

Stable isotopes and changing paradigms on soil nitrogen and carbon biogeochemistry

J.A. Carreira¹, B. Viñegla¹, M.C. Blanes¹, R. García-Ruiz¹

(1) Área de Ecología. Dpto. de Biología Animal, Vegetal y Ecología. Facultad de Ciencias Experimentales. Universidad de Jaén. Campus Las Lagunillas Edif. B-3, 23071-Jaén, España

➤ Recibido el 9 de junio de 2010, aceptado el 26 de julio de 2010.

Carreira, J.A., Viñegla, B., Blanes, M.C., García-Ruiz, R. (2010). Stable isotopes and changing paradigms on soil nitrogen and carbon biogeochemistry. *Ecosistemas* 19(3):14-23.

Many perceptions on ecosystem biogeochemistry are based on flow diagrams in which element pools (boxes) are interconnected by abiotic and biotic mechanisms controlling transformations of chemical species and flows among the pools (arrows). Because of the ability of stable isotopes to integrate such processes over time and space, they have played a central role in our current understanding of nutrient cycling, particularly in the cases of N and C. Most fluxes and transformations involved in terrestrial nutrient cycling cross over or take place in soil compartments. We here review the development of new paradigms in soil nitrogen (and carbon) cycling research, to which stable isotopes contributed through three main approaches: (i) as integrators of nutrient input/output budgets from broad ecosystems compartments or "black-boxes", (ii) as tracers to unravel specific processes and end-member pools operating within these black-boxes, and (iii) as markers or indicators of nutrient use, availability and deficiency to plants. New challenges and future perspective to that respect are also discussed.

Key words: nutrient cycling, isotope techniques, conceptual models, new perspectives.

Carreira, J.A., Viñegla, B., Blanes, M.C., García-Ruiz, R. (2010). Isótopos estables y cambios de paradigma sobre la biogeoquímica del nitrógeno y del carbono en el suelo. *Ecosistemas* 19(3):14-23.

Muchos de los modelos conceptuales e ideas desarrollados en el ámbito de la biogeoquímica se basan en diagramas de flujo que representan las reservas de nutrientes como "cajas" interconectadas por "flechas" que indican los procesos abióticos y bióticos que regulan su transformación y flujo entre reservas. El uso de isótopos estables permite integrar dichos procesos en el espacio y en el tiempo, y por ello han jugado un papel central en la comprensión de los ciclos de nutrientes como el N y el C en los ecosistemas, y en particular en el suelo. Aquí revisamos el proceso de cambio de paradigmas que ha tenido lugar respecto al reciclado de estos nutrientes en el suelo; un proceso en el que los isótopos estables han contribuido significativamente a través de tres aproximaciones principales: (i) como integradores en balances de entrada/salida que consideran como "cajas negras" a compartimentos amplios del ecosistema, (ii) como trazadores para revelar procesos intermedios que operan dentro de las "cajas negras" y el destino final de los nutrientes, y (iii) como marcadores o indicadores de uso, disponibilidad y estrés por nutrientes en las plantas. También se discuten los retos actuales y las perspectivas de futuro a este respecto.

Palabras clave: ciclos de nutrientes, técnicas isotópicas, modelos conceptuales, nuevas perspectivas.

Introduction

Most perceptions and conceptual models on ecosystem biogeochemistry are based on flow diagrams in which element pools, represented by boxes, are interconnected by arrows which denote the physical or biological processes controlling transformations of chemical species and flows among the pools, as shown in **Figure 1**. Because of the ability of stable isotopes to integrate such transfers over time and space, it is not surprising they are regarded as especially suitable tools, and have indeed played a central role, in disentangling nutrient cycling mechanisms (Nadelhoffer and Fry, 1994). Therefore, the use of stable isotopes has a long tradition in the field of biogeochemistry, particularly for the cases of the N and C cycles.

