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Review Paper

The chemical and functional characterization of soil N and its biotic components

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ABSTRACT

The aim of this review is to describe and discuss the concepts that have been employed to interpret N mineralization–immobilization in soil, and how N turnover is related to the characteristics of organic N and the biota conducting the transformations. A brief survey of the period before the arrival of electronic searches became available provides access to the classical literature that can help interpret today's challenges. Classical (acid hydrolysis) and modern spectrometry and spectroscopy techniques indicate that protein N is the prevalent component of organic N in soil. The presence of heterocyclic N can indicate its abiotic, partial synthesis as in fire-affected soils. Clays and pedogenic oxides can protect organic N against microbial degradation. The evidence for such protection is mostly based on *in vitro* studies involving pure clays, and proteins and their relevance to field conditions requires further work. The proteomic approach, with extraction and characterization of proteins stabilised by soil colloids (structural proteomics) might give further insights into this area. Functional proteomics can improve our understanding of the degradation of organic pollutants and organic debris as well as identifying the molecular colloquia between microorganisms and between soil biota and plant roots. Subdivision of organic N into sub-pools has helped to interpret mechanistic studies and modelling of N dynamics. Uncomplexed organic matter, obtained by physical fractionation procedures, is considered a labile pool. The interpretation of N mineralization measurements is affected by immobilization during microbial attack especially in high-C environments. Transfer of materials among particle size fractions and changes in microbiological properties of aggregates also can occur during fractionation procedures. Classical mineralization–immobilization turnover (MIT) does not always occur since microorganisms (and plants) can take up amino acid N with intracellular deamination. Protozoa, due to their grazing activities, can influence not only N mineralization but also the composition of rhizosphere–plant growth stimulating communities. Differences between N-poor and N-rich microsites, occurring in the same soil, can markedly affect the competition for N between plants and microorganisms especially the nitrifiers. The use of molecular techniques has allowed the identification of unculturable microorganisms and functional genes in the N cycle. Archeae are probably capable of oxidising NH_4^+ to NO_3^- and anerobic ammonia oxidation (Ammonox) bacteria have been identified in biofilms and probably also occur in soils. The use of nitrate as an electron acceptor is encoded by specific gene clusters but nitrate reduction also occurs in dissimilatory nitrate reduction.

1. Introduction

Nitrogen (N) dynamics in the soil–plant system are complex and difficult to study because the element is present in different oxidation forms, as soluble and gaseous compounds and as both organic and inorganic compounds that may be associated with soil minerals. It is characterised by a great range of different oxidation and reduction reactions and by exchanges with both the atmospheric and liquid phase. The N in the soil–plant system is prevalent in the organic phase. In water and in the atmosphere, the element (N_2) largely prevails over the inorganic and organic N compounds. There is a large amount of inorganic N primarily as NH_4^+-fixed by clays and some geological materials (Stevenson, 1986; Voroney and Derry, 2008). Humification processes and interaction with minerals control its long-term sequestration in the organic phase.. Ecological studies have been revolutionized by molecular and proteomic techniques during the last decade. These have allowed the detection of new species active in N reactions in soil and the determination of the expression of some of the responsible genes.

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We discuss the concepts employed in interpreting N mineralization–immobilization in soil, and how N turnover is related to both the chemical and functional characteristics of organic N. Recent findings on microorganisms involved in transformation of inorganic N, using molecular techniques in soil, will be discussed because they represent an example of how the modern techniques can change the picture of microbial N transformations in soil. We also discuss the application of proteomics that provide a better understanding of microbial synthesis of protein N in soil. This helps interpret soil enzyme activity and provides a better understanding of organic N formation and mineralization. We start this review with a short historical summary of the subject, before the arrival of electronic searches, to help use some of the classical literature to interpret today's challenges. The great wealth of early information and papers could not be covered and we apologize to authors of the great majority that were left out.

2. Review

2.1. The basics of nitrogen research

Lavoisier and his collaborators coined both the words *azote* and *nitrogene* for the newly discovered major component of the atmosphere and analysed nitrogenous constituents such as ammonia, saltpeter, and a multitude of plant types (Lawes et al., 1862; Aulie, 1970). The transfers and transformations of N and plant uptake remained a mystery until Bousingault's classic study of N incorporation into plants. Dumas and Boussingault in 1844 (Aulie, 1970) in their volume entitled "The Chemical and Physiological Behavior of Organic Nature" wrote "The atmosphere is a mysterious link that connects the animal with the vegetable kingdom and the vegetable with the animal kingdom". Justus von Liebeg in the book "Organic chemistry in its Application to Agriculture and Physiology" stressed the role of ammonia as the major component of the transfer mechanism between the atmosphere, precipitation, plants and animal excreta (Paul, 1976). The interchanges between Liebeg, with his ammonia theory, and Boussingault, Lawes, Gilbert, Pugh and Berzelius, who stressed N transformations, led to some interesting reading about the N cycle and its early researchers that is well worthwhile delving into (Aulie, 1970). Lawes et al. (1862) published detailed accounts of the uptake of N by plants grown continuously in rotations and with or without manure. This literature should be available in many libraries and is well worth an evening's perusal. The controversy resulted in efforts to measure nitrates and ammonia in air and rainfall. The investigations of plants grown under controlled conditions recognized the possibility that plants also could lose N. The difficulty of obtaining quantitative data for ammonia absorption was and still is a problem today (Viets, 1974; Binkley, 2002) and can be referred to as the study of occult N.

The latter part of the 19th century saw the establishment of the biological role of many of the nutrient cycling processes by Schloesing and Muntz and Warington (nitrification), Wollny (organic matter decomposition), Heilregel and Wilfarth (N fixation), Goppelsroeder and Gayon and Dupetit (N reductions and denitrification) (Waksman, 1952). The designation of the period between 1890 and 1910 as the golden age of soil microbiology can be attributed to the fact that at least one organism for the major transformations of the N cycle was isolated by early soil microbiologists such as Winogradsky and Beijerinck (Waksman, 1932). The hope, as enunciated by Caron and Löhnis, that a knowledge of the organisms would lead to the possibility of the management of the N cycle (Löhnis and Fred, 1923) however, did not come to fruition because of a lack of knowledge of their complex ecology, such as interactions between the organisms and their environment and the fact that so few of the soil organisms are culturable. Because of the availability of good microscopes, it was realized early in the 20th century that plate counts accounted for only ~1% of the bacteria observed using a microscope (Waksman, 1952). This difference was rationalized by the interesting statement that organisms that did not grow on a gelatin plate could not be involved in the N cycle and were therefore not worthy of study (Paul, 2007).

Norman (1946) pointed out that the belief that soil organisms could be specifically used to increase soil fertility hindered early microbiological analysis of N cycling. He also pointed out the problems of cumulative errors in balance sheets and the lack of adequate statistics. The observation that there are few specialists in the soil population applies directly to today's discussion on soil biodiversity. His discussion of the accumulation of nitrite in acidic soils is equally applicable to the present molecular analysis of the role of Archaea in nitrification and their possible role in the production of N_2O as a greenhouse gas (Prosser and Embley, 2002).

The first third of the 20th century saw the development of a good understanding of decomposition of plant products, the use of legume inoculants, a knowledge of the climatic factors affecting microbial growth and soil organic matter (SOM) decomposition and the role of the C:N ratio in mineral N accumulation (Waksman and Tenney, 1927; Jensen, 1929). The observation that added substrates that decompose rapidly supply nutrients but usually do not build up SOM led Löhnis (1926) to suggest the priming phenomena that was (Broadbent and Bartholomew, 1948) and still is (Fontaine et al., 2004; Blagodatskaya and Kuzyakov, 2008) the subject of controversy when tracers are added to soils. It is also the basis of present discussions, in present climate change policy, as to whether N additions and added plant residues result in the sequestration of soil C (Khan et al., 2007). The quality of the plant residues relative to turnover and possible priming and the effect of steady state conditions of SOM under specific conditions must also be considered. The possibility of soil population turnover was postulated by Richards and Norman in 1931. Harmsen and van Schreven (1955) suggested that the basic principles underlying N mineralization and immobilization were then understood. They, however, then pointed out that the relationships between C and N and the effects of environmental factors had to be determined for each soil type indicating the need to establish underlying principles.

