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Abiotic immobilization of nitrate in two soils of relic *Abies pinsapo*-fir forests under Mediterranean climate

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Abstract Evidence for abiotic immobilization of nitrogen (N) in soil is accumulating, but remains controversial. Identifying the fate of N from atmospheric deposition is important for understanding the N cycle of forest ecosystems. We studied soils of two *Abies pinsapo* fir forests under Mediterranean climate seasonality in southern Spain—one with low N availability and the other with symptoms of N saturation. We hypothesized that biotic and abiotic immobilization of nitrate (NO_3^-) would be lower in soils under these forests compared to more mesic temperate forests, and that the N saturated stand would have the lowest rates of NO_3^- immobilization. Live and autoclaved soils were incubated with added $^{15}NO_3^-$ (10 μg N g^{-1} dry soil; 99% enriched) for 24 h, and the label was recovered as total dissolved-N, NO_3^-, ammonium (NH_4^+), or dissolved organic-N (DON). To evaluate concerns about possible iron interference in analysis of NO_3^- concentrations, both flow injection analysis (FIA) and ion chromatography (IC) were applied to water extracts, soluble iron was measured in both water and salt extracts, and standard additions of NO_3^- to salt extracts were analyzed. Good agreement between FIA and IC analysis, low concentrations of soluble Fe, and 100% (±3%) recovery of NO_3^- standard additions all pointed to absence of an interference problem for NO_3^- quantification. On average, 85% of the added $^{15}NO_3^-$ label was recovered as $^{15}NO_3^-$, which supports our hypothesis that rates of immobilization were generally low in these soils. A small amount (mean = 0.06 μg N g^{-1} dry soil) was recovered as $^{15}NH_4^+$ in live soils and none in sterilized soils. Mean recovery as DO^{15}N ranged from 0.6 to 1.5 μg N g^{-1} dry soil, with no statistically significant effect of sterilization or soil type, indicating that this was an abiotic process that occurred at similar rates in both soils. These results demonstrate a detectable, but modest rate of abiotic immobilization of NO_3^- to DON, supporting our first hypothesis. These mineral soils may not have adequate carbon availability to support the regeneration of reducing microsites needed for high rates of NO_3^- reduction. Our second hypothesis regarding lower expected abiotic immobilization in soils from the N-saturated site was not supported. The rates of N deposition in this region may not be high enough to have swamped the capacity for soil NO_3^- immobilization, even in the stand showing some symptoms of N saturation. A growing body of evidence suggests that soil abiotic NO_3^- immobilization is common, but that rates are influenced by a combination of factors, including the presence of

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plentiful available carbon, reduced minerals in anaerobic microsites and adequate NO_3^- supply.

Keywords Ferrous-wheel hypothesis · Retention · Iron · ^{15}N · Nitrogen saturation status · Nitrogen cycle

Introduction

Atmospheric deposition of nitrogen (N) to terrestrial ecosystems has increased in recent decades as a result of human activity (Galloway et al. 2004; UNEP and WHRC 2007). Inputs of N may initially stimulate forest productivity, but N accumulation in forest ecosystems can lead to N saturation. Nitrogen saturation has been defined in several ways (Agren and Bosatta 1988; Aber et al. 1989, 1998; Stoddard 1994; Binkley and Hogberg 1997), but can be considered most generally as the long-term removal of N limitations on biotic activity, accompanied by a decrease in N retention capacity (Magill et al. 1996), and eventually resulting in increases in ecosystem N loss and forest decline (Aber et al. 1998).

Results from recent fertilization experiments in temperate forests suggest that soils, rather than plants, are the dominant long-term sink for added N, and, by inference, for chronic N atmospheric inputs (Gundersen et al. 1998; Nadelhoffer et al. 1999). The degree to which N deposition stimulates tree growth remains controversial (Magnani et al. 2007; Sutton et al. 2008). The soil sink is related to immobilization mechanisms, which also remain poorly understood. Recent evidence indicates a potential for abiotic N incorporation into soil organic matter. This evidence stems from experiments that have reported a rapid disappearance of added ^{15}N-nitrate ($^{15}NO_3^-$) to non-sterile and sterile soils (Davidson et al. 1991; Dail et al. 2001; Huygens et al. 2008). Rapid incorporation of the $^{15}NO_3^-$ added into a form of dissolved organic nitrogen (DON) has been demonstrated for soil organic horizons of temperate forests (Dail et al. 2001; Perakis and Hedin 2001). Dail et al. (2001) used two sterilization methods to demonstrate that the fast conversion of $^{15}NO_3^-$ to $DO^{15}N$ within minutes of addition to sterilized organic horizons was abiotic. Although Perakis and Hedin (2001) did not use a sterilization treatment, the rapidity with which they observed conversion of $^{15}NO_3^-$ to $DO^{15}N$, suggests that an abiotic mechanism might have been responsible. To date, no proven mechanism exists for abiotic retention of NO_3^- in soil organic matter. The 'ferrous wheel hypothesis' postulates that iron Fe(II) in anaerobic microsites could catalyze NO_3^- reduction to nitrite (NO_2^-), which then reacts quickly with dissolved organic carbon (DOC) (Davidson et al. 2003).