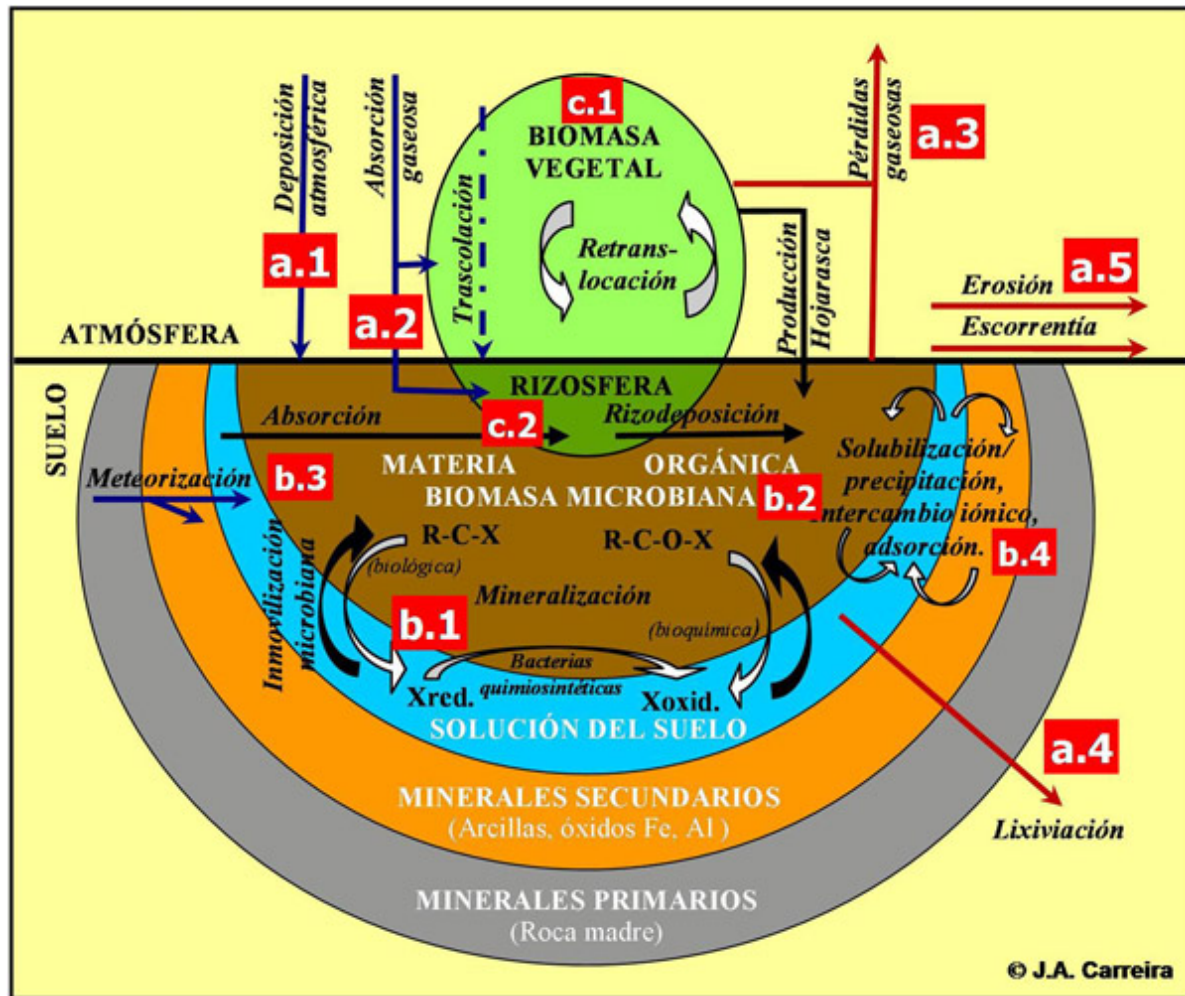


Figure 1. Schematic representation of the main pools and fluxes involved in terrestrial nutrient cycling. Red boxes with letters and numbers inside indicate the processes isotope research has had a significant contribution to unravel (see main text). Blue arrows represent major primary sources of nutrient inputs to the ecosystem; red arrows, pathways of nutrient loss. The internal cycle can be divided into three major circuits: the soil-plant pathway (plant absorption, retranslocation and litter production); the soil-microbes pathway (soil enzymes activity, microbial mineralization of organic matter and immobilization of mineral nutrients, and microbial chemosynthesis –e.g., nitrification–); and the soil solution-mineral phase or geochemical pathway (ionic exchange, precipitation/solubilisation, adsorption/desorption, and other abiotic nutrient retention mechanisms).

The majority and the most important fluxes and transformations involved in nutrient cycling of terrestrial ecosystems cross over or take place in soil compartments. Soil is a complex, porous media, in which organizational features at the microscale, as well as the activity of a huge functional variety of microscopic organisms and exogenous enzymes, are key to understand the outcome of biogeochemical processes at broader scales. Nevertheless, the opaque and tri-phasic nature of soils, in comparison for instance with pelagic systems, renders them as study cases where a detailed and mechanistic investigation of nutrient cycling is a particularly difficult task. For this reason, diagrams representing terrestrial nutrient cycling customarily treat the soil as a black box for which just element inputs (e.g., atmospheric deposition, litter production) and outputs (e.g., leaching, gaseous emissions, erosional losses) and a few “pooled” nutrient pools (e.g., total and roughly defined labile fractions) can be specified at the most.

In this context, stable isotopes may once more be foreseen as useful tools to help overcome the difficulties. Indeed, pioneering ^{15}N tracer experiments were conducted as early as in the 1940s, soon after availability of the first mass spectrometers, to study the fate of added N in agricultural soils. Similarly, the principles of the ^{15}N pool dilution technique were also first applied in soil science research a long time ago. For instance, by using this approach and considering only two broad pools (mineral and organic N), Kirkham and Bartholomew (1954) were able to separate mineral N production and consumption in soils (“b.1”

in **Fig. 1**). Therefore, the possibility of calculating gross rates of soil N mineralization and microbial immobilization have been here for more than half a century. Despite this, the widespread application of isotope techniques in soil nutrient cycling studies started only in the last 15-20 years. Until the 90's, most estimates of N mineralization and nitrification were net measurements, which were calculated from the change in the size of all or a part of the inorganic pool in soil samples incubated over prolonged periods (Hart et al., 1994). Surprisingly, it was known that net and gross rates were not correlated at least in some ecosystems (Stark and Hart, 1997; see also **Fig. 2**). The view of plants as poor competitors for mineral N compared to microbes was also proposed decades ago (Bartholomew and Clark 1950), and it settled. All this made researchers to conceive N availability to plants as the N 'left over' once microbial demand is met, and strongly shaped the prevalent paradigm in terrestrial N cycling... until recently. Soil biogeochemistry is currently involved in a throughout revision of the traditional ways of thinking (e.g., Schimel and Bennett, 2004, Zak et al., 2006, Kemmitt et al., 2008, Van der Heijden et al., 2008, Gärdenäs et al., 2010). The recent generalization in the use of isotope techniques has had a very significant contribution to this.

