evidence for the presence of phosphatase activity associated to clays (Ladd et al. 1993).

However, it is well established that the contents of organic matter and microbial biomass depend on the clay content of soil (Ladd et al. 1996). Already Jenny in 1941 concluded that the clays retard the decomposition of organic matter by comparing soils differing in clay content but with similar climate, vegetation and management regimes. The addition of ^{14}C-labelled simple (such as glucose) or complex (vegetable remains) substrates to soils with varying clay content has led to the understanding that the clays do not affect the prime degradation of the substrate but the successive stabilisation of the microbial degradation products and the synthesised microbial biomass (Ladd et al. 1996). Differences in the dynamics of C and N turnover were assessed by studies based on fractionation procedures of soils treated with ^{14}C- and ^{15}N-labelled compounds. Extracellular N organic compounds produced by microorganisms were preferentially associated with clay particles during early periods of microbial degradation of ^{14}C- and ^{15}N-labelled wheat straw or ^{14}C-labelled glucose added with ^{15}N-NH_4^+ (Ladd et al. 1996). As shown in Chap. 7, the bibliography on the properties of protein–clay complexes prepared in the laboratory is extensive.

4.3 Extraction of Enzymes from Soil

A cathepsin-like activity was measured in a soil extract in 1954 (Antoniani et al. 1954). However, according to Skujins (1967), Fermi and Sabramanian were the first to measure enzyme activities (protease and aminase activities, respectively) in a soil extract. Urease was isolated from a surface forest soil with phosphate buffer at pH 6 and purified in the United States by Briggs and Segal (1963). In Australia, Ladd (1972) determined protease and peptidase activities in several pasture and wheat soil extracts by using 0.1 M Tris-borate buffer at pH 8.1 as an extractant. Then, in the 1970s and 1980s, lyase, oxidoreductase and hydrolase activities were monitored in soil extracts (Nannipieri et al. 1996). Several procedures for the extraction of enzymes from soil have been proposed and they are generally based on the use of salt solutions as extractants. Phosphate, acetate, citrate, tris(hydroxymethyl)aminomethane (tris), tris-borate, borate, etc., have been used to extract enzymes from soil, as reviewed by Shcherbakova et al. (1981), Tabatabai and Fu (1992) and Nannipieri et al. (1996). It was realised that it is not acceptable to purify enzymes extracted from soil because the extracted enzymes can be a mixture of enzymes from different sources (Nannipieri et al. 1996). Indeed, enzymes can originate from a multitude of sources from the variety of microbial species inhabiting soil, and from plants and fungi (Torsvik et al. 1996; Nannipieri et al. 2003). It was suggested that enzyme extraction from soil should give high yields of extracted enzymes to allow the characterisation of the properties of the extracted enzyme complexes (Nannipieri et al. 1974, 1980, 1996). In addition, an efficient extraction procedure for immobilised enzymes in soil should avoid the lysis of microbial cells (and thus the consequent release of intracellular enzymes), the formation of artefacts during soil extraction, such as the adsorption or entrapment of the extracted enzymes within the solubilised complexes, and interaction between the extractant and the inorganic components to give enzyme-like activity (Nannipieri et al. 1996). For example, the citrate can interact with manganese to form complexes showing laccase-like activity as a result of soil extraction (Leonowicz and Bollag 1987). Nannipieri et al. (1974) used sodium pyrophosphate at neutrality to extract ureases from a humic podzol without breaking microbial cells, by considering that this solution had been used to extract organic matter under mild conditions (Bremner and Lee 1949). Sodium pyrophosphate was also used to extract urease from coarse textured solodic, reddish-chocolate clay loam, grey-brown podzolic, red-yellow podzolic and alpine humus soils (Lloyd 1975). The same extractant was used to extract β-glucosidase and catalase from an Umbric Dystrochrept soil (Perez Mateos et al. 1988; Busto and Perez Mateos 1995), and phosphatase, casein- and

benzoylargininamide-hydrolysing proteases from Mollisol, Histosol and Alfisol soils (Nannipieri et al. 1980).

Sodium hydroxide, the common solution used to extract organic matter from soil at alkaline pH values (12–13), was used to extract malathion esterase from a clay-loam soil; the enzyme was then purified by a procedure involving addition of salts ($MnCl_2$), with precipitation by $(NH_4)_2SO_4$, dialysis and ion chromatography. It was shown that the extracted enzyme was a glycoprotein (Getzin and Rosefield 1971; Satyanarayana and Getzin 1973). However, alkaline conditions should be avoided in studies on enzyme–soil colloid complexes because enzymes are denatured and lysis of microbial cells can occur.

Extensive characterisation of extracted enzymes from soil has been mainly carried out by the groups of Mayaudon in Belgium and Nannipieri and Ceccanti in Italy with the aims of studying properties and state of the organic-enzyme complexes and the processes responsible for their formation (Nannipieri et al. 1996). The two groups used different extraction procedures and thus probably studied different enzyme complexes.

Laccases, polyphenol oxidases, phosphatases, phosphodiesterases, arylsulphatases, cellulases, xylanases, β-glucosidases, invertases and proteases, extracted by shaking a pasture soil with phosphate-EDTA at pH 7–8 for 1 h (Mayaudon et al. 1973; Batistic et al. 1980), were supposed to be fungal glycoenzymes protected against proteolysis by their entrapment in lipopolysaccharides synthesised by Gram-negative bacteria (Mayaudon 1986). It was also hypothesised that these multienzymatic lipopolysaccharide complexes were linked through Ca bridges to the humic matrix and their extraction by phosphate occurred because EDTA chelated these Ca ions (Fig. 4.2). According to Mayaudon (1986), the interactions between the fungal enzymes and the bacterial lipopolysaccharides occur after partial hydrolysis of the latter once released into soil environment.