The tracer era began in the mid 1930s when Rittenberger constructed the first mass spectrometer. The first soil tracer experiment was conducted by Norman and Werkman (1943) who reported that the decomposition, in soil, of soybean residues, labelled with ^{15}N fertilizer, resulted in only 20% plant uptake of the tracer. Most of the rest of the ^{15}N residue-N was incorporated into SOM. The review by Winsor (1958) noted the preferential utilization of ammonia by microorganisms, the usefulness of tracers to measure plant uptake and net N mineralization–immobilization rates, the calculation of the priming effect and the usefulness of using mathematical equations to describe N transformations (Kirkham and Bartholomew, 1955). Gainey's (1936) suggestion that SOM is composed of an active and passive fraction was further developed by Jansson (1958) as shown in Fig. 1. This shows the relationship between the internal N cycle and the input of mineral N to soil. The concept of an active fraction is common in today's models, and still the subject of research attempting to find a soil test for potentially available N (Bundy and Meisinger, 1994). The slow turnover rate of the N incorporated into SOM corresponds most closely to the dynamics of the slow pool of SOM. The significance of fixed NH_4^+ was considered by Jansson (1958) as was the fact that nitrification is outside the internal N cycle. Later it was shown that fertilizer, tracer ^{15}N added to soil decreased in availability with time (Legg et al., 1971) and the majority of N was immobilized as amino acids (Cheng and Kurtz, 1963; Stewart et al., 1963) and amino sugars (Isiriah and Keeney, 1973). The incorporation of ^{13}C and ^{14}C both at enhanced

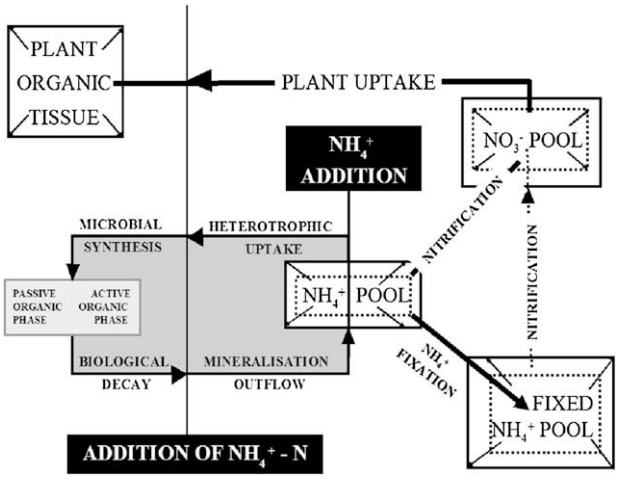

Fig. 1. Nitrogen reactions in the soil–plant system and the central role of mineralization–immobilization turnover. (Redrawn from Jansson and Persson, 1982.)

concentrations and at natural background concentrations led to breakthroughs in the measurement of the degradation of individual compounds (Jenkinson and Rayner, 1977) and the role of substrate quality and abiotic factors on decomposition (Scharpenseel and Schiffman, 1977; Ladd et al., 1981). The formation of organic N includes the fungal production of humic molecules by secondary metabolism induced by the addition of simple molecules such as glucose and nitrogen (Haider et al., 1975).

The fumigation–incubation method to determine microbial biomass of soil (Jenkinson and Powlson, 1976) was later modified to the fumigation–extraction method, which allows determining the native and labelled C, N, P and S present in microbial biomass. (Jenkinson, 1988). Microbial biomass C averages 2–5% of the soils C with a C:N ratio (~ 5:1), that is narrower than that of the soil humus components. Microbial biomass is both a measurable pool of nutrients that reacts to management and a transformation station through which decomposition products pass (Anderson and Domsch, 1989; Wardle, 1992). Paul and Juma (1981) calculated the turnover, expressed as the half life, of microbial biomass N to be 24 weeks (Median Residence Time-MRT = 35 weeks). Microbial biomass N was determined by the fumigation–incubation method. The active fraction of non-biomass N was measured by the total N and ^{15}N mineralized during laboratory incubation assuming that the recently immobilized ^{15}N was uniformly distributed within the active-N pool but not within the other N pools. Accordingly the non-active fraction was said to have a half life of 77 weeks (MRT = 111 weeks). Stabilised organic N released during the latter part of an extended incubation was determined to have a half life of 27 years. Old organic N related to that associated to resistant C by ^{14}C dating and assuming a constant C:N ratio for this fraction was determined to have a half life of 600 years (MRT = 870 years).

Only a portion of the microbial biomass appears to be active at any one time. Paul and Voroney (1980) found that ~12% of the microbial biomass C responded by growth to a ^{14}C-labelled substrate. The new growth had a decay rate of 1.3×10^{-1} days (MRT of 7.7 days) while the unlabeled biomass had a decay rate of 5.5×10^{-3} days (MRT of 182 days). This, as well as the study by Amato and Ladd (1992), indicates that there are soil-dependant control mechanisms for the turnover of the resting biomass.

It is only now that we are starting to recognize the complex, competitive interactions that ensure the longevity of native soil populations and at the same time make it difficult for introduced organisms, whether desirable or pathogens, to survive and function in the soil milieu. We still cannot manage the organisms except by indirect means such as substrate supply, host plants and to some extent cultivation or the lack thereof. A major worry today relates to what climate change will do to the organisms and processes we study.

2.2. Chemical structure and functional organic N pools

Nitrogen distribution, after acid hydrolysis, has been used to characterise indigenous and ^{15}N added as fertilizer or plant residues. The indigenous amino acids (Tables 1 and 2) comprised 36–39% of the organic N whereas the ^{15}N amino acids comprised 50–75% of the ^{15}N label after plant growth. Amino sugars, coming largely from fungi, accounted for 9.9% of the ^{15}N in a fertilized field but 13% where cellulose, creating immobilization of N by fungi, was added. Bacteria contained a low amount of amino-sugar ^{15}N as shown by Table 1 and amino sugars were not further formed on incubation of the bacterial cells for 100 days indicating little fungal growth on the labelled cell material. The unidentified, soluble N, where bacteria were added, probably could be partly attributed to nucleic acids. Neidhard et al. (1990) stated that 55% of the dry weight of *Escherichia coli* was proteinaceous and 23% was RNA–DNA. The unidentified N decreased significantly during 100 days of soil incubation while the proportion of ^{15}N accounted for as amino acids increased indicating that it is the amino compounds that are stabilised in SOM.

Ladd and Brisbane (1967) showed that one third of the N released from humic acids by acid hydrolysis could also be freed by the enzyme pronase, thus demonstrating that a large proportion of the amino acid N is present as protein–peptide N associated with humic compounds. Other analytical approaches used to identify organic N fractions such as Curie-point-pyrolysis gas chromatography/mass spectrometry (Cp Py-GC/MS) and pyrolysis-field ionization mass spectrometry (Py-FIMS) (Schulten et al., 1997), ^{15}N nuclear magnetic resonance spectroscopy (^{15}N NMR) (Knicker, 2007), and X-ray photoelectron spectroscopy and X-ray absorption near-edge structure (XANES) spectroscopy (Leinweber et al., 2007) confirm that proteinaceous materials are the most abundant organic N component.