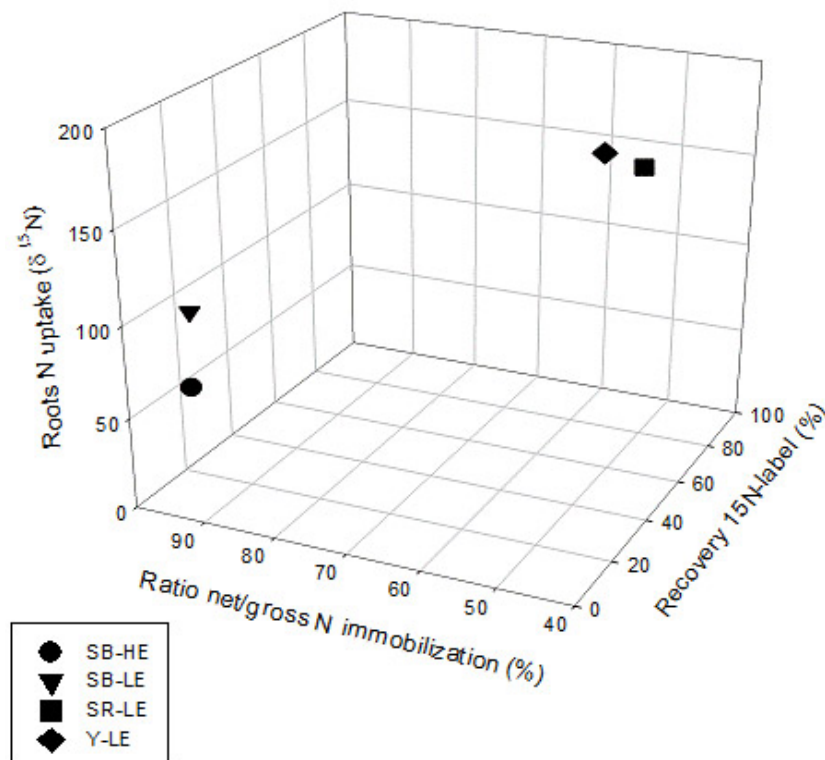


Figure 2. Recovery of added ^{15}N from soil samples one year after labelling application (%), soil net to gross N mineralization ratio (%), and tree root ^{15}N uptake rates in *Abies pinsapo* stands with different land-use history and atmospheric N deposition inputs. See Table 1 for the reference and characteristics of the different stands. Some data are estimates.

The combined-use of this three types of indicators allow to define multiple possible N states of ecosystems that go beyond the simplistic view of N-limited or N-saturated status (modified from Brumme and Khannza, 2008): (1) those at equilibrium, but N unsaturated and N limited or co-limited (long-undisturbed ecosystems that will respond to additional N input by increasing N accumulation), (2) those not at equilibrium and accumulating N (N is still limiting), including ecosystems that were affected by major perturbations other than N loading in the past and are currently recovering and at mid-to-late aggradative phases, (3) those at equilibrium but N saturated and not N limited (additional N input is matched by N output), and (4) those not at equilibrium and losing N (because of recent disturbances or changes induced by N input or other factors).

We here review this process of changing paradigms, focusing in the case of soil nitrogen cycling, but with some incursions in the case of C cycling. Regarding the application of stable isotopes in biogeochemical studies, three main approaches can be distinguished that have contributed to the improvement of conceptual and mechanistic models on elemental cycling within and among ecosystems. Isotopes are used as: (i) integrators for assessing nutrient input/output budgets when whole ecosystems or broad compartments within them are considered as "black-boxes", (ii) tracers to unravel the specific processes and end-member pools operating within these black-boxes, especially in the soil compartment, and (iii) markers or indicators of nutrient use, availability and deficiency to plants.

Stable isotopes in assessing input/output nutrient budgets

Pollutant sources of N inputs to ecosystems can be inferred from the isotope signature of atmospheric deposition (Driscoll et al. 2003) (“a.1” in **Fig. 1**). ^{15}N isotope techniques have also been widely adopted for estimating N inputs by biological fixation. They are based upon differences in ^{15}N dilution in tissues of fixing and reference non-fixing plants (Högberg, 1997) (“a.2” in **Fig. 1**). The formers have access to atmospheric N_2 , a source with low ^{15}N abundance compared to soil N or ^{15}N enriched fertilizers, which are the only sources for the reference plants. Thus, there are two main variations of the technique: One makes use of the natural ^{15}N abundance in available soil N, and the other involves enrichment of soil N by addition of ^{15}N -enriched fertilizers. The main problems associated with these methods are that their application theoretically requires that all test (“ N_2 -fixing”) and control plants accumulate N from the soil with the same ^{15}N enrichment, and how to ensure that the $^{15}\text{N}/^{14}\text{N}$ ratio in plants tissues accurately reflects that in the soil, which is variable in time and with soil depth. Thus, special care must be paid in selecting reference plants, e.g. how to ensure that they are not actually fixing N_2 , are absorbing most of its N from the same zone as the fixing plants, and in the same pattern with time (Danso et al., 1993).

Both ^{15}N natural abundance and enrichment methods are also valuable to help evaluate losses of N from ecosystems (“a.3” to “a.5” in **Fig. 1**). The natural abundance approach has the advantage of not disrupting the actual concentration and dynamic of elements in the system. However, enrichment methods allow capturing the contemporary status of the system without the inconveniences that the long-term memory of current natural abundance signals poses into the data interpretation. For instance, soil ^{15}N natural abundance was higher in a N-limited, low elevation, serpentine Pinsapo-fir forest site that had experienced marginal agricultural uses and intensive grazing and logging until the 1950s, than in a comparable N-saturated one currently receiving high atmospheric deposition inputs but which had not been as much disturbed by these activities in the past (**Table 1**). Natural abundance of ^{15}N is a long-term integrator of variations in the openness of the N cycle along environmental, perturbation and land-use history gradients, since the light isotope is preferentially lost (e.g., Austin and Vitousek, 1998; Eshetu and Högberg, 2000). Disturbed sites show elevated soil ^{15}N values compared to less disturbed and natural forest ecosystems, and surface soil layers have depleted ^{15}N abundance relative to the lower horizons (Fry, 2006).