The enzymatic preparations extracted by pyrophosphate were rich in humic matter; these humic-enzyme complexes were fractionated by gel chromatography only after exhaustive ultrafiltration of the soil extract with sodium pyrophosphate at pH 7.1 (Ceccanti et al. 1978). The compounds with molecular weight lower than 10,000 were discarded and the retained material was separated by ultrafiltration into two fractions of molecular weight higher (A_I) and lower (A_{II}) than 100,000. Gel chromatography of these two fractions (A_I and A_{II}) gave three and two urease active fractions differing in molecular weight, respectively (Ceccanti et al. 1978). The gel chromatography of the highest molecular weight fraction (A_I) with sodium pyrophosphate as eluent gave three peaks of phosphatase activities and one peak of each protease (casein and benzoylargininamide-hydrolysing proteases) activity, whereas the gel chromatography of the lowest molecular

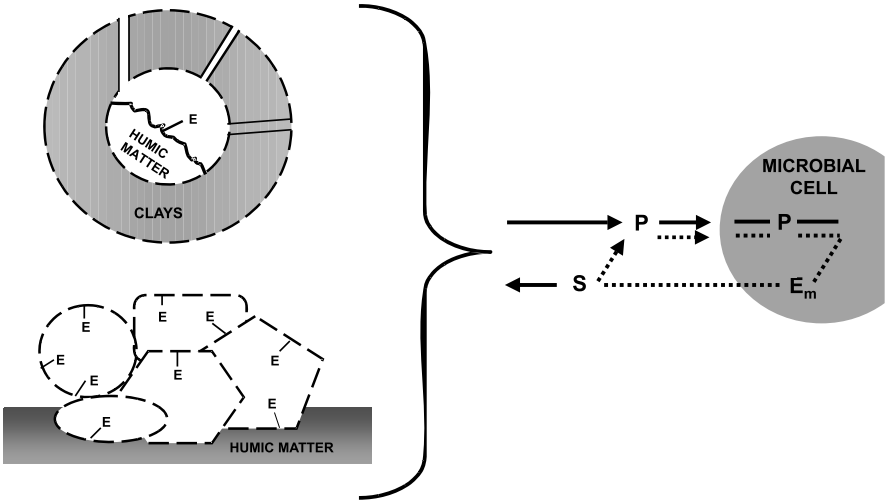

Fig. 4.2. Possible role of stabilised enzymes (model by Burns above; model by Mayaudon below) in microbial ecology (modified by Burns 1982). *P* Product; *S* substrate; E_m induced microbial enzyme; *E* enzymes

fraction (A_{II}) using water as eluent gave one peak of each enzyme activity (Nannipieri et al. 1985).

Characterisation of soil extracts by pyrolysis-gas chromatography (Py-GC) showed that 0.1 M sodium pyrophosphate at pH 7.1 extracted condensed humic substances, glycoproteins, intact or partially decomposed carbohydrates (Bonmati et al. 1988). Soil extracts were active against three different protease substrates: *N*-benzoyl-L-argininamide, specific for trypsin, *N*-benzyloxycarbonyl-L-phenylalanyl L-leucine (ZPL), specific for carboxypeptidases, and casein, essentially non-specific.

Characterisation of A_I and A_{II} fractions by elemental analysis, Py-GC and isoelectric focusing (IEF) showed that both fractions had a C/N ratio higher than a pure protein and properties similar to humic and fulvic acids of the same soil (Ceccanti et al. 1986). In addition, the A_I fraction was richer in carbohydrates and in highly condensated humus than the A_{II} fraction. Total activity of these hydrolases was generally increased after ultrafiltration or gel chromatography, probably as the result of separation of inhibitory humic constituents and enzymes (Nannipieri et al. 1985). Phosphatase and urease active fractions with higher molecular weight were more resistant to thermal denaturation and proteolysis than the enzymatically active fractions with lower molecular weight (Nannipieri et al. 1978, 1982, 1988). It was hypothesised that these data validated the hypothesis by Burns et al. (1972a) who proposed that hydrolases (ureases) were entrapped within or-

ganic or organo-mineral complexes and surrounded by a humic network with pores large enough to allow the passage of substrates and products of the hydrolytic reaction, but not the passage of high molecular weight compounds such as proteases (Fig. 4.2; Nannipieri et al. 1996).

Usually, studies on enzyme extraction from soil and characterisation of enzyme complexes have mainly focused on hydrolases (urease, phosphatase, β-glucosidase, etc.) for their important role in nutrient cycling and for the extensive bibliography on properties of the respective model complexes such as those with clays (see Chap. 7). It is important to underline that the present knowledge in the field derives above all from studies carried out in the 1970s and 1980s. In addition, it is well established that artefacts can occur during the extraction of enzyme complexes from soil.

4.4 The Role of Stabilised Enzymes in Soil Microbial Ecology

According to Burns (1982) enzymes entrapped by humic colloids are essential for the successful competition of microbial cells in the hostile soil extracellular environment. Particularly the stabilised enzymes can catalyse the transformation of high molecular substrates into products that can be taken up by near microbial cells and can act as inducers or chemoattractants. In the first case the products induce the microbial cell to synthesise and release the specific extracellular enzyme into the extracellular soil environment; in the second case, the microbial cells will move towards the substrate.