There is still some uncertainty in the identification and measurement of heterocyclic N (Kogel-Knabner, 2006; Olk, 2008). The use of GC/MS, Cp Py-GC/MS and Py-FIMS (Schulten et al., 1997) indicated that heterocyclic N accounted for up to 35% of organic N; it however might be formed as an artefact during heating in Py-MS estimates. Its presence has been detected by ^{15}N NMR analysis in fire affected but not in fire unaffected soils (Knicker, 2007). Jokic et al. (2004a,b) using N *K*-edge XANES detected the presence of heterocyclic N compounds in soil and showed its abiotic formation. The "supramolecular association" of self-assembling heterogeneous and relatively small molecules has been proposed to occur during humification in soil (Piccolo, 2002).

Table 1
Comparison of indigenous nitrogen and ^{15}Nitrogen distribution (%) in a field and two incubation studies.

N type	Amino acids	Amino sugars	NH_4^+ after hydrolysis	Unidentified Soluble	Acid insoluble
Field ^{15}N application – growing season US corn belt (Allen et al., 1973)					
Indigenous	36	8.0	18	16	22
^{15}N	59	9.9	11	10	10
Incubated with cellulose + ^{15}N–8 weeks – Japan (Kai et al., 1973)					
Indigenous	39	16	12	15	21
^{15}N	50	13	14	18	5.2
Incubated with ^{15}N-labelled bacterial cells – Canada (Chu and Knowles, 1966)					
Indigenous	37	12	7	24	28
^{15}N 0 days	55	4	1	33	7
100 days	75	3	0.4	17	6

Table 2
Recovery of urea-N in soil under a sorghum–wheat rotation as percentage of the applied amount (from Nannipieri et al., 1999).

	Soil depth (cm)	Foggia		Rieti Piedifiume		Rieti Casabianca	
		Sorghum (%)	Wheat (%)	Sorghum (%)	Wheat (%)	Sorghum (%)	Wheat (%)
Soil							
Total N	0–20	25.9 ± 6.6	47.5 ± 6.2	33.6 ± 2.6	60.3 ± 5.6	63.5 ± 2.7	49.0 ± 12.9
	20–40	12.0 ± 1.4	45.3 ± 4.1	15.1 ± 3.3	8.4 ± 4.4	32.8 ± 1.3	13.4 ± 0.8
Non-exch.	0–20	0.09 ± 0.1	2.1 ± 1.1	14.0 ± 3.2	5.3 ± 0.4	24.6 ± 1.6	14.9 ± 3.8
NH_4^+–N	20–40	0.05 ± 0.0	1.5 ± 0.2	4.0 ± 0.3	0.1 ± 0.0	6.8 ± 0.6	3.0 ± 0.2
Exch.	0–20	0.04 ± 0.0	0.4 ± 0.0	0.06 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	0.2 ± 0.0
NH_4^+–N	20–40	0.01 ± 0.0	0.5 ± 0.0	0.01 ± 0.0	0.03 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
NO_3^-–N	0–20	2.0 ± 0.6	10.4 ± 0.0	1.2 ± 0.3	4.7 ± 1.4	2.0 ± 0.4	2.5 ± 0.6
	20–40	1.8 ± 0.2	10.4 ± 1.4	0.4 ± 0.0	0.3 ± 0.0	7.9 ± 0.0	0.7 ± 0.2
Microb.	0–20	0.8 ± 0.1	5.3 ± 1.3	4.7 ± 0.3	4.7 ± 0.4	5.0 ± 0.3	4.4 ± 1.2
Biomass-N	20–40	0.8 ± 0.2	3.5 ± 1.4	0.2 ± 0.0	1.1 ± 0.4	0.1 ± 0.0	1.8 ± 0.7
Plant							
Stalks		16.2 ± 0.7		16.3 ± 2.3		9.5 ± 2.6	
Straw			15.3 ± 4.4		7.8 ± 1.5		9.9 ± 1.0
Grain		12.3 ± 0.1	14.9 ± 1.5	23.2 ± 2.0	18.6 ± 2.7	11.1 ± 1.7	16.9 ± 2.8

The prevalence of amine N and the absence of appreciable amounts of heterocyclic N in measurements with ^{15}N NMR analyses have been taken as evidence that organic N is biologically formed in soil since it is difficult to abiotically form amide bonds (Lutzow et al., 2006). However, ^{15}N NMR spectroscopy is insensitive for detecting some organic N compounds due to the low ^{15}N abundance of soil (Vairavamurthy and Wang, 2002). The acid-insoluble N, shown in Table 1, has been partially identified as phenyl amino acids (Piper and Posner, 1972) and it can also include proteinaceous materials, that are non-hydrolysable due to their binding or occlusion by pedogenic oxides or tightly associated with clays (Kleber et al., 2007), and highly alkylated and substituted N heterocyclics, which are structural components of humic compounds (Leinweber and Schulten, 1998). The non-hydrolysable N fraction is a resistant but not an inert N fraction (Chu and Knowles, 1966; Kai et al., 1973).

The soil C:N ratio, once corrected for undecomposed plant residues, approaches a ratio of 8:1 in most soils. The C:N ratio, as well as the proportion of both C and N that are non-hydrolysable, decreases with depth in most soils (Stevenson, 1986) indicating that in the constituents of deeper soil some other factor is responsible for their great mean residence times. An extensive number of studies on the dynamics of N have related to the effects of C and N content, temperature (Stanford et al., 1975), residue particle size (Sims and Frederick, 1970), aggregation and soil type (Monreal et al., 1981; Sorensen, 1981; Christensen and Bech-Anderson, 1989), wetting, drying and grinding (Sorensen, 1974), waterlogging and anaerobiosis (Tusneem and Patrick, 1971; Yoneyama and Yoshida, 1977), and living plants (Huntjens, 1971). The N transformations in deserts have been particularly interesting because these soils often have quite narrow C:N ratios (Nishita and Haug, 1973). The significance of abiotic processes is often greater in decomposition in arid soils than the biotic effects (Parton et al., 2007). Plants inputs to forest soils, have wide C:N ratio and high lignin contents (Knowles and Chu, 1969; Overein, 1972). Mineral N accumulation can occur at C:N ratios much wider than in grassland and agricultural soils with more easily decomposed plant residues (Vitousek and Matson, 1985; Schimel, 1988; Nadelhoffer et al., 1995; Barrett and Burke, 2002).

Measured C:N ratios are narrower in high clay soils and in subsoils even after correction for clay-fixed NH_4^+ (Stevenson, 1986). This is especially true for recently formed microbial products that are associated with fine clays (Sorensen, 1981). Both C and N concentrations and the C:N ratio of particles decreases with the increase in particle density in different soils (Sollins et al., 2006). The low C/N ratio of organic layers on surfaces of particles of higher density in soil may be due to the adsorption of proteins and peptides with hydrophilic and hydrophobic domains. According to the "onion" layering hypothesis, proteins–peptides adsorbed by clay particles, could interact with organic matter through their hydrophobic domains (Sollins et al., 2006). Recent theories on the nature of organo-mineral bonds add further information on the role of clays in the stabilization of organic matter including amino acids (Kleber et al., 2007) and the attachment of microbial cells to particles involves the secretion of proteins or the action of surface-active microbial proteins (Bitton and Marshall, 1980; Dufrene et al., 1999).