Study site	Site reference	Parent Material	Distance to industrial area (km)	Bulk mineral N deposition [§] (kg N ha ⁻¹ yr ⁻¹)	Net nitrate throughfall [§] (kg N ha ⁻¹ yr ⁻¹)	N status	Natural abundance $\delta^{15}\text{N}$ (‰)	^{15}N -label recovery (%)
Yunquera-LE ¹	Y-LE	Calcareous	~70	4.9	6.7 ^a	Limited	+3.41 ^a	47.2 ± 9.0
Sierra Real-LE ¹	SR-LE	Serpentine	~60	3.5	3.7 ^a	Limited	+1.36 ^a	n.d.
S. Bermeja-LE ²	SB-LE	Serpentine	~35	7.8	14.4 ^a	Saturated	+0.31 ^c	12.6 ± 6.1
S. Bermeja-HE ²	SB-HE	Serpentine	~35	10.4	n.d.	Saturated	+1.35 ^a	11.3 ± 1.5

Table 1. Mean natural ^{15}N abundance in 0-20 cm soils, and label recovery from 0-20 cm soils one year after autumn ^{15}N applications to the soil surface soils of relict, Mediterranean-fir, aggrading forests located along an atmospheric N deposition gradient in Southern Spain, which differ in previous land-uses. Different superscript letters within columns stands for significant differences at the $\alpha=0.05$ level.

LE: low elevation stands, 1200 m above sea level. HE: high elevation stands, 1400 m asl.

1: Sites with a previous land-use history of marginal agriculture and/or intensive grazing, logging and fire. 2: Sites which have a long history of forestry use.

§: Source: Liétor (2002), Salido (2007), Torres-Cañabate (2006).

On the other hand, the use of ^{15}N -labelled compounds in budgeting the N cycle has allowed the refinement of general hypothesis on controls over forest nutrient cycling such as the Nutrient Retention Hypothesis (Vitousek and Reiners, 1975) and the Nitrogen Saturation Hypothesis (Aber et al., 1989). In both cases, isotopes fostered a shift from a plant productivity-based view of controls over N retention, to one which gives more weight to microbial and abiotic retention mechanisms occurring in the soil and litter layers. For instance, studies with labelled nitrogen at the NITREX sites in Europe (Tietema et al., 1998) and at the Harvard Forests in USA (Nadelhoffer et al., 2004) allowed to validate the suggestion from previous non-labelled fertilisation experiments that soil retention, rather than primary productivity, is critical to explain the high resilience to the onset of N leaching usually shown by forests subjected to chronic atmospheric deposition. Nevertheless, this result, which comes from studies in which ^{15}N was added onto the forest floor, have recently been challenged by Dail et al. (2009). These authors used a novel procedure to directly apply ^{15}N to the forest canopy, which better emulate natural N deposition processes. They

report that plants, which retained 29 and 61% of a 20 kg N ha⁻¹ year⁻¹ addition in above- and belowground parts, and not soils, were the most important sink for the added N, at least in the short-term (3 years).

Despite the potential shown by stable isotopes to infer the input/output budgets in the N cycle, this is strongly constrained by problems in closing the mass balance. Incomplete and even poor recovery is a common feature of many ecosystem ¹⁵N tracer studies (Gundersen, et al., 1998, Nadelhoffer et al., 1999). This is often explained by unmeasured or partially measured fluxes such as denitrification and leaching from the soil, and by direct volatilization and photolysis of N compounds. There are also large uncertainties associated with small enrichments of ecosystems pools with high N content, such as the forest floor, soils and bark (Nadelhoffer et al., 2004).

Isotopes for opening the soil black-box

Beside the ability to integrate over time and space the many processes involved in nutrient cycling, thus allowing for the evaluation of the net outcome from ecosystem black-boxes, stable isotopes are invaluable in the partitioning of the specific processes and end-member pools operating within them (“b.1” to “b.4” in **Fig. 1**). In this context, stable isotopes can be used as tracers when one or various sources are marked, so that their fates and transformations can be followed. Additionally, isotope dilution methods, in which a pool that is the product of the transformation of interest is labelled, allow the gross rate of the process to be estimated through measuring how rapidly the added isotope is diluted by influx of the natural isotope into the pool overtime. For instance, by labelling the soil NH₄⁺ pool, gross N mineralization-immobilization and nitrification rates can be calculated (e.g., Davidson et al., 1991) (“b1” in **Fig. 1**). This allowed the characterization of the role of heterotrophic and chemoautotrophic microorganisms in the retention and mobility of N within soils. More recently, attention is being called into dual isotopic tracers (¹³C and ¹⁵N) techniques in order to cope with the difficult challenge of clarifying interactions between roots and microorganisms to improve our understanding of C and N cycling within the soil black-box. For instance, in one of the latest reviews aimed at the identification of main knowledge gaps, Gårdenäs et al. (2010) point out to that approach as the way to investigate how living roots alter the decomposition rate of soil organic matter by the ‘priming’ effect of rhizodeposition (“b2” in **Fig. 1**). This topic is a poorly understood link between vegetation and soil C and N cycling, which is considered critical to improve global-scale C models.

The development of mobile equipments which combine analytical and multiplexer devices now permits the simultaneous measurement of soil-atmospheric gas exchange and its isotopic signature in situ. For instance, continuous measurement in the field of the rates and δ¹³C signal of CO₂ efflux from control, root-exclusion and root plus mycorrhizal-exclusion treatments have allowed the partitioning of the root, extra-radical mycorrhizal and soil heterotrophic components of forest soil respiration (Heinemeyer et al., 2007). These authors found that ectomycorrhizal mycelium, whose activity is simplistically included in the ‘autotrophic’ root respiration term with previous methods, can contribute substantially more to soil CO₂ flux than do roots (~25% versus ~15%). More importantly, in contrast to the soil heterotrophic fraction, ectomycorrhizal respiration showed no sensitivity to soil temperature change, but it was responsive to moisture and appeared to be highly dependent on assimilate supply from the trees. This directly challenges current approaches for modelling the responses of soil respiration to climate warming which consider the soil as a single heterotrophic compartment and that soil CO₂ efflux only show temperature-dependency.