Both Mayaudon's and Burns' models (Fig. 4.2) involve the entrapment of the stabilised enzymes by organic polymers or organo-mineral complexes, with the surrounding networks having pores large enough to permit the passage of low molecular weight substrates and products but not the passage of the high molecular weight proteases, which should catalyse the proteolytic hydrolysis of the stabilised enzyme. This view has been criticised by Ladd and Butler (1975) for extracellular enzymes acting on high molecular weight substrates because such type of protection would also not permit the detection of the stabilised enzymes.

According to Burns (1982) it is difficult to prove the validity of any theory interpreting processes at the microenvironment scale in soil because we are not able to set up model experiments simulating such a reality. An interesting technique to study spatial interactions between microorganisms, enzymes and their substrates was set up by Hope and Burns (1985). Petri dishes were prepared with carboxylmethylcellulose (CMC) agar; after solidification, a ring of agar was removed and replaced by a barrier ring prepared with soil fractions or soil. Cellulase was pipetted inside the bar-

rier ring and the extent of diffusion of the enzyme outside the barrier was measured by precipitating any unhydrolysed CMC. Clay minerals such as bentonite, with a high cation-exchange capacity and a high unit surface area, limited the diffusion of cellulase, whereas kaolinite, with properties opposite to bentonite, had no effect on the diffusion of the enzyme.

4.5 Proteomics

Proteomics, a term coined in 1995, aims to obtain a global and integrated view of the biology of cells by studying all the proteins of a cell rather than each one individually (Wasinger et al. 1995; Wilkins et al. 1995). As stated in Sect. 1, there are many more proteins than genes in cells because of post-transcriptional modifications. This implies that the gene characterisation is not sufficient to assess the types and functions of proteins in the organism.

Complete draft sequences are now available for the genomes of many eubacteria, several archaebacteria, several unicellular eukaryotes, several plants and animals. As these sequences have accumulated it has become increasingly apparent that new methods are needed to exploit the information that they contain. Three types of proteomics have been applied in biology (Graves and Haystead 2002). *Protein expression proteomics* concerns the quantitative study of protein expression between samples that differ by some variables. This approach has allowed the determination of specific proteins in signal transduction or in disease processes. *Structural proteomics* aims to identify all proteins of a protein complex (e.g. in subcellular organelles) assessing their location and characterising all protein–protein interactions. *Functional proteomics*, a broad term for many specifically directed proteomic approaches, allows the study and characterisation of a selected group of proteins and can provide important information about protein signalling, disease mechanisms or protein–drug interactions. Thus, the proteomics approach can concern either the entire proteome or subproteomes.

In any approach, the analytical method should resolve the protein mixture into its individual components so that each protein can be identified and characterised. The predominant method used for the separation of proteins is two-dimensional (2-DE) polyacrylamide gel electrophoresis where proteins are separated by two distinct properties: the net charge in the first dimension and the molecular mass in the second dimension (Graves and Haystead 2002). The immobilisation of a pH gradient on the gel has increased the resolution and reproducibility of the technique (Bjellqvist et al. 1993; Gorg et al. 2000). In particular, 2-DE has the ability to resolve proteins that have been subjected to post-translational modifications, because these

protein modifications confer a change in protein mass and charge (Graves and Haystead 2002). The main application of the technique is the comparison between two samples so as to determine qualitative and quantitative differences concerning the protein expression. Indeed, the appearance or disappearance of spots can provide information about differential protein expression, while the intensity of the spots can give quantitative information about protein expression levels (Graves and Haystead 2002). A recent advance in 2-DE is represented by difference gel electrophoresis (DIGE) where two proteins fluorescently tagged with two different dyes are run on the same 2-D gel (Unlu et al. 1997). After the run, fluorescence imaging of the gel is used to create two images which are superimposed to identify pattern differences. Thus this approach avoids comparison of several 2-D gels.

The drawbacks of 2-DE are: (1) the technique is labour-intensive and time-consuming; and (2) the complete resolution of all proteins does not occur on a single 2-D gel because the protein mixture is too complex to be completely resolved (Graves and Haystead 2002). Large or hydrophobic proteins will not enter the gel during the first dimension, and the pH range of the gel does not permit proteins with isoelectric points (Ip) lower than pH 3 and higher than pH 10 (Gorg et al. 2000) to be resolved. In addition, the gels only show abundant proteins and not low-copy proteins.

An alternative to 2-DE is to digest a protein mixture to peptides by trypsin and then to purify the peptides before analysis by mass spectrometry (MS). The disadvantage is the cost of the instrumentation, the time employed in the analysis and the computing power to deconvolute the data obtained (Graves and Haystead 2002). This approach presents the advantage that it is able to analyse a greater number of proteins than the 2-DE method. In addition, the mass spectrum of the unknown protein can be compared with theoretical mass spectra produced by computer-generated cleavage of proteins in the database (Graves and Haystead 2002).

Another promising alternative to 2-DE is the use of an isotope-coded affinity tag (ICAT) which allows quantitative profiling between different samples without the use of electrophoresis (Gigy et al. 1999). Protein samples are treated with two chemically identical reagents that differ only in the mass as a result of the isotope composition. The ICAT reagent consists of a biotin affinity group, a linker region that can incorporate heavy (deuterium) or light (hydrogen) atoms, and a thiol-reactive end group for linkage to cysteine residues of proteins. This reagent permits the quantification of the expression level of proteins (Graves and Haystead 2002). For example, two sets of populations of cells in a different state can be differentiated because they are labelled with either a light or heavy form of the ICAT reagent, and the difference in peak heights between heavy and light peptide ions directly correlates with the difference in protein abun-

dance in the cells. In addition, the protein can be identified after hydrolysis by trypsin, purification of labelled peptides by avidin chromatography by virtue of the biotin tag, and analysis by MS.