Clay strongly affects stabilization of organic N directly (Ladd et al., 1996) and through the formation of aggregate-protected particulate and non-particulate organic matter (Gulde et al., 2008; Yoo and Wander, 2008). The bibliography on the interactions between proteins and clay particles is extensive (Stotzky, 1986; Nielsen et al., 2006), but most of these studies have been carried out *in vitro* using purified clay particles and purified proteins. The relevance of these studies to field conditions is questionable since both proteins and clay particles are rarely present as pure components. Proteins are released after cell death and lysis into the extracellular soil environment whereas surface-reactive particles such as clays are covered by Fe–Al oxyhydroxides and/or humic substances (Nielsen et al., 2006). Organic N is not the only N form by which N is stabilised in soil. Ammonium-fixing clays can also stabilise N as NH_4^+ (Stevenson, 1986). The percentage of ^{15}N enriched urea applied at 100 kg N ha^{-1} to three soils under sorghum–wheat rotation and present as non-exchangeable NH_4^+–N in the 0–40 cm soil layer at sorghum harvest was lower than 1% in a soil containing 45.6% non-ammonium-fixing clays. It was 18.0% in a soil with 15.6% ammonium-fixing clays and 31.4% in a soil with 50% ammonium-fixing clays (Nannipieri et al., 1999).

Mechanistic studies on cycling involving organically bound nutrients are often based on the subdivision of SOM into conceptual pools often described from C research and adapted to N on the basis of their C:N ratios (Parton et al., 1987; Paustian, 2001). Modelling has used a range of kinetics and differing degrees of substrate availability and protection with fairly well understood controls by abiotic factors (Plante and Parton, 2007; Cabrero et al., 2008). In addition to microbial biomass N, modellers have proposed several sub-pools for the non-living organic N: labile, chemically protected, physically protected, recalcitrant, etc. (Olk and Gregorich, 2006). Dissolved organic N is an easily measurable N pool that can be used to monitor bioavailable N in soil (Cookson et al., 2007).

Uncomplexed, organic C obtained by physical fractionation procedures is considered a labile pool. It is difficult to translate these into N pools since the content of this fraction is generally not related to N mineralization because of N immobilization occurring in the high-C fractions. Studies with particle size fractionation have shown that sand-size particles are enriched with plant polymers, silt-size particles with aromatic compounds and clay-size particles with microbial products (Lutzow et al., 2006). Interpretation of data from such studies requires caution because material transfer among particle size fractions and changes in microbiological properties of aggregates can occur during the specific fractionation procedures (Olk and Gregorich, 2006).

The history of N identification based on acid hydrolysis and separation of the hydrolysate into amino acids, amino sugars, resultant NH_4^+ and unknown N needs to be re-examined in light of the modern information that the majority of the soil N is proteinaceous in nature and often associated with minerals. The acid hydrolysis procedure itself requires redefinition in that although it is useful in obtaining an old C fraction it also contains some modern materials that are decomposable. The fact that acid hydrolysis changes the SOM so that more of it can be later pyrolyzed and released as amino acid products that had previously been stabilised by sesquioxides also must be used in further interpretation of soil N chemistry. Since nucleic acids comprise a significant proportion of microbial cells, their role as a soil N pool as well as in molecular studies also requires further research. The presence of heterocyclic N should also be evaluated to assess the importance of abiotic processes in humification. Nowadays the scientific debate on humification does not consider the intracellular production of humic molecules *in vitro* by some soil microorganisms reported by Haider et al. (1975) The stabilization of proteins and peptides by soil colloids has been investigated by using pure molecules whereas the involvement of other cell constituents, released together with proteins and peptides after cell lysis, has been neglected. These cell constituents affect the adsorption and persistence of extracellular DNA by clay minerals (Pietramellara et al., 2009). The development of an array of analytical methods determining the N content of meaningful organic pools and the determination of the active microbial biomass N is needed to better understand and quantify the N dynamics in soil.

2.3. Nitrogen mineralization–immobilization: reconciling classical and new views

The N mineralization–immobilization turnover (MIT), that is the conversion of organic N to NH_4^+ and the reverse process, that are both carried out by soil microorganisms, has been considered to have a major influence on the amount of bioavailable N in soil (Myrold and Bottomley, 2008). The similarity of controls in different soils as well as the similar proportions of C:N in most soils after the removal of plant constituents have lead to numerous attempts to measure mineralizable or potentially available N as an indicator for soil fertility (Clark and Roswell, 1981; Bundy and Meisinger, 1994), for interpreting ecosystem functioning (Schimel and Bennett, 2004) and for assessing soil quality (Drinkwater et al., 1996). Chemical methods, such as hot water extraction, heating in 2 M KCl, or absorbance of $NaHCO_3$ soil extracts at 200 nm light wavelength offer rapid, but not universally accepted, techniques for predicting bioavailable N. Biologically based-techniques, such as the short-term incubation that utilizes the naturally-occurring microbial enzyme systems to release the mineralizable N under anaerobic or aerobic incubations at various temperatures has been used. Isotopic dilution of ^{15}N that can take into account both mineralization and immobilization (Duxbury et al., 1989), has shown promise. The lack of universal adoption can be attributed to the cost and length of the period of analysis and inconsistencies with results from actual field requirements under differing climatic conditions where the plant needs, as well as the soil N supplying capacity vary (Mosier et al., 2004).

Hart et al. (1994) published a comparison of gross and net N mineralization–immobilization rates with microbial biomass C and N. They included a good literature review of forest-soil N. The effect of anthropogenic N on forest soils has been studied in detail (Aber et al., 1993; Frey et al., 2004). Nitrogen mineralization and immobilization are considered as single-step processes in the holistic approach but they are multi-enzyme processes (Ladd and Jackson, 1982). For example, mineralization of protein N to NH_4^+–N involves the release of amino acid N. This is first catalysed by proteases and then, the conversion of amino acid N to NH_4^+–N, is catalysed by amino acid dehydrogenases or amino acid oxidases. Urease activity is sometimes considered an indicator of N mineralization in soil despite the review by Ladd and Jackson (1982). However, it would be an indicator of urea hydrolysis in urea-fertilized soils. In contrast to most mineral soils from temperate regions, where first order kinetics adequately describe C and N mineralization, kinetics of tundra soils generally follow zero order kinetics in that substrate rarely becomes limiting with time and the breakdown of labile constituents is tied to lignin decomposition (Weintraub and Schimel, 2003).

Genetically engineered microorganisms, based on fusing gene reporters, such as *lux* (emission of light), *gfp* (green fluorescent proteins with emission of fluorescence) and *ina*Z (ice nucleation proteins with ice nuclei formation), with an inducible gene promoter (Lei et al., 2006) have been used to detect many compounds in soil including available N (Sørensen et al., 2006; Sørensen and Nybroe, 2006). DeAngelis et al. (2005), using two bacterial biosensors for detecting NO_3- availability, reported significantly lower NO_3- availability in the rhizosphere of wild oats than in bulk soil. The competition between roots and the whole-cell bioreporters could be attenuated by soil amendment with NO_3^-. Microbial biosensors now have tripartite systems for the simultaneous detection of bioavailable C, N and P (Standing et al., 2003).

Both N mineralization and N immobilization processes occur simultaneously and thus net N mineralization is measured unless the ^{15}N dilution technique is used to determine gross rates of the two processes (Murphy et al., 1999). Net mineralization can be fitted either to hyperbolic or first order equations (Stanford and Smith, 1972; Juma and Paul, 1984) and in most mineral soils it is affected by immobilization during early incubation; thus longer term incubations more realistically describe the release of soil N. The addition of ^{14}C and ^{15}N-labelled compounds absorbed to different-size fractions has shown greater decomposition rates in the presence of plants especially when absorbed to fine clays (Cortez and Hambed, 1992). Sanchez et al. (2002) found similar data when maize grown on soils, with previously incorporated legume residue, released more N than fallow management. This was confirmed when subsequent incubation, after the field treatment, showed a lower mineralizable-N pool in the soils that had previously grown a maize crop.