Continuing with the soil carbon cycling, the recent proposition by Kemmitt et al. (2008) of the “Regulatory Gate” hypothesis has challenged the classical and long held theories in soil microbiology proposed by Winogradsky, of the existence of autochthonous and zymogenous microbial populations (Winogradsky, 1924). The “Regulatory Gate” hypothesis states that the rate limiting step of soil organic matter (SOM) mineralization is governed by abiotic processes that convert non bioavailable SOM into bioavailable SOM, thus being independent of microbial biomass size, community structure or specific activity. Again, the subsequent debate is calling for isotopes techniques as the necessary methodological approach to resolve the controversy and to show that the “Regulatory Gate” is also (micro)biologically driven (see, for instance, Paterson, 2009).

Regarding the N cycle, non-isotopic incubation methods that allow estimating net process rates helped to develop the classic paradigm that shaped the view of most researchers examining soil N processes during the last century: that N mineralization is the core point of the soil N cycle and the process controlling N availability to plants (Aber and Melillo, 2001). In contrast to conventional incubation methods, isotopic dilution ones can take into account both mineralization and microbial immobilization. Studies using ¹⁵N showed substantial rates of gross mineralization and nitrification in systems where little NH₄⁺ and NO₃⁻ accumulates during net mineralization assays (Davidson et al., 1992, Stark and Hart, 1997), which highlights the limitations of net rates as measures of N cycling dynamics. In addition, when plants are removed from the system, as is the case during net-mineralization assays, the N that would otherwise have been taken up by plants is immobilized by the microbes, therefore resulting in low or negative net-mineralization estimates. Consequently, over the course of the 1990s, thinking about net mineralization became more refined with the developing awareness of the complex dynamics of gross mineralization/ immobilization reactions (Hart et al., 1994). These concerns led to more focused research testing those

assumptions. For instance, in terms of the soil processes involved in the development of the N saturation syndrome in forest ecosystems, it is now considered that the suppression of immobilisation of deposited NO_3^- rather than stimulation of NO_3^- production is the dominant process determining the onset of NO_3^- leaching. This idea came from the combination of field non-labelled N addition studies and ^{15}N tracer experiments. The formers showed that leaching losses increased in response to NO_3^- additions rather than to total inorganic-N inputs (Emmett et al., 1998). Using isotope labelling methods, the NO_3^- leached was found to be predominantly the NO_3^- applied during the same year (Tietema et al., 1998). The simultaneous consideration of net rates derived from conventional and gross rates from isotopic assays (e.g., gross/net mineralization and nitrification ratios) have also provided of more sensitive indicators of the ecosystem N status (Emmett, 2007) (see also **Fig. 2**).

The use of stable isotopes is also being invoked as the salvation table to deal with the testing of new ideas coming from later revisions of the N saturation hypothesis. One of them is the role suggested for the so called “mycorrhizal loop” (Aber et al., 1998), which has been proposed to explain unexpected results from large-scale experiments of N addition to forests in Europe (e.g., NITREX) and in the USA. The initial hypothesis (Aber et al., 1989) was that plant uptake would be the main sink, resulting in increased primary production, and recycling of N through litter and humus to the mineral N available pool. This mechanism, if free heterotrophic microbes are assumed to be C or energy limited in most soils, will saturate soon; thus resulting in increased nitrate leaching. However, the huge rates of N retention by soils that were found in such experiments led to question the assumption. Therefore, available soil C should be meeting demands from microbial growth in order to account for the reported rates of N immobilisation. In this case, large increases in soil respiration should occur concomitantly. However, they were not observed. As a consequence, the revisited hypothesis of the microbial loop, that mycorrhizal assimilation of C derived directly from plant photosynthates will drive the retention of added N, came into play.

Isotopes have not only served to clarify and to quantify previously known arrows and pool transformations within the soil black-box. More interestingly, they are contributing to add novel processes to it and to assess their relevance. A recent and still controversial example of a newly proposed abiotic N retention mechanism is the process of NO_3^- fixation to organic matter through its previous reduction to nitrite by Fe^{2+} in anaerobic microsites (the ferrous-wheel hypothesis), which was first shown to occur in organic horizons of temperate forests (Davidson et al., 2003). We have recently demonstrated it also takes place even in mineral soils subjected to seasonally-dry conditions such as in Mediterranean-type climate regions, and that its intensity does not depend on the N saturation status of the system as is typical for abiotic retention mechanisms (Torres-Cañabate et al., 2008). Examples of novel biotic N transformations that isotopes are helping to put into the context of soil ecosystems are heterotrophic nitrification, anaerobic ammonium oxidation (anammox), and dissimilatory nitrate reduction to ammonium (DNRA). For instance, Silver et al. (2005) have shown with the aid of ^{15}N that DNRA is a significant mechanism for N retention in tropical systems that reduce the soil NO_3^- -concentration and at the same time keeps the N in an available form.

Isotopes to uncover plant nutrient use and deficiency, and the outcome of plant-microbial competition

Natural abundance of ^{15}N has been used to compare plant species patterns of N uptake (see review by Dawson et al., 2002) (“c.1”, **Fig. 1**). Isotope studies are also changing the classical view that plants use only inorganic materials for their nutrition (Schimel and Bennett, 2004). Many studies had revealed that plant use of organic N forms, either by direct uptake from the soil solution or indirectly through mycorrhizal symbiosis, is a common feature in tundra and boreal ecosystems (e.g., Chapin et al., 1993). However, more recently, strong evidences are accumulating that indicate this phenomenon is not restricted to cold environments, and that it is much more widespread than previously thought at least in nutrient-poor ecosystems (Dawson et al., 2002). Additional suggestive evidence comes from ^{15}N time-course research showing that although soil mineral N does not accumulate over the monitoring period in such ecosystems, indicating that N availability remains limited, plants do accumulate ^{15}N over time, suggesting they do “win” microbial immobilisation some of the competition events for N turning over in the soil (e.g. Hodge et al., 2000). Also pointing out into this direction, we have shown that microbial retention of ^{15}N added to soils of N-saturated *Abies pinsapo* forests is much lower when tree roots are present compared to root-exclusion treatments, provided that the non-nitrogen (phosphorus) tree nutritional limitation induced by excess N is alleviated through compensatory fertilisation (**Table 2**). This suggests plants may “win”, and that the reduced capacity of the plant sink for chronic N deposition is the result of a shift from N limitation of primary production to limitation by other nutrients rather than the consequence of an intrinsically poor competitive ability of tree roots against microbes for available N (Blanes et al., submitted).