Another promising technique is microarray systems involving miniature chips and MS, which allow the analysis of a great number of proteins (see Chap. 5).

4.6 Soil Proteomics

It is now recognised that also in soil microbiology there is a need to go beyond DNA analysis for a better understanding of soil functionality (Nannipieri et al. 2003). By characterising both DNA and RNA one might be able to determine the actual species effectively involved in the processes being measured. Different protocols involving the simultaneous extraction of RNA and DNA from soil have recently been published (Duarte et al. 1998; Griffiths et al. 2000; Hurt et al. 2001; Weinbauer et al. 2002; see also Chap. 3). Further research is required for the efficient removal of humic substances and residual DNA without partial degradation of the RNA so as to reliably use RNA for microbial community analysis (Smalla 2004). However, as mentioned above, there is a need to combine the proteomics approach with nucleic acid characterisation to better understand soil functions.

Microbial proteomics has mainly concerned axenic cultures whereas environmental applications are still in their infancy (Graves and Haystead 2002). A correct application of proteomics to soil should consider the complexity of the system; only 4% of total N is present in microbial biomass whereas 30–45% of the soil N is present as extracellular proteins (Stevenson 1986). Thus, the study of gene expression by soil microbiota is made complex by this high background of protein N stabilised by soil colloids which is not directly related to microbial activity.

By considering the distribution of organic N in soil the author would suggest an arbitrary classification of soil proteomics into *functional proteomics* and *structural proteomics* (Fig. 4.3). The former should reveal differences in protein expression by soil microflora in the presence of pollutants and organisms (fauna, plants and other microorganisms) interacting with the target microbial species, changes in agricultural or forest management and changes in environmental conditions. It should evidence any protein responsible for homologous cell-to-cell interactions, such as quorum sensing, and proteins involved in the heterologous cell-to-cell interactions, such as symbiosis and competition. It should reflect microbial activity and it should be combined with DNA and mRNA analyses to link the expression of specific gene sequences to the respective protein function. One possible

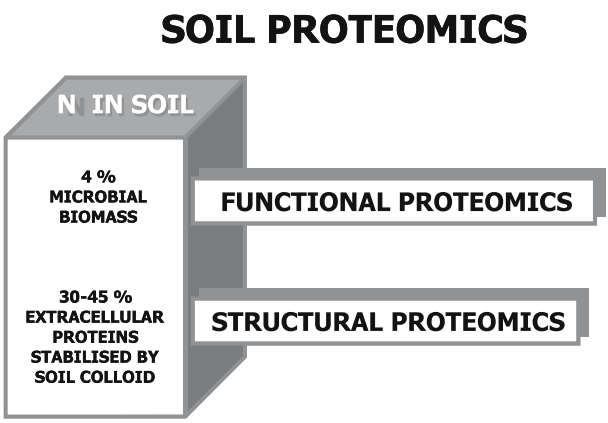

Fig. 4.3. Possible proteomics approaches in soil

problem may be the presence of different proteins (from diverse microbial sources) with the same function. In contrast, *structural proteomics* should characterise proteins adsorbed and stabilised by soil colloids so as to understand mechanisms responsible for such stabilisation in situ. Indeed, most of our knowledge on this subject derives from laboratory experiments involving interactions of a single pure protein with a single colloid (clay mineral or humic fraction). This is not the situation *in situ* because, for example, "dirty" proteins (due to the presence of other cellular components) interact with "dirty" clays (clays covered by hydroxides, oxides and/or humic molecules). So far only the adsorption of pure proteins versus "dirty" clays has been compared (Stotzky 1986; Fusi et al. 1989; Nannipieri et al. 1996).

Murase et al. (2003) found that the clearest electrophoretic patterns in sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) were obtained when extracellular proteins were extracted from an Entisol with phosphate buffer at pH 6.0. Protein bands ranged from 35 to 68 kDa and a homology search in the Protein Information Resource International Database, through the GenomeNet (http://fasta.genome.ad.jp/), allowed the identification of a homologue of thermostable cellulase produced by the thermophilic fungi *Humicola insolens* and *H. grisea* (Murase et al. 2003). By considering the extracting solution used, Murase et al. (2003) probably investigated free extracellular and intracellular proteins released by cell lysis during the extraction. Indeed, phosphate at pH 6.0 is a poor extractant of organic matter from soil and the extraction of stabilised proteins requires more efficient extractants and higher pH values, as discussed above. By increasing the pH of the phosphate extractant, Murase et al. (2003) increased the amount of extracted organic matter from soil resulting in SDS-PAGE patterns with fuzzy protein bands and tailing.

Another successful application of proteomics to soil was shown by Singleton et al. (2003) who showed that Cd pollution decreased the total amount of protein extracted by 35% and increased the production of small molecular weight proteins (< 21 kDa), probably indicative of a microbial response to metal exposure (see Chap. 5). Proteins were extracted after four repeated freeze/thaw cycles to kill and lyse cells in the presence of a protease inhibitor cocktail.

An autoclaving method with citrate buffer at pH 8.0 was used to extract glomalin from soil (Wright and Upadhyaya 1996, 1998; Rilling et al. 2002). Properties and the supposed role of glomalin in soil are discussed in Chap. 5.