These results have been called into question by recent findings of Colman et al. (2007), who suggested that a possible iron interference in NO_3^- quantification by flow injection analysis (FIA, using the NH_4Cl/EDTA buffer method) could lead to underestimation of NO_3^- concentrations. Consequently DON concentrations would be overestimated, because DON is calculated by subtracting ammonium (NH_4^+) and NO_3^- from total dissolved nitrogen (TDN). Colman et al. (2007) concluded that the abiotic NO_3^- retention reported by other authors was an artifact of the method used to quantify NO_3^-. In response, Davidson et al. (2008) recommended using analyses of iron concentrations and standard additions of NO_3^- to soil extracts to identify possible iron interference of NO_3^- analyses. Hence, we applied these methods and we also used ion chromatography of water extracts to evaluate this potential source of error.

In the present study we investigated the potential for abiotic NO_3^- immobilization in soils under forests of *Abies pinsapo* (Boiss.), a relic fir endemic in the Mediterranean climate region of southern Spain. As remnants of temperate-like ecosystems covering broader areas during last glaciations (Carrión et al. 2003), stand-dynamics and some other biogeochemical features in *Abies pinsapo* forests still resemble those in typical temperate conifer forests (Liétor et al. 2002, 2003). Thus, *Abies pinsapo* fir forests have been used as an experimental model of temperate-like conifer forests that are currently subjected to a warmer and seasonally drier (summer drought) climate (Viñegla et al. 2006). Also pertinent to the present study are previous observations in these forests indicating N saturation symptoms (Table 1) in a stand where net N throughfall was 12.2 kg N ha^{-1} $year^{-1}$ and nitrate leaching was considerable (8.2 kg N ha^{-1} $year^{-1}$) and detectable even during the growing season, while another stand with net throughfall of 2.8 kg N ha^{-1} $year^{-1}$ and no leaching during the growing season (6.4 kg N ha^{-1} $year^{-1}$), remains N limited (Salido 2007). Our ability to model and to predict the consequences of atmospheric N deposition in *Abies pinsapo* fir forests, and, by inference, to

Table 1 Mean (SD) values for physical and chemical properties of the top mineral soil (0–5 cm), for mineral N bulk deposition and for N status of *Abies pinsapo* leaves in the studied sites

	Yunquera (Y) (N-limited)	Bermeja (B) (N-saturated)	Method reference
Mean bulk N deposition (kg DIN-N ha^{-1} $year^{-1}$)	8.64[b]	4.96[b]	Volume-weighted monthly mean DIN-N Open-top pluviometer
Lithology	Calcareous	Serpentinitic	
Soil classification	Typic Haploxerept[a]	Oxiacuic Hapludoll[a]	SSS (1999)
Pedoclimate	Mesic-xeric[a]	Mesic-udic[a]	SSS (1999)
Bulk density (g cm^{-3})	1.6 (0.1)	1.4 (0.9)	Blake and Hartge (1986) (excavation method)
pH (1:1 soil:H_2O)	7.2 (0.1)	5.7 (0.5)	McLean (1982)
Sand (%)	34.3 (6.3)	56.9 (3.0)	
Silt (%)	42.2 (6.7)	30.6 (0.7)	Gee and Bauder (1986) (Pipet method)
Clay (%)	23.5 (5.2)	12.5 (3.2)	
Cation exchange capacity (mmol kg^{-1})	278.2 (42.9)	344.9 (40.6)	Rhoades (1982) (saturation with NaOAc)
Exchangeable Ca (mmol kg^{-1})	108.6 (14.5)	58.2 (4.0)	
Exchangeable Mg (mmol kg^{-1})	15.3 (13.9)	40.6 (5.6)	Thomas (1982) (NH_4OAc extraction)
Exchangeable K (mmol kg^{-1})	6.1 (0.5)	3.9 (0.4)	
Exchangeable Na (mmol kg^{-1})	4.3 (0.8)	3.6 (0.3)	
$CaCO_3$ equivalent (%)	3.6[a]	0.0[a]	Nelson (1982) (pressure calcimeter)
Organic C (%)	4.8 (0.2)	9.3 (2.3)	Nelson and Sommer (1982) (Walkley-Black procedure)
Total N (mg N g^{-1})	3.5 (0.3)[b]	4.4 (0.5)[b]	CNHS Analyzer
C/N	16.9 (0.7)[b]	20.8 (0.9)[b]	
NH_4^+ (μg N g^{-1})	8.2[c]	15.4[c]	Keeney and Nelson (1982)
NO_3^- (μg N g^{-1})	3.5[c]	25.3[c]	(KCl extraction)
Potential net N mineralization (μg N g^{-1} day^{-1})	1.5[c]	2.3[c]	Hart et al. (1994) (aerobic incubation)
Potential net N nitrification (μg N g^{-1} day^{-1})	1.2[c]	2.25[c]	
Potential N–N_2O production rate (nmol g^{-1} h^{-1})	2[b]	10[b]	Klemedetsson et al. (1977)
N in 1 year leaves (mg g^{-1})	17.4 (3.4)[a]	22.2 (4.0)[a]	
N in 2 years leaves (mg g^{-1})	15.1(1.6)[a]	19.2 (2.2)[a]	CNHS Analyzer
C/N in 1 year leaves	42.5 (3.2)[a]	36.5 (5.0)[a]	
C/N in 2 years leaves	46.0 (3.8)[a]	38.3 (4.6)[a]	