The MIT process does not always occur in soil because microorganisms can take up amino acids, with intracellular deamination and release of surplus NH_4^+ (Barak et al., 1990; Barraclough, 1997); the uptake of low molecular weight organic compounds, such as amino acid, by soil microorganisms was called "direct route" (Barraclough, 1997) or "direct pathway" (Manzoni et al., 2008). For this reason, the formation of dissolved organic N (DON) rather than NH_4^+ production can control N dynamics in soil (Cookson et al., 2007). Urea uptake by soil microorganisms with intracellular hydrolysis and successive immobilization of NH_4^+, as determined by a ^{13}C-urea tracer technique by-passed the N mineralization–immobilization

pathway and was very high and exceeded the gross N mineralization (Nielsen et al., 1998). The uptake of low molecular weight organic N compounds by plants can occur in Arctic tundra (Kielland, 1994), boreal (Jones and Kielland, 2002), alpine (Raab et al., 1999), wetland (Henry and Jefferies, 2002), desert (Schiller et al., 1998) ecosystems and in other systems where N supplies are limited (Schimel and Bennett, 2004). It can also occur in N fertilized soils, when the availability of the added fertilizer N markedly decreases after a few days due to microbial N immobilization (Nannipieri et al., 1990). Plants infected by mycorrhizae can use both low (amino acids, amino sugars and peptides) and high molecular weight organic (proteins) N compounds as N sources (Schimel and Bennett, 2004). In many ecosystems, the critical process is not N mineralization but the depolymerization of N-containing compounds (Fig. 2) due to the activity of enzymes, such as extracellular proteases released by microorganisms (Chapin et al., 2002).

Once organic N is formed by soil microorganisms, three processes can occur: i) organic N can be mineralized to NH_4^+–N, which is then released in the extracellular soil environment (Jansson and Persson, 1982; Barraclough, 1997); ii) organic N can be released after cell death or microbial stress (Schimel and Bennett, 2004); iii) mineral N can be released after micro/meso-faunal grazing (the microbial loop) (Clarholm, 1994). Bacterial-grazing protozoa and bacterial-feeding nematodes can cause NH_4^+ excretion, with increases in bioavailable N in soil. They can also change the composition of bacterial communities, with selection of plant growth promoting rhizobacteria in rice and tomato rhizosphere and consequent stimulation of root growth, promotion of a different root architecture and better nutrients uptake (Fig. 2) (Kreuzer et al., 2006; Mao et al., 2006). Soil microorganisms are usually better competitors than plants for available N in soil. About 40% of ^{15}N enriched urea applied to a sward containing 20% *Dactylis glomerata*, 20% *Lolium*, 40% *Medicago sativa* and 20% *Lotus corniculatus* was immobilized as microbial biomass N after 9 days (Nannipieri et al., 1990). The fertilizer N taken up by the grass and legume was quantitatively lower than the microbial N immobilization, amounting to 12.3% of the applied urea-N 45 days after urea application.

Depolymerization of organic N, competition between plants and microorganisms and differences among microsites have been incorporated into a conceptual model describing the behavior of bioavailable N in terrestrial ecosystems N (Schimel and Bennett, 2004). According to this model, N cycling in microsites depended on the presence of available N and four different situations were hypothesised: i) N-poor ecosystems, such as boreal, arctic and alpine ecosystems, are characterised by no N mineralization to NH_4^+, uptake of low molecular weight organic N compounds by both plant and soil microorganisms, and better competition of soil microorganisms than plants for bioavailable N; ii) bioavailable N can increase in some microsites and thus there are rich and poor N microsites. The presence of negatively charged particles can limit NH_4^+ diffusion; iii) the number of microsites with available N prevails over the N-limited microsites and most of soil microorganisms use local organic N substrates rather than depending on N diffusing from other microsites. This situation can decrease the competition between plants and microorganisms and plants can have access to NH_4^+–N mineralized by microorganisms. The NH_4^+ still prevails over NO_3^- since both plants and soil microorganisms limit the supply of NH_4^+ to nitrifiers; iv) both bioavailable N and NH_4^+ are present in non-limiting concentrations; NH_4^+ can be used by nitrifiers, NO_3^- becomes the dominant mineral N form and plants also take up NO_3^-. This situation can occur in agricultural systems and N-rich forests when the amount of mineral N increases in soil; plant uptake of organic N is less important than in the other cases since high cellular NH_4^+ concentration can inhibit amino acid uptake as it occurs in conifers (Persson and Nasholm, 2002).

The model proposed by Schimel and Bennett (2004) did not consider the effects of the composition of microbial communities involved in N reactions. Delays in nitrification can occur when *Nitrosospira* cluster 3b and *Nitrosomonas*, but not *Nitrosomonas* cluster 3a are the prevailing nitrifiers in soil (Webster et al., 2005). Microsite effects and the internal portions of aggregates or spatial heterogeneity have been associated with nitrification and denitrification, as well as with general soil heterogeneity (Frey, 2007). Interpretation of the patterns, determination of soil biodiversity (Bardgett et al., 2005) and the role of scale and soil particle size on soil processes (Lavelle et al., 2004; Standing et al., 2007) are being examined in an increasing number of ecosystems (Sessitch et al., 2001; Schimel et al., 2005; Standing et al., 2007; Manzoni et al., 2008).

Soil was compared by Kubiena (1938) to a city whose architecture is destroyed after soil sampling and breakdown of soil aggregates (Fig. 3). Any determination involving soil extracts, such as the characterization of DNA for evaluating the structure of microbial

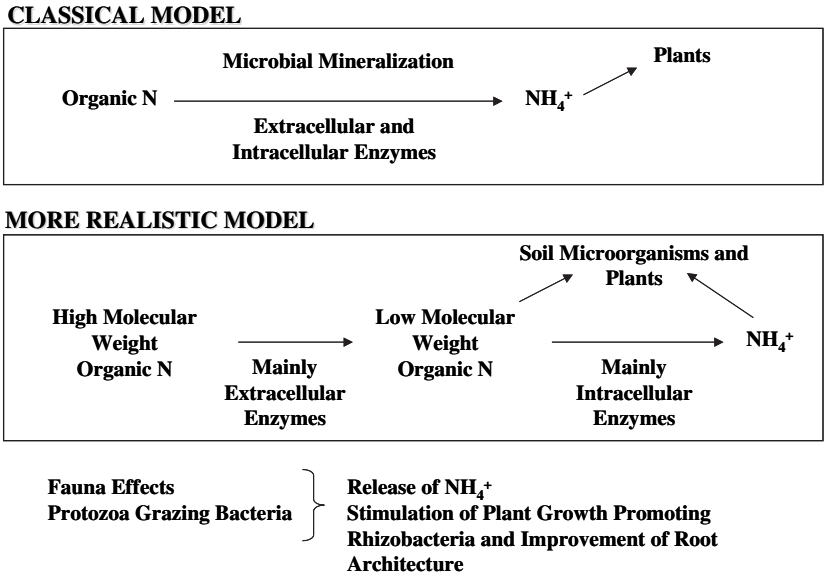

Fig. 2. Classical versus more realistic models concerning N mineralization.