Proteins of dissolved organic matter (DOM) have been characterised by MS-based proteomics (Schulze et al. 2005). Their phylogenetic origin and metabolic functions were assessed by using an extensive database. The approach involves removal of humic acids and small molecules from soil solution by gel filtration, proteins separation by SDS-PAGE, trypsin digestion of protein gel slices, extraction of tryptic peptides from gel slices, with their desalting and separation by nanoflow liquid chromatography prior to analysis by MS. About 50% of the proteins of forest soil DOM originated from bacteria and the number of identified proteins decreased with depth and with the amount of DOM. A protease inhibitor cocktail was not used during DOM sampling at different soil depths and active proteases might have affected the protein composition of the sample.

The MS-based proteomics was applied to characterise soil enzymes involved in C reactions (Schulze et al. 2005). After air-drying and sieving (< 2 mm) of soil, discrete organic particles were eliminated by flotation, and settled soil material was washed with deionised water and then air-dried. Proteins were separated from inorganic material by 10% HF, and, after neutralisation, they were separated from the other organic molecules by gel filtration and SDS-PAGE before being analysed as reported above.

The use of ultrasonic treatment proposed by Ogunseitan (see Chap. 5) can increase the extraction efficiency of the phosphate extractant but it also favours the solubilisation of stabilised proteins because it breaks soil aggregates. Thus, a mixture of microbial and stabilised proteins is obtained in the soil extractant. The same drawback can be present in the procedure by Schulze et al. (2005) to extract enzymes from soil by using HF solution which is a strong acid extractant. As discussed above, extracts with only microbial proteins should be obtained if the aim is to study protein expression (functional proteomics). Given the large background of stabilised proteins in soil, a possible approach can be to separate microbial cells from soil particles followed by the extraction of proteins from the microbial cells. Possible drawbacks of the cell extraction method might be the low extraction yields and possible changes in the composition of

protein mixture during the extraction procedure. An advantage might be the possibility to use techniques set up with axenic cultures, such as the ICAT technique. It might be possible to label proteins, DNA and mRNA molecules, with the possibility to distinguish and separate labelled from unlabelled molecules. Labelling of nucleic acids with stable isotopes is discussed in Chap. 10. Methods based on freeze/thaw or autoclaving, used by Singleton et al. (2003) and Wright and Upadhyaya (1996), may mainly extract intracellular or ectoproteins since these techniques cause cell lysis, and the extraction of proteins associated with soil colloids is low by the buffer solutions used (Nannipieri et al. 1996). However, the addition of a protease inhibitor cocktail is needed because proteases are active during cell lysis in soil, as shown by Renella et al. (2002), and they can hydrolyse the target protein. In addition, contaminants co-extracted with proteins from soil can be present and they should be eliminated by purification procedures before protein analysis. This procedure might cause the loss of specific proteins or non-proteic components essential for the function of the target protein. Anyway, each extraction method should also show a recovery yield of known proteins added to soil and extracted by the method used so as to determine the extraction efficiency of the employed procedure. Indeed, the quantitative recovery of the target protein is essential to obtain consistent data.

So far there are no studies on structural proteomics with precise identification of proteins really involved in the adsorption or binding with soil colloids. Humic-enzyme complexes have been extracted from soil, partially purified and characterised as reported above but their organism origin, and thus the type of enzyme, has not been determined. Enzyme-humic complexes, extracted from soil and partially purified, have been fractionated by IEF. There are also a few reports of soil structural proteomics. Cacco and Maggioni (1976) extracted acetylnaphthyl esterase from an A_1 horizon of an Alpine podzol using the procedure of Burns et al. (1972b). Briefly, they carried out a first extraction with 0.25 M phosphate buffer (pH 7.0), 2 M urea, 4 M NaCl, 0.013 M ethylenediaminetetraacetate and 0.06 M mercaptoethanol, at 4 °C for 4–5 h; a second extraction of the soil sediment with 0.25 M phosphate (pH 7) for 3 h; a third and fourth extraction with 0.05 M phosphate for 3 and 4 h, respectively. The pooled soil extract was dialysed against 5 mM mercaptoethanol and concentrated by dialysing it against polyethylene glycol before IEF. Both bands at Ip values of 5.7 and 6.5 showed enzyme activity and contained humic molecules, whereas the bands at Ip 4.7 and 5.7 only showed enzyme activity (Cacco and Maggioni 1976). No enzyme activity was observed below Ip 4.7.

IEF of humic matter and proteases (benzoyl-L-arginimide-hydrolysing proteases) of soil extracts (obtained with sodium pyrophosphate at pH 7.1) and derivative fractions (A_I and A_{II}, see above) obtained by ultrafiltration

was carried out by Ceccanti et al. (1989) on 5% (w/v) polyacrylamide gel tubes (0.5 × 8 cm) using carrier ampholytes at pH 4–6 at a final concentration of 2%. Densitometric scanning of the focused organic matter was carried out at 460 nm with a densitometer, and gel-trapped humic bands were analysed for the protease activity. The largest proportion of protease activity in the extract was located in a broad organic-matter peak at Ip 4.44. About 50% of the enzyme activity was found in the remaining humic bands, whose Ip values ranged from 4.93 to 4.06. However, the highest specific activity (enzyme activity/organic C) characterised the most acidic band. The IEF of the (A_I) fraction (with molecular weight higher than 100,000) revealed four enzymatically active bands with about 50% of the total protease activity of fraction concentrated in the band at Ip 4.65.