[a] Liétor et al. (2003); $CaCO_3$ values correspond to the Ah horizon (0–10/12 cm)

[b] Salido (2007), Salido et al. (in review)

[c] Liétor et al. (2002); Concentration figures are the annual average (11 samplings, $n = 5$ per site)

conserve this endemic and protected species, depends upon our understanding of the mechanisms of N cycling within these ecosystems, including transformations of NO_3^-.

In this context, we amended live and sterilized soil samples from both forest stands, using $^{15}NO_3^-$, with the aim of investigating the importance of abiotic NO_3^- retention in soils of *Abies pinsapo* fir forests. We hypothesize that soils under *Abies pinsapo* fir forests would show less N retention capacity overall than reported in more mesic temperate forest ecosystems. Mediterranean ecosystems have a limited capacity to retain N inputs (Riggan et al. 1985; Fenn et al. 1996; Fenn and Poth 1999), principally due to high

nitrification rates, short growing seasons, and temporal asynchrony between hydrological loss fluxes and plant N demand, mainly as a consequence of summer-dry periods followed by intense autumn rains (Fenn et al. 1998; Meixner et al. 2001). A second hypothesis is that rates of abiotic immobilization of soil NO_3^- would be higher in the N limited stand than in the N saturated site. Recent studies suggests a decrease in NO_3^- abiotic retention with N enrichment in temperate forests soils (Bernston and Aber 2000; Corre et al. 2007).

Material and methods

Site description

Abies pinsapo Boiss. is an endemic and relic species, now restricted to small patches in north-facing slopes (1,100–2,000 m above sea level) of western coastal mountain-ranges in southern Spain: Sierra de las Nieves Natural Park and Los Reales de Sierra Bermeja Natural Site in Málaga, and Sierra de Grazalema Natural Park in Cádiz, with a total area of approximately 2,350 ha (Ruíz de la Torre 1994). These stands are remnants of temperate-like coniferous forests that covered a much broader area during the last ice-age periods. The *Abies pinsapo* forests are currently subjected to Mediterranean climate with hot and dry summers (mean summer monthly temperature higher than 22°C) and temperate winters (mean monthly temperature higher than 5°C). Precipitation is high (ranging from 1,000 to 2,000 mm per year) but rainfall seasonality is distinctly Mediterranean, with more than 80% of it falling between October and May, followed by a long summer drought.

Aggrading stands of *Abies pinsapo* fir forests showing either N saturation (Sierra Bermeja, site "B", 5º12′7′′E, 36º29′26′′N) or N limitation (Yunquera in Sierra de las Nieves, site "Y", 4º57′52′′E, 36º43′31′′N) symptoms have been identified (Liétor et al. 2002; Salido 2007). The B site exhibits higher rates of potential net N mineralization, net nitrification and denitrification, and higher values of extractable NO_3^- and NH_4^+ concentrations, than the Y site (Table 1). The N status of *Abies pinsapo* leaves is also significantly higher in the B site (Table1). These site differences have been attributed to the induction of a N-saturation state in the Bermeja forest due to N atmospheric deposition (Liétor et al. 2002).

Both stands, approximately 30 km apart, are comparable in terms of topography (40% average north-facing slope, between 1,150 and 1,200 m of elevation). The soil in site Y is a well-drained, Ah (0–12 cm)–Bw (12–24/33 cm)–2C (24/33–40 cm), Typic Haploxerept (SSS 1999) developed on calcareous materials (limestones and dolomites). The soil in site B, where ultrabasic serpentines and peridotites dominate, is a well-drained, Ah1 (0–10 cm)–Ah2 (10–70 cm)–B (>70 cm), Oxiacuic Hapludoll. Other basic properties of the soil at each stand are shown in Table 1.

Soil collection, preparation and sterilization

The experiment was conducted on Ah horizon soil samples (0–10 cm) collected in both sites in the dry season (July 2006). Important rates of abiotic retention of the added NO_3^- have been reported in the O horizon (organic horizon) of temperate soils (Dail et al. 2001; Perakis and Hedin 2001; Davidson et al. 2003; Sotta et al. 2008). Nevertheless, the soil profile of *Abies pinsapo* fir forests lacks a clear O horizon (other than a thin L/F litter layer). Three Ah horizon samples were taken from each site, with each one being a composite of three to four sub-samples taken randomly within 1 ha of sampling area. Soil samples were air dried and then sieved (<2 mm) before they were shipped to the Woods