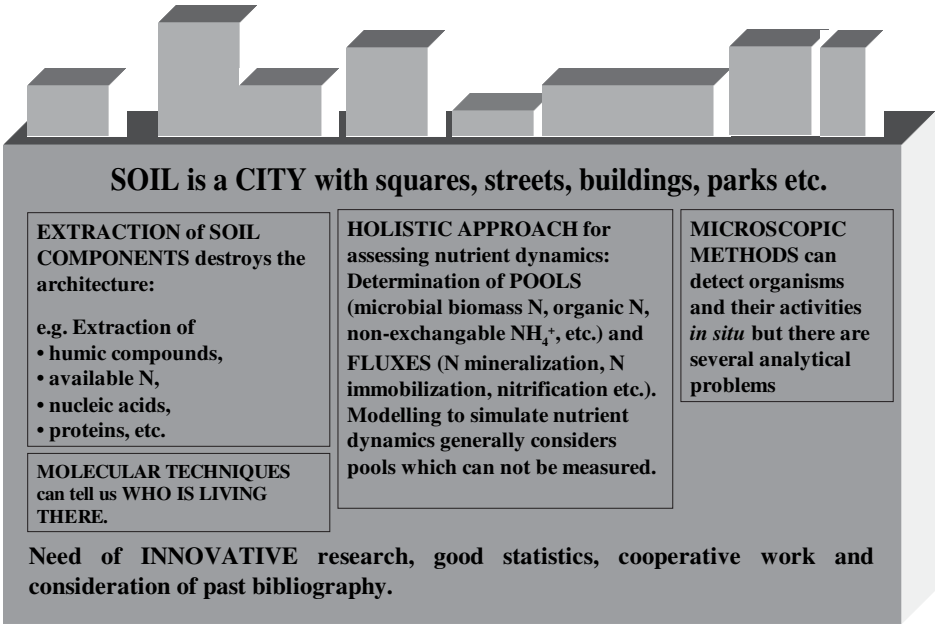

Fig. 3. Soil has been considered by Kubiena (1938) like a city whose overall architecture is destroyed after sampling and handling the soil sample to be analysed. All analytical approaches (chemical extraction, holistic approach to study the dynamic of soil nutrients, and microscopic analysis) present problems.

communities, does not permit the localization of the measured variables in the soil matrix. The holistic approach requires monitoring the dynamics of a nutrient in soil and evaluating its distribution in the main pools. The use of stable isotope probes that separate labelled from unlabeled DNA can distinguish active from non-active microbial biomass and measure microbial growth. However, it is not possible to identify "hot spots" of microbial activity. Fluorescence *in situ* hybridization (FISH) has been used to detect soil microorganisms in undisturbed soil samples (Eickhorst and Tippkötter, 2008). Scanning electron microscopy and transmission electron microscopy of soil sections treated with cytochemical or histochemical methods have been also used to detect the localization of microorganisms, fauna, enzymes, etc. in the soil matrix (Foster and Martin, 1981; Ladd et al., 1996). Bacteria are often largely confined to the periphery of degrading plant residues. Organic deposits become highly electron dense as their size decreases making it impossible to determine their origin (Foster and Martin, 1981). Acid phosphatase activity has been detected in small (7×20 nm) fragments of microbial membranes, roots, mycorrhizae, etc. of soil. The presence of naturally-electron dense soil components (minerals) and soil components reacting with OsO_4 (humus) does not allow the detection of extracellular proteins associated with clay minerals and humic components, respectively, by electron microscopy. (Ladd et al., 1996).

In spite of 70 years of effort, one of the major goals of N research, that of being able to predict N mineralization, bioavailable N and fertilizer N needs, has not been adequately achieved. The over application of both fertilizer N and natural N supplies is leading to major pollution of the earth's water and atmospheric resources. Some of the answers lie in better education and policy enactment, but a better understanding of plant and microbial needs also is required. This involves better modelling of N mineralization–immobilization reactions under varying climatic conditions. It also involves better understanding of plant needs and ongoing research will be required. There are a number of indications that plant–microbial–faunal interactions could be better managed to supply soil organic N (Coleman et al., 2004). Maize plants and their associated soil biota, in the presence of mineralizable soil N have been shown to be able to obtain significant amount of extra N (Sanchez et al., 2002). This merits further investigation but past history shows that a successful approach, where the N needs for plant growth can be satisfied by mineralizable N, requires a better understanding of the basic concepts involved and better management of the plant soil system. Soil processes and microbial growth occur in many microsites of the soil matrix and methods that completely alter and destroy the soil architecture in order to study either of them must be improved. Biosensory organisms that can be detected *in situ* can be used (Nannipieri and Smalla, 2006).

2.4. The proteomic approach to soil N studies

Molecular techniques for detecting DNA sequences have been extensively used in soil (Nannipieri and Smalla, 2006). There are many factors affecting gene expression and thus DNA measurements, by themselves, are not indicative of soil processes. Monitoring the expression of gene sequences at both transcription and translational levels still has methodological problems. The analysis of the target mRNA represents one opportunity to analyse the activity of the target gene. Despite the fact that extraction procedures of soil RNA and DNA are similar in principle, successful extraction and characterizations of mRNA from soil has lagged that of DNA due to problems such as activity of nucleases and fast turnover rate of prokaryotic mRNA (Bakken and Frostegård, 2006). However, several methods are now available to characterise mRNA and thus to measure gene expression in soil (Krsek et al., 2006). The first study linking the presence and expression of genes to the measured enzyme activity (chitinase activity) in soil was carried out by Metcalfe et al. (2002). Primers targeted to a gene fragment from family 18 (one of the two main groups in which chitinases have been classified according to amino acid similarities within the catalytic domain of the enzyme molecule) were used to determine the diversity and origin of chitinases after analysis of DNA extracted from a pasture soil. The addition of sludge to the pasture soil increased the chitinase activity and the number of actinobacteria but decreased the diversity of chitinase enzymes. Extraction of transcripts was unsuccessful, probably due to the adsorption of mRNA by soil colloids, and the target enzyme proteins were not

monitored. The interrelation of DNA, RNA, protein and substrate activity has been called the meta approach.

Multiple protein isoforms can be synthesised by a single gene because mRNA molecules can be subjected to post-transcriptional control such as alternative splicing, polyadenylation and mRNA editing (Graves and Haystead, 2002). Microbiologists have extensively used protein measurements (proteomics) and substrate measurements or metabolites (metabolome). This is now being augmented by much better methodology. Protein expression in pure culture has been extensively measured by 2-dimensioal gel electrophoreisis. The availability of high efficiency, ionization mass spectrometers has resulted in the development of rapid and sensitive protein identification from solution (Pandey and Mann, 2000).

The characterization of proteins, synthesised during gene expression, is probably the best approach for monitoring gene expression in soil (Nannipieri, 2006). The application of proteomic studies to soil needs to consider two protein sources. Intracellular proteins could be extracted from soil microbial populations. The characterization of extracellular proteins, protected against proteolysis by their association with soil colloids, should give further insights into mechanisms responsible of such stabilization and thus verify the validity of theories such as the "onion" theory discussed earlier (Section 2.2). The two approaches have been termed *soil functional* proteomics and *soil structural* proteomics, respectively (Nannipieri, 2006). The extraction of intracellular proteins from soil populations is complicated by the large background of extracellular organic N. Indeed, microbial N accounts, for an average, for 4% of organic N in soil whereas the majority of the total organic N in soil is present at least partially, as extracellular protein N or peptides N stabilised by soil colloids, as discussed earlier. A successful extraction of intracellular proteins from soil should inhibit proteases, avoid the adsorption of proteins once released after cell lysis by soil colloids and be representative of the status of microorganisms inhabiting soil. The manipulation of the sample prior to extraction should not alter microbial physiology.

The study of functional proteomics can improve our understanding of degradation of organic pollutants and organic debris, nutrient cycling, blockage of inorganic pollutants, molecular colloquia between microorganisms, between plant roots and microorganisms and between plant roots (Nannipieri, 2006). As it is done for the extraction of nucleic acids from soils, separation of microbial cells from soil particles and successive cell lysis with release of proteins or cell lysis *in situ* with extraction of proteins is an alternative. The microbial extraction prior to lysis should give purer samples but with lower yields. In addition, it was considered to give artefacts due to possible changes in the physiology of microorganisms during the extraction (Nannipieri, 2006) making it difficult to relate the microbial proteins to the effects of the studied factors (i.e. stresses, agriculture management, etc.). Maron et al. (2008) used the indirect extraction approach to show that copper or mercury pollution of soil stimulated the synthesis of protein with molecular weights ranging from 20 to 50 kDa; some of these proteins, such as those of heavy metal efflux pumps, were involved in heavy metal resistance mechanisms.