4.7 Conclusions

In spite of the fact that both clays and humic matrix can adsorb or entrap enzymes, only humic-enzyme complexes have been extracted from soil and characterised (Mayaudon 1986; Nannipieri et al. 1996). Sodium pyrophosphate at neutrality has been used to extract several enzymes from soil. Probably, these enzymes are entrapped in organic complexes surrounded by a network with pores large enough to allow the passage of substrates and reaction products but not that of proteases. This model is not valid for enzymes acting on high molecular weight substrates because if their activity is detected they would also be accessible to proteases (Ladd and Butler 1975). It is noteworthy that the extracted enzymes have not been characterised for their organism origin and thus their precise nature is not known.

A possible relationship between immobilised enzymes, exogenous substrates and soil microorganisms has been hypothesised by Burns (1982). However, this relationship has never been verified because it is still problematic to set up model experiments simulating processes at the microenvironmental scale.

It is now established that also in soil microbiology there is a need to go beyond DNA analysis for better characterization of microbial functions in soil (Nannipieri et al. 2003). The characterisation of both DNA and RNA might allow the determination of the actual species effectively involved in the processes being measured; however, there is a need to measure specific functions and carry out the proteomics approach to better characterise microbial functions. The potential applications of soil proteomics in soil are very promising because we could obtain a better understanding of microbial cell-environment interactions, such as responses of microbial cells to stresses, homologous cell-to-cell interactions, such as quorum sensing,

and heterologous cell-to-cell interactions, such as symbiosis and predation. A correct application of proteomics to soil involves consideration of the complexity of the system; particularly, the fact that only 4% of total N is present in microbial biomass whereas 30–45% of the soil N is present as extracellular stabilised proteins (Stevenson 1986) which cannot be related to microbial activities (Nannipieri et al. 2002). Only proteins associated with microbial cells should be considered for characterising microbial functions in soil and how they respond to changes in agricultural management, pollution, presence of plants, etc. Here this approach has been called *soil functional proteomics* to differentiate it from the characterisation of proteins adsorbed by clay minerals or entrapped by humic molecules (*soil structural proteomics*). Given the large background of stabilised proteins in soil a possible approach can be to separate microbial cells from soil particles followed by the extraction of proteins from microbial cells. Possible drawbacks of the cell extraction method might be the low extraction yields and possible changes in the composition of protein mixture during the extraction procedure. Advantages might be the possibility to use techniques, such as the ICAT technique (Gigy et al. 1999), set up with axenic cultures. Methods based on freeze/thaw and autoclaving have been used to extract proteins from Cd-polluted soil (Singleton et al. 2003) and glomalin from soil (Wright and Upadhyaya 1996, 1998), respectively. Probably, these procedures mainly extract intracellular or ectoproteins, due to cell lysis and due to the low extraction of proteins associated with soil colloids by the buffer solutions used (Nannipieri et al. 1996). However, the addition of a protease inhibitor cocktail is needed because proteases are active during cell lysis in soil, as shown by Renella et al. (2002), and they can hydrolyse the target protein. In addition, contaminants co-extracted with proteins from soil can be present and they should be eliminated by purification procedures before protein analysis. This procedure might cause the loss of specific proteins and/or non-proteic components essential for the function of the target protein. Anyway, each extraction method should also show a recovery yield of known proteins added to soil and extracted by the method used so as to determine the extraction efficiency of the employed procedure. Indeed, the quantitative recovery of the target protein is essential in order to obtain consistent data.

References

Antoniani C, Montanari T, Camoriano A (1954) Investigations in soil enzymology. I. Cathepsin-like activity. A preliminary note. Annali Facoltà di Agraria, Università di Milano 3:99–101

Atlas RM, Bartha R (1981) Microbial ecology. Fundamentals and applications. Addison-Wesley, Reading, Massachusetts

Batistic L, Sarkar JM, Mayaudon J (1980) Extraction, purification and properties of soil hydrolases. Soil Biol Biochem 12:59–63

Bjellqvist P, Pasquali C, Ravier F, Sanchez JC, Hochstrasser D (1993) A nonlinear wide-range immobilised pH gradient for two-dimensional electrophoresis and its definition in a relevant pH scale. Electrophoresis 14:1357–1365

Bonmati M, Ceccanti B, Nannipieri P (1988) Protease extraction from soil by sodium pyrophosphate and chemical characterization of the extracts. Soil Biol Biochem 14:2113–2125

Bremner JM (1965) Organic forms of nitrogen. In: Black CA (ed) Methods of soil analysis, part 2. American Society of Agronomy, Madison, WI, pp 1238–1255

Bremner IM, Lee H (1949) Studies on soil organic matter. II. The extraction of organic matter from soil by neutral reagents. J Agric Sci 39:274–279

Briggs MH, Segal L (1963) Preparation and properties of a free soil enzyme. Life Sci 1:69–72

Burns RG (1982) Enzyme activity in soil: location and a possible role in microbial ecology. Soil Biol Biochem 14:423–427

Burns RG (1986) Interactions of enzymes with soil mineral and organic colloids. In: Huang M, Schnitzer M (eds) Interactions of soil mineral with natural organics and microbes. Special publication 17. Soil Science Society of America, Madison, WI, pp 429–451

Burns RG, Pukite AH, McLaren AD (1972a) Concerning the location and persistence of soil urease. Soil Sci Am Proc 36:308–311

Burns RG, El-Sayed MH, McLaren AD (1972b) Extraction of a urease-active organo-complex from soil. Soil Biol Biochem 4:107–108

Busto MD, Perez-Mateos M (1995) Extraction of humic β-glucosidase fractions from soil. Biol Fertil Soils 20:77–82