Altitude (m a.s.l)	P treatment plots	¹⁵ N retention by the soil (microbial immobilization) (% respect to total added label)			
		0-5 cm samples		5-15 cm samples	
		Roots excluded	Roots present	Roots excluded	Roots present
1200	Non-fertilized	28.3	23.6	28.8	20.9
	P-fertilized	*42.6	23.3	27.1	19.2
1400	Non-fertilized	14.4	12.6	16.8	12.2
	P-fertilized	*25.0	14.9	23.0	19.1

Table 2. Percentage of the applied ¹⁵N label recovered by the end of the growing season from 0-5 cm and 5-15 cm soil samples (microbial immobilization) where the roots had been excluded (-R) or were present (+R), collected from both non-fertilised (-P) and P-fertilized (+P) plots located at two elevations in N-saturated *Abies pinsapo* stands from Sierra Bermeja (Málaga, Spain). Asterisks indicate significant differences between the -R and the +R treatments at a given plot and elevation ($\alpha=0.05$).

Isotopes have also provided of an easy root bioassay to evaluate plant nutrient stress and deficiency ("c.2", **Fig. 1**). The bioassay, which is much more sensitive than standard soil nutrient availability indexes or foliar analyses, measures isotope uptake rates by field-collected, excised roots under standard lab conditions. Uptake rates show a negative exponential relationship with the degree plant nutrient demand is being satisfied by soil nutrient supply; roots from nutrient-deficient plants showing a "hunger" uptake response (Jones et al., 1991). The bioassay have been successfully applied, for instance, in the screening of N to other elements unbalances of tree nutrition in European forests subjected to atmospheric N deposition (Harrison et al., 1999), and it was key to support the hypothesis that the induction of deficiency by nutrients others than N is a widespread mechanisms triggering the threshold-shift from a N-limited to a N-saturated status in forests (Carreira et al., 2000).

Ongoing challenges and future perspectives

As reported above, all the three approaches for using stable isotopes in studies of ecosystem nutrient cycling and availability to plants have limitations. However, since they provide complementary information, their combined use is promising. An example of this potential refers to the case of determining the N status of terrestrial ecosystems (**Figure 2**), an issue that seems to be more complex than the simplistic view of either N-limited or N-saturated states (Connell and Thornley, 2000; Brumme and Khannza, 2008).

We have reviewed how stable isotopes have had a relevant role in past and recent changes in the perceptions on controls over N and C cycling. The common view that plants use only inorganic N "left over" by soil microbes, which allowed to cleanly divide the N cycle into two pieces (the leaky/un-leaky, net mineralization/immobilisation loop, and the plant uptake, use and return pathway), have been substituted in the last few years by a new paradigm. It considers that the limiting step in N cycling is driven by depolymerization of N-containing polymers by microbial and mycorrhizal extracellular enzymes, and that plant uptake of organic N is a "circuit" rather than a "short-circuiting" of the N cycle (Schimel and Bennett, 2004). This new conceptual model is still lacking important soil pathways such as heterotrophic nitrification and DNRA. And it must incorporate the specific mechanisms underlying the outcome of plant-microbe competition and soil-microsite phenomena. The new biogeochemical challenge for stable isotope research is to help unravelling these three major new perspectives of N cycling research: depolymerization, competition, and microsites. Notably, these are the same type of key issues feeding up the controversy between defenders and detractors of the Regulatory Gate hypothesis on soil C cycling (e.g., Kuzyakov et al., 2009). ¹⁵N and ¹³C tracing modelling would further help to integrate simultaneously occurring N and C pools and pathways, to optimize parameter estimation, and to evaluate their importance in a full-ecosystem context (see, for instance, Müller et al., 2007).

Finally, hidden beneath these nutrient pools and fluxes diagrams are the genetic attributes and the activities of the individual organisms that give rise to population dynamics and community-level interactions, and that ultimately drive biogeochemical processes, even unknown ones. The recent advances and widespread application of biomolecular techniques have rendered such topic treatable, leading to a molecular dawn in Biogeochemistry (Zak et al., 2006). Also in this field, isotope applications go hand-to-hand with genomic and proteomic. For instance, stable isotope probing (SIP) avoids the need for an a priori knowledge of the functional genes that mediate a particular biogeochemical pathway (related DNA sequences may not be available in molecular databases). SIP uses an isotopically enriched substrate (e.g. ¹³C or ¹⁵N) to directly label the DNA of organisms participating in that pathway. The resulting "heavier" DNA can be separated in a density-gradient from that of the whole community finger-print, thus offering potential for species-specific delineation of microbial roles in N and C cycling (Radajewski et al., 2000). On the other hand, functional genes, as well as others, are mechanistically linked to biogeochemical

processes via the enzymes that they produce, the activity of which can be assessed by the use of stable isotope tracers. Proteomics offers the potential to identify the functions and source organisms for enzymes in soil (Schulze et al., 2005), with the added benefit that proteins offer the potential to simultaneously track fluxes of C and N (and P and S also), if coupled to labelling approaches. In situ isotope labelling of substrates, in conjunction with culture-independent molecular techniques which allow tracing to a particular microbial group, constitutes a promising new approach to link functions with identity in soil N and C cycling studies (Hirsch et al., 2010).