Other reports on soil proteomics are based on the direct extraction method. Singleton et al. (2003) extracted proteins from soil with bead-beating or freezing–thawing cycles of soil suspended in Tris–HCl buffer containing a protease inhibitor cocktail, sucrose dithiothreitol and EDTA. They showed that cadmium pollution of soil decreased the content of extracted proteins, as determined by the Bradford method, but stimulated the synthesis of low molecular weight (<21 kDa) proteins. These proteins were thought to be metallothioneins produced by eukaryotic microorganisms in response to the Cd pollution, and partially hydrolysed proteins released after cell death and lysis. Muranase et al. (2003) obtained clear patterns in SDA-PAGE of proteins extracted by phosphate buffer at pH 6.0 and detected a homologue of a thermostable cellulase of the thermophilic fungi of the genus *Humicola*. In addition to bead-beating or freezing–thawing cycles of soil suspended in buffers, Ogunseitan (2006) has proposed sonication of phosphate–EDTA soil suspensions.

Autoclaving of citrate buffer soil suspensions has been used to determine glomalin-related proteins, glycoproteins said to play an important role in soil structure (Wright and Upadhyaya, 1996, 1998). However, both colorimetric (Bradford method) and immunological (ELISA) techniques can react with not only proteins but also phenolic compounds and litter and humic components (Rosier et al., 2006; Whiffen et al., 2007; Roberts and Jones, 2008). Schindel et al. (2007) found that NMR spectra of glomalin-related proteins were similar to those of humic substances and not to those of typical glycoproteins. Also in this case, those reports generated before the arrival of the electronic search systems have been neglected because in the sixties it was established that colorimetric methods determining components of soil extracts could give artefacts (Stevenson, 1986).

Proteins have been extracted from dissolved organic matter of forest soil, purified by gel filtration, with removal of humic acids, phenolic compounds and small molecules, and concentrated by ethanol before SDS-PAGE (Schulze, 2004). After silver staining, each protein band was cut, digested by trypsin and mixtures of tryptic peptides separated by nanoflow liquid chromatography prior to analysis by mass spectrometry. The phylogenetic origin of proteins was evaluated and bacterial proteins were classified as ribosomal, transcription, membrane and as enzyme proteins according to their function. The power of the analytical MS tool was also shown by detecting chlorocatechol dioxygenases, enzymes involved in the degradation of 2,4-D (2,4-dichlorophenoxy acetic acid), after mass spectrometry analysis of tryptic peptides obtained from excised bands of SDS-PAGE gels, obtained by electrophoresis of protein preparations extracted by 0.1 M NaOH from 2,4-D treated soils and successively purified (Benndorf et al., 2007).

The proteomic approach builds on the rich legacy of enzyme studies in soil. The enzyme studies however have not delivered on the initial hope that being able to directly monitor the agents of a change in soil would lead us to a better understanding and possibly even improved management of our rich agricultural and ecosystem resources. Among the reasons for this has been the protection of enzymes by the soil matrix such that they persist for extended periods and the complexity of the matrix such that the enzymes could be separated from their substrate. The new automated techniques that have accelerated the analysis of hundreds of samples in a short time period is greatly expanding our knowledge and the tie in of proteomics with other molecular techniques will hopefully result in the breakthroughs that those of us long in this field have been hoping for.

2.5. New reactions and new microbial species

Most of the research on microbial N transformations in soil has been focused on a limited set of microbial pathways: i) symbiotic and asymbiotic N fixation; ii) N mineralization–immobilization turnover; iii) autotrophic nitrification; iv) heterotrophic denitrification. Recent studies suggest that the picture of microbial N transformations is probably more complex due to the following other processes that may occur in soil:

i) Nitrifier denitrification, that is the oxidation of NH_4^+ to NO_2^- followed by the reduction of NO_2^- to NO, N_2O and N_2; this process is carried out by the ammonia-oxidising bacteria (AOB) and the enzymes involved are similar to those of denitrifiers (Wrage, 2007; Norton, 2008).

ii) Anaerobic ammonia oxidation (Anammox) involves oxidation of ammonia to N_2 with reduction of nitrite (Jetten, 2001); this process has been shown to occur by ^{15}N-labelling experiments in different water treatment facilities and environments but not in soil (Schmid et al., 2005). So far, four anammox bacterial genera have been identified and they can be used as alternative N removal processes. Dr Marc Strous and Professor Mike Jetten (both at University of Technology, Delft, Netherlands) have proposed that the anammox bacterial species can reduce nitrite to hydroxylamine, which can condense with ammonium to hydrazine (N_2H_4); the formed hydrazine is eventually oxidised to N_2 and the released electrons are used to reduce nitrite. The enzyme capable of oxidising hydroxylamine and hydrazine has been extracted from anammox bacteria and accounts for about 10% of the total protein content of the cell. The anammox bacteria can coexist with AOB and nitrite-oxidising bacteria (NOB) in biofilms of oxygen-limited anammox reactors (Kindaichi et al., 2007) and it seems reasonable to hypothesise their presence in soil under anaerobic conditions. Further research should use molecular techniques with polymerase chain reaction (PCR) amplification of specific genes extracted from soil. A potential application of anammox bacteria might be to control odors (from ammonia) generated from the storage and spreading of animal wastes.

Autotrophic nitrification, that is the oxidation of ammonia to nitrate in soil, is due to the activity of autotrophic ammonia- and nitrite-oxidisers whereas heterotrophic nitrification by heterotrophic bacteria and fungi, is the oxidation of either organic-NH_2 or NH_4^+ with the use of organic Carbon as an energy source; this process is supposed to occur under acidic conditions (Kowalchuk and Stephen, 2001; Prosser and Embley, 2002; Jordan et al., 2005). Unculturable AOB can now be determined by extracting DNA from soil and by successive PCR amplification of the *amoA* gene (Kowalchuk and Stephen, 2001), which is responsible for the synthesis of one of three subunits of ammonia monooxygenase (AMO), the enzyme catalyzing the conversion of ammonium to hydroxylamine. The other two subunits are synthesised by *amoB* and *amoC* genes (Nicol and Schleper, 2006).

It has been recently shown that Archaea could be also ammonia oxidisers (AOA) after: i) the extraction from soil of 16S rRNA with a group Archaea along side genes encoding potential homologues of bacterial amo A and B subunits and a neighbouring *amoC*-like gene (Nicol and Schleper, 2006); ii) the increase in Archaea *amoA* expression in the presence of ammonia; and iii) the identification of genes encoding potential ammonia permease, urease and urea transporters in some Archaea species (Nicol and Schleper, 2006). Archaea *amoA* genes copies and their activities, measured by reverse transcription quantitative PCR studies and complementary DNA analysis using novel cloning-independent pyrosequencing technology, largely prevailed over copies and activities of bacteria *amoA* genes in both pristine and arable soils (Leininger et al., 2006). Future research should discover Archaea genes encoding hydroxylamine oxido-reductase, the enzyme catalyzing the conversion of hydroxylamine to nitrite, evaluate the role of Archaea in nitrifying activity, determine the effects of soil properties on both abundance and activity of AOA and AOB, and evaluate the capacity of AOA to produce N_2O as it occurs in AOB since N_2O is a greenhouse gas with 296 times the global warming potential of CO_2 (Houghton et al., 2001).