Cacco G, Maggioni A (1976) Multiple forms of acetyl-naphthyl esterase activity in soil organic matter. Soil Biol Biochem 8:321–325

Ceccanti B, Nannipieri P, Cervellli S, Sequi P (1978) Fractionation of humus-urease complexes. Soil Biol Biochem 10:39–45

Ceccanti B, Alcaniz-Baldellou JM, Gispert-Negrell M, Gassiot-Matas M (1986) Characterization of organic matter from two different soils by pyrolysis-gas chromatography and isoelectric focusing. Soil Sci 142:83–90

Ceccanti B, Bonmati-Pont M, Nannipieri P (1989) Microdetermination of protease activity in humic bands of different sizes after analytical focusing. Boil Fertil Soils 7:202–206

Colledge M, Scott JD (1999) AKAPs: from structure to functions. Trends Cell Biol 9:216–221

Duarte GF, Rosado AS, Seldin L, Keijzer-Wolters AC, van Elsas JD (1998) Extraction of ribosomal RNA and genomic DNA from soil for studying the diversity of the indigenous microbial community. J Microbiol Methods 32:21–29

Erdmann VA, Szymanski M, Hochberg A, deGroot N, Barciszewski (1999) Collection of mRNA-like non-coding RNAs. Nucleic Acids Res 27:192–196

Foster R (1985) In situ localization of organic matter in soils. Questiones Entomologicae 21:609–633

Foster R, Martin JK (1981) In situ analysis of soil components of biological origin. In: Paul EA, Ladd JN (eds) Soil biochemistry, vol 5. Marcel Dekker, New York, pp 75–110

Fusi P, Ristori GG, Calamai L, Stotzky G (1989) Adsorption and binding of protein on "clean" (homoionic) and "dirty" (coated with Fe oxyhydroxides) montmorillonite, illite and kaolinite. Soil Biol Biochem 21:911–920

Getzin LW, Rosefield I (1971) Partial purification and properties of a soil enzyme that degrades the insecticide malathion. Biochim Biophys Acta 235:442–453

Gigy SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol 17:994–999

Gorg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W (2000) The current state of two-dimensional electrophoresis with immobilised pH gradients. Electrophoresis 21:1037–1053

Graves PR, Haystead TAJ (2002) Molecular biologist's guide to proteomics. Microbiol Mol Biol Rev 66:39–63

Griffiths RI, Whiteley AS, O'Donnell AG, Bailey MJ (2000) Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA and rRNA-based microbial community composition. Appl Environ Microbiol 66:5488–5491

Hope CFA, Burns RG (1985) The barrier-ring plate technique for studying extracellular enzyme diffusion and microbial growth in model soil environments. J Gen Microbiol 131:1237–1243

Hurt RA, Qiu X, Wu L, Roh Y, Palumbo AV, Tiedje JM, Zhou J (2001) Simultaneous recovery of RNA and DNA from soils and sediments. Appl Environ Microbiol 67:4495–4503

Jenkinson DS (1988) Determination of microbial biomass carbon and nitrogen in soil. In: Wilson JR (ed) Advances in nitrogen cycling in agricultural ecosystems. CAB International, Wallingford, pp 368–386

Jenny H (1941) Factors of soil formation. McGraw-Hill, New York

Kirschner M (1999) Intracellular proteolysis. Trends Cell Biol 9:M42–M45

Knicker H, Fründ R, Lüdemann H-D (1993) The chemical nature of nitrogen in native soil organic matter. Naturwissenschaften 80:219–221

Krishna RG, Wold F (1993) Post-translational modification of proteins. Adv Enzymol Relat Areas Mol Biol 6:265–298

Ladd JN (1972) Properties of proteolytic enzymes extracted from soil. Soil Biol Biochem 4:227–237

Ladd JN, Butler JHA (1975) Humus-enzyme systems and synthetic organic polymer enzyme analogs. In: Paul EA, Mc Laren AD (eds) Soil biochemistry, vol 4. Marcel Dekker, New York, pp 143–194

Ladd JN, Foster RC, Skjemstad JO (1993) Soil structure: carbon and nitrogen metabolism. In: Brussard L, Kooinstra MJ (eds) Workshop on methods of research on soil structure. Geoderma 56:401–434

Ladd JN, Foster RC, Nannipieri P, Oades JM (1996) Soil structure and biological activity. In: Stotzky G, Bollag J-M (eds) Soil biochemistry, vol 6. Marcel Dekker, New York, pp 23–78

Leonowicz A, Bollag J-M (1987) Laccases in soil and feasibility of their extractions. Soil Biol Biochem 19:237–242

Lloyd AB (1975) Extraction of urease from soil. Soil Biol Biochem 7:357–358

Mayaudon J (1986) The role of carbohydrates in the free enzymes in soil. In: Fuchsman CH (ed) Peat and water. Elsevier Applied Science, New York, pp 263–309

Mayaudon J, El Halfawi M, Chaivignac MA (1973) Properties of diphenol oxidase extracted from soil. Soil Biol Biochem 5:369–383

Murase A, Yoneda M, Ueno R, Yonebayashi K (2003) Isolation of extracellular protein from greenhouse soil. Soil Biol Biochem 35:733–736

Nannipieri P (1994) The potential use of soil enzymes as indicators of productivity, sustainability and pollution. In: Pankhurst CE, Doube BM, Gupta VVSR, Grace PR (eds) Soil biota. Management in sustainable farming systems. CSIRO, East Melbourne, pp 238–244