References

- Aber, J.D., Nadelhoffer, K.J., Steudler, P., Melillo, J.M. 1989. Nitrogen saturation in northern forest ecosystems. *BioScience* 39:378-386.
- Aber, J., McDowell, W., Nadelhoffer, K., Magill, A., Berntson, G., Kamakea, M., McNulty, S., Currie, W., Rustard, L., Fernandez, I. 1998. Nitrogen saturation in forest ecosystems: hypothesis revisited. *BioScience* 48:921-934.
- Aber, J.D., Melillo, J.M. 2001. *Terrestrial ecosystems*. Second edition. Harcourt Academic Press, San Diego, California, USA.
- Austin, A.T, Vitousek, P.M. 1998. Nutrient dynamics on a precipitation gradient in Hawaii. *Oecologia* 113:519-29.
- Bartholomew, W.V., Clark, F.E. 1950. Nitrogen transformations in soil in relation to the rhizosphere microflora. *Transaction of the Fourth International Congress of Soil Science* 2:112-113.
- Brumme, R., Khanna, P.K. 2008. Ecological and site historical aspects of N dynamics and current N status in temperate forests. *Global Change Biology* 14:125-141.
- Carreira, J.A., García-Ruiz, R., Liétor, J., Harrison, A.F. 2000. Changes in soil phosphatase activity and P transformation rates induced by application of N- and S-containing acid-mist to a forest canopy. *Soil Biology and Biochemistry* 32:1857-1865.
- Chapin, F.S., Moilanen, L., Kielland, K. 1993. Preferential use of organic nitrogen for growth by a non-mycorrhizal arctic sedge. *Nature* 361:150-153.
- Connell, M.G.R., Thomley, J.H.M. 2000. Nitrogen states in plant ecosystems: a viewpoint. *Annals of Botany* 86:1161-1167.
- Dail, D.B., Hollinger, D.Y., Davidson, E.A., Fernandez, I., Sievering, H.C., Scott, N.A., Gaige, E. 2009. Distribution of nitrogen-15 tracers applied to the canopy of a mature spruce-hemlock stand, Howland, Maine, USA. *Oecologia* 160:589-599.
- Danso, S.K.A., Hardarson, G., Zapata, F. 1993. Misconceptions and practical problems in the use of ¹⁵N soil enrichment techniques for estimating N₂ fixation. *Plant and Soil* 152:25-52.
- Davidson, E.A., Hart, S.C., Firestone, M.K. 1992. Internal cycling of nitrate in soils of a mature coniferous forest. *Ecology* 73:1148-1156.
- Davidson, E.A., Hart, S.C., Shanks, C.A., Firestone, M.K. 1991. Measuring gross nitrogen mineralization, and nitrification by ¹⁵N isotopic pool dilution in intact soil cores. *European Journal of Soil Science* 42:335-349.
- Davidson E.A., Chorover, J., Dail, B.D. 2003. A mechanism of abiotic immobilization of nitrate in forest ecosystems: the ferrous wheel hypothesis. *Global Change Biology* 9:228-236.
- Dawson T.E., Mambelli, S., Plamboeck, A.H., Templer P.H., Tu K.P. 2002. Stable isotopes in plant ecology. *Annual Reviews in Ecology and Systematic* 33:507-59.
- Driscoll, C.T., Whitall, D., Aber, J., Boyer, E., Castro, M., Cronan, C., Goodale, C.L., Groffman, P., Hopkinson, C., Lambert, K., Lawrence, G., Ollinger, S. 2003. Nitrogen pollution in the northeastern United States: Sources, effects, and management options. *Bioscience* 53:357-374.
- Emmett, B.A., Reynolds, B., Silgram, M., Sparks, T.H., Woods, C. 1998. The consequences of chronic nitrogen additions on N cycling and soil water chemistry in a N saturated Sitka spruce stand, North Wales. *Forest Ecology and Management* 101:165-175.