Under anaerobic conditions, nitrate can be used as an electron acceptor by anaerobic, facultative bacteria with formation of gaseous intermediates (NO and N_2O) and N_2 as the final product, through five reactions catalysed by dissimilatory nitrate reductase (Nar), dissimilatory nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase (N_2Or) (Tiedje, 1994). These enzymes are encoded by specific gene clusters; however, the first reduction from NO_3^- to NO_2^- is not specific to denitrification since it also occurs in the dissimilatory nitrate reduction to NH_4^+, and thus primers based on genes of the other denitrification enzymes are used to determine unculturable denitrifiers (Philippot, 2002). Dissimilatory reduction of NO_3^- to NH_4^+ usually occurs when there is an excess of available carbon with respect to the NO_3^- concentration and it is carried out by both anaerobic and facultatively anaerobic bacteria (Takaya, 2002).

For over a century, denitrification has been considered a process carried out by prokaryotes until it was identified that some fungal species, such as *Fusarium oxysporum*, can use nitrate as electron acceptors in the absence of oxygen (Takaya, 2002). The enzymes responsible for the dissimilatory reduction of nitrate to N_2O are coupled with ATP production and thus they have a different location in the cell with respect to those involved in the nitrate dissimilatory reduction by bacterial cells. These fungal species contain both a cytochrome P450nor (P450nor) catalyzing the reduction of NO to N_2O and a flavohemoglobin (fhb) converting NO to nitrate and acting as a detoxifying system (NOD). After the discovery of fungal denitrification, dissimilatory mechanisms were discovered in actinomycetes, considered to be strictly aerobic (Kumon et al., 2002). By using specific bacterial (streptomycin) and fungal (cycloheximide) inhibitors and the ^{15}N gas flux method, Laughlin and Stevens (2002) concluded that fungi were responsible for most of N_2O evolved from temperate grassland soils from Northern Ireland. However, data from biocides studies should be considered with a certain caution due to both the non-target effects of these compounds on soil microflora and their degradation in soil (Badalucco et al., 1994).

The organisms and enzymes involved in the oxidation and reduction of mineral N are limited in number relative to the large populations involved in mineralization–immobilization. Molecular techniques that do not require culturing have recently identified the nitrifying Archaea and the anammox bacteria. That these exist and will be found in soils is without doubt. Also bacteria have been the dominant players. The possible role of fungi in denitrification and of Archaea in nitrification expands the role of these organisms in the many questions concerning biodiversity. The hope for controlling negative environmental effects of the N cycling organisms has been based to some extent on the limited number of organisms and reactions involved. Nature, without disturbance, operates primarily through the organic-ammonium phase of N uptake and transformations. We need to strive to go as much as possible back to that mode of operation. That means not adding more mineral or organic fertilizer than is absolutely necessary at the time of plant uptake. It also means encouraging our systems to operate as much as possible by short circuiting the nitrification process. Without this disease of disturbance and excess N, there is little loss to the environment and plants especially with mycorrhizas can effectively operate on low molecular weight organic N and NH_4^+. Denitrification is the major process retuning fixed N back to the atmosphere. When the reaction goes to completion with the production of N_2 there are no toxic intermediates. This process is especially active in sewage treatment plants and wetlands that intercept nitrate from soils to streams and to some extent groundwaters. Available sources of energy (carbon) and anaerobic conditions drive the reaction to N_2 without toxic intermediates and these conditions should be employed where possible.

3. A look to the future on the basis of what we now know about nitrogen today

The 170 years since the first detailed analysis of soil N processes was initiated by Bousingault, Lawes, Gilbert and Liebig (Aulie, 1970; Paul, 2007) have seen the identification of the major processes and

organisms involved. The golden age of microbiology at the beginning of the last century identified at least one organism involved in nitrification, denitrification, N mineralization and both symbiotic and asymbiotic fixation and the realization that *in toto* these processes completed the N cycle. The processes of decomposition of both litter and soil organic matter, immobilization and nutrient interactions together with meaningful examination of C:N ratios were established by 1950. The advent of tracers especially ^{15}N together with better analytical techniques and mathematical descriptions of the various interactions led to analytical description of the individual processes, measurements of inputs and losses and the understanding that immobilized mineral N enters the slow pool of soil organic matter. The recognition of enzymes and of N in the stabilization of soil organic matter, with a fairly constant C:N ratio, as well as the concept of fixed ammonium in the clay fraction led to further understanding and a holistic approach to N cycling. The use of ^{15}N dilution made it possible to measure both gross and net mineralization and immobilization processes but the extensive number of required measurements with sophisticated equipment in soils with great microsite variability makes this approach difficult for routine analysis even today (Myrold and Bottomley, 2008).

Bioavailable N is an important concept in soil fertility and ecosystem functioning (Griffin, 2008). Both chemical and biological methods have been used to determine bioavailable N, but they are not universally adopted probably due to inconsistencies in N mineralization and immobilization turnover and differences in field requirements under differing climatic conditions where the plant needs, as well as the soil N supplying capacity, vary (Mosier et al., 2004). New approaches such as biosensors have promise for measuring soil N available to microorganisms at the microscale level and they may assist in interpreting and verifying models interpreting microscale functioning (Schimel and Bennett, 2004). These measurements are done with soil slurries and thus under conditions differing from those *in situ*.

Losses of N from soil to the atmosphere as N_2 completes the N cycle initiated when N is fixed either by the Haber Bosch process, biological fixation or combustion and lightning. However, there also are transfers to the environment as NH_3, N_2O and NO_X. Transfers to low N soils and vegetation can result in enhanced growth especially in a CO_2 enhanced atmosphere. They can also lead to changes in the structure and function of the soil microbial community (Frey et al., 2004) and overall ecosystem responses as shown for the mountainous soils and vegetation undergoing the effects of N enrichment (Baron et al., 2000). Most native systems without disturbance exist as NH_4^+-organic N uptake systems. It is only in the presence of excess N that nitrification occurs producing gaseous intermediates and water pollution. Much of the present loss of N from the soil can be attributed to excess fertilization and poor manure management (Raun and Schepers, 2008). Education and policy implementation should be used to correct some of this. Further research to stop nitrification and enhance organic uptake of N could supply great benefits to society.

The use of molecular techniques has characterised the movement of our science into the 21st century and this period could possibly be called a second golden age of soil microbiology. These techniques allowed determining unculturable species in soil and the measurement of the role of soil biodiversity as well as functional genes in the N cycle. Microbial diversity is probably less important when studying N mineralization and N immobilization processes than when studying processes such as nitrification, which are carried out by a limited group of microbial species (Norton, 2008). There are many organisms involved in the decomposition of organic matter, N mineralization and N immobilization processes; a reduction in any group of species has little effect on overall macroscale processes because the surviving microorganisms can carry out the reactions (Nannipieri et al., 2003). Microbial composition and diversity should be important at the microscale level where only a few species are present. Additional insights into N cycling can be obtained by studying microbial synthesis of protein N, which is the main organic N component in soil. A better knowledge of microbial synthesis of proteins (soil functional proteomic) can improve the understanding of the synthesis of organic N in soil whereas the characterization of proteins protected against microbial degradation by their interactions with surface-reactive particles or their inclusion within humic component (soil structural proteomic) can give insights on the stabilization of organic N in soil (Nannipieri, 2006). Although most soil N is now thought to be of an amide nature a better characterization of heterocyclic N, which is thought to arise during abiotic synthesis such as in char formation, is required.

Molecular and proteomic techniques are becoming ever more easy to apply to the soil and its matrix components. Further advances in some of these techniques are essential for better understanding the localization of microbial processes such as N immobilization and mineralization in the soil matrix and for testing the validity of models describing the functioning of these processes at microscale levels. However, new approaches that we have not even mentioned are also needed. We agree with Andren et al. (2008) who pointed out the need for imaginative research, good statistics and cooperative work for solving problems that still hinder understanding today. We also argue that the future is best built on a good understanding of the past and the detailed reading and understanding the original and past literature in the field is essential for successful future breakthroughs.

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