Nannipieri P, Ceccanti B, Cervelli S, Sequi P (1974) Use of 0.1 M pyrophosphate to extract urease from podzol. Soil Biol Biochem 6:359–362

Nannipieri P, Ceccanti B, Cervelli S, Sequi P (1978) Stability and kinetic properties of humus-urease complexes. Soil Biol Biochem 10:143–147

Nannipieri P, Ceccanti B, Cervelli S, Sequi P (1980) Extraction of urease, phosphatase, proteases, organic carbon and nitrogen from soil. Soil Sci Soc Am J 44:1011–1016

Nannipieri P, Ceccanti B, Conti C, Bianchi D (1982) Hydrolase extracted from soil: properties and activities. Soil Biol Biochem 14:257–263
Nannipieri P, Ceccanti B, Bianchi D, Bonmati M (1985) Fractionation of hydrolase-humus complexes by gel chromatography. Biol Fert Soils 1:25–29
Nannipieri P, Ceccanti B, Bianchi D (1988) Characterization of humus-phosphatase complexes extracted from soil. Soil Biol Biochem 20:683–691
Nannipieri P, Sequi P, Fusi P (1996) Humus and enzyme activity. In: Piccolo A (ed) Humic substances in terrestrial ecosystems. Elsevier, Amsterdam, pp 293–328
Nannipieri P, Kandeler E, Ruggiero P (2002) Enzyme activities and microbiological and biochemical processes in soil. In: Burns RG, Dick RP (eds) Enzymes in the environment. Activity, ecology and applications. Marcel Dekker, New York, pp 1–33
Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G (2003) Microbial diversity and soil functions. Eur J Soil Sci 1–16
Newman A (1998) RNA splicing. Curr Biol 8:R903-R905
Perez Mateos M, Gonzales Carcedo S, Busto Nunez MD (1988) Extraction of catalase from soil. Soil Sci Soc Am J 52:408–411
Renella G, Landi L, Nannipieri P (2002) Hydrolase activity during and after the chloroform fumigation of soil as affected by protease activity. Soil Biol Biochem 34:51–60
Rilling MC, Wright SF, Eviner VT (2002) The role of arbuscular mycorrhizal fungi and glomalin in soil aggregation: comparing effects of five plant species. Plant Soil 238:325–333
Sarkar N (1977) Polyadenylate of mRNA in prokaryotes. Ann Rev Biochem 66:173–197
Satyanarayana T, Getzin LW (1973) Properties of a stable cell-free esterase from soil. Biochemistry 12:1566–1572
Schulten H-R, Schnitzer M (1998) The chemistry of soil organic nitrogen: a review. Biol Fertil Soils 26:1–15
Schulze WX, Gleixner G, Kaiser K, Guggenberger G, Mann M, Schulze E-D (2005) A proteomic fingerprint of dissolved organic carbon and of soil particles. Oecologia 142:335–343
Shcherbakova TA, Mas'ko AA, Galushko NA, Kuprevich VF (1981) Extraction of organomineral complexes with enzymatic activity from soil. Pochvovedeniye (Translated) 4:71–78
Singleton I, Merrington G, Colvan S, Delahunty JS (2003) The potential soil protein-based methods to indicate metal contamination. Appl Soil Ecol 23:23–52
Skujins J (1967) Enzymes in soil. In: McLaren AD, Peterson GH (eds) Soil biochemistry, vol 1. Marcel Dekker, New York, pp 371–414
Smalla K (2004) Culture-independent microbiology. In: Bull AT (ed) Microbial diversity and bioprospecting. ASM Press, Washington, DC, pp 88–99
Stevenson FJ (1986) Cycles of soil. Carbon, nitrogen, phosphorus, sulfur and micronutrients. John Wiley & Sons, New York
Stotzky G (1986) Influence of soil minerals and metabolic processes, growth adhesion, and ecology of microbes and viruses. In: Huang M, Schnitzer M (eds) Interactions of soil mineral with natural organics and microbes. Special Publication 17. Soil Science Society of America, Madison, WI, pp 305–428
Tabatabai A, Fu M (1992) Extraction of enzymes from soils. In: Stotzky G, Bollag J-M (eds) Soil biochemistry, vol 7. Marcel Dekker, New York, pp 197–227
Torsvik VL, Sørheim R, Goksøyr J (1996) Total bacterial diversity in soil and sediment communities – a review. J Ind Microbiol 17:170–178
Unlu M, Morgan ME, Minden JS (1997) Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 18:2071–2077

Wasinger VC, Cordwell SJ, Cerpa-Poljak A, Yan JX, Gooley AA, Wilkins MR, Duncan MW, Harris R, Williams KL, Humphery-Smith I (1995) Progress with gene product mapping of the *Mollicutes: Mycoplasma genitalium*. Electrophoresis 16:1090–1094

Weinbauer MG, Fritz I, Wenderoth DF, Höfle MG (2002) Simultaneous extraction from bacterioplankton of total RNA and DNA suitable for quantitative structure and function analyses. Appl Environ Microbiol 68:1082–1087

Wilkins MR, Sanchez, JC, Gooley AA, Appel RD, Humphery-Smith I, Hochstrasser F, Williams KL (1995) Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. Biotechnol Gen Engin Rev 13:19–50

Wright SF, Upadhyaya A (1996) Extraction of an abundant and unusual protein from soil and comparison with hyphal protein of arbuscular mycorrhizal fungi. Soil Sci 161:575–586

Wright SF, Upadhyaya A (1998) A survey of soils for aggregate stability and glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi. Plant Soil 198:97–107