- Emmett, B.A. 2007. Nitrogen saturation of terrestrial ecosystems: Some recent findings and their implications for our conceptual framework. *Water, Air and Soil Pollution* 7:99-109.
- Eshetu, Z., Högberg, P. 2000. Effects of land use on ^{15}N natural abundance of soils in Ethiopian highlands. *Plant and Soil* 222:109-117.
- Fry, B. 2006. *Stable Isotope Ecology*, 308 pp. Springer, New York. USA.
- Gärdemas, A.I., Agren, G.I., Bird, J.A., Clarholm, M., Hallin, S., Ineson, P., Kätterer, T., Knicker, H., Nilsson, S.I., Näsholm, T., Ogle, S., Paustian, K., Persson, T. Stendahl, J. 2010, (en prensa). Knowledge gaps in soil carbon and nitrogen interactions – From molecular to global scale. *Soil Biology and Biochemistry*. doi: 10.1016/j.soilbio.2010.04.006.
- Gundersen, P., Emmett, B.A., Kjonaas, O.J., Koopmans C.J., Tietema A. 1998. Impact of nitrogen deposition on nitrogen cycling in forests: A synthesis of NITREX data. *Forest Ecology and Management* 101:37-56.
- Harrison A.F., Carreira, J.A., Poskitt, J.M., Robertson, S.M.C., Smith, R., Hall, J., Hornung, M., Lindley, D.K. 1999. Impacts of pollutant inputs on forest canopy condition in the U.K.: possible role of P limitation. *Forestry* 72:367-377.
- Hart, S.C., Stark, J.M., Davidson, E.A., Firestone, M.K. 1994. Nitrogen mineralization, immobilization, and nitrification. In: Weaver, R.W., et al. (eds.), *Method of soil analysis, Part 2, Microbiological and Biochemical properties*, pp.: 985-1018, Soil Science Society of America Book Series, nº 5. Wisconsin. USA.
- Hart, S.C., Nason, G.E., Myrold, D.D., Perry, D.A. 1994. Dynamics of gross nitrogen transformations in an old-growth forest: the carbon connection. *Ecology* 75:880-891.
- Heinemeyer, A., Hartley, I.P., Evans, S.P., Carreira J.A., Ineson, P. 2007. Forest soil CO_2 flux: uncovering the contribution and environmental responses of ectomycorrhizas. *Global Change Biology* 13:1786-1797.
- Hirsch, P.R., Mauchline, T.H., Clark, I.M. 2010. Culture-independent molecular techniques for soil microbial ecology. *Soil Biology and Biochemistry* 42:878-887.
- Högberg, P. 1997. ^{15}N natural abundance in soil-plant systems. *New Phytologist* 137:179-203.
- Hodge, A., Robinson, D., Fitter A. 2000. Are microorganisms more effective than plants at competing for nitrogen? *Trends in Plant Science* 5:304-308.
- Jones, H.E., Quarmbay, C., Harrison, A.F. 1991. A root bioassay test for nitrogen deficiency in forest trees. *Forest Ecology and Management* 42:267-282.
- Kemmitt, S.J., Lanyon, C.V., Waite, I.S., Wen, Q., Addiscott, T.M., Bird, N.R.A., O'Donnell, A.G., Brookes, P.C. 2008. Mineralization of native soil organic matter is not regulated by the size, activity or composition of the soil microbial biomass—a new perspective. *Soil Biology and Biochemistry* 40:61-73.
- Kirkham, D., Bartholomew, W.V. 1954. Equations for following nutrient transformations in soil, utilizing tracer data. *Soil Science Society of America Proceedings* 18:33-34.
- Kuzyakov, Y., Blagodatskaya, E., Blagodatsky, S. 2009. Comments on the paper by Kemmitt et al. (2008) 'Mineralization of native soil organic matter is not regulated by the size, activity or composition of the soil microbial biomass – A new perspective' [Soil Biology and Biochemistry 40:61-73]: The biology of the Regulatory Gate. *Soil Biology and Biochemistry* 41:435-439.
- Liétor, J. 2002. *Patrones de disponibilidad y limitación por nutrientes como indicadores de estado en masas de pinsaparr (Abies pinsapo, Boiss.)*. PhD Thesis, University of Jaén, Spain. 276 pp.
- Müller, C.T., Rütting, T., Kattge, J., Laughlin, R.J., Stevens, R.J. 2007. Estimation parameters in complex ^{15}N tracing models via Monte Carlo sampling. *Soil Biology and Biochemistry* 39:715-726.

- Nadelhoffer, K.J., Fry, B. 1994. Nitrogen isotope studies in forest ecosystems. In: Lajtha, K., Michener, R. (eds.), *Stable Isotopes in Ecology and Environmental Science*. pp. 23-44. Blackwell Scientific Publications, Boston, MA, USA.
- Nadelhoffer, K.J., Downs, M.R., Fry, B. 1999. Sinks for ^{15}N -enriched additions to an oak forest and a red pine plantation. *Ecological Applications* 9:72-86.
- Nadelhoffer, K.J., Colman, B.P., Currie, W.S., Magill, A., Aber, J.D. 2004. Decadal-scale fates of ^{15}N tracers added to oak and pine stands under ambient and elevated N inputs at the Harvard Forest (USA). *Forest Ecology and Management* 196:89-107.
- Paterson, E. 2009. Comments on the regulatory gate hypothesis and implications for C-cycling in soil. *Soil Biology and Biochemistry* 41:1352-1354.
- Radajewski, S., Ineson, P., Parekh, N.R., Murrell, J.C. 2000. Stable-isotope probing as a tool in microbial ecology. *Nature* 403:646-649.
- Salido, M.T. 2007. *Evaluación del estado de saturación de nitrógeno en masas de pinsapo (*Abies pinsapo* Boiss.) en un gradiente geográfico del sur de la Península Ibérica: patrones de entrada, circulación interna y salida de nitrógeno en el ecosistema*. PhD Thesis, 179 pp. University of Jaén, Spain.
- Schimel, J.P., Bennett, J. 2004. Nitrogen mineralization: challenges of a changing paradigm. *Ecology* 85:591-602.
- Schulze, W.X., Glexner, 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.
- Silver W.L., Thompson, W.A., Reich, A., Ewel, J.J., Firestone, M.K. 2005. Nitrogen cycling in tropical plantation forests: potential controls on nitrogen retention. *Ecological Applications* 15:1604-1614.
- Stark, J.M., Hart, S.C. 1997. High rates of nitrification and nitrate turnover in undisturbed coniferous forests. *Nature* 385:61-64.
- Tietema, A., Emmett, B.A., Gundersen, P., Kjønnaas, O.J., Koopmans, C.J. 1998. The fate of ^{15}N -labelled nitrogen deposition in coniferous forest ecosystems. *Forest Ecology and Management* 101:19-27.
- Torres-Cañabate, P. 2006. *Efectos del cambio climático sobre la susceptibilidad de bosques a la deposición de Nitrógeno: Bosques relictos de *Abies pinsapo* (Boiss.) como modelo experimental*. Master Thesis, 50 pp. University of Jaén. Spain.
- Torres-Cañabate, P., Davidson, E.A., Bulygina, E., García-Ruiz, R., Carreira, J.A. 2008. Abiotic immobilization of nitrate in soils of relict *Abies pinsapo*-fir forests under Mediterranean climate. *Biogeochemistry* 91:1-11.
- Van der Heijden, M.G.A., Bardgett, R.D., van Straalen, N.M. 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters* 11:296-310.
- Vitousek, P.M., Reiners, W.A. 1975. Ecosystem succession and nutrient retention: a hypothesis. *Bioscience* 25:370-381.
- Winogradsky, S. 1924. Sur la microflora autochtone de la terre arable. *Comptes rendus hebdomadaires des seances de l'Academie des Sciences (Paris)* D 178, 1236-1239.
- Zak, D.R., Blackwood, C.B., Waldrop, M.P. 2006. A molecular dawn for biogeochemistry. *Trends in Ecology and Evolution* 21:288-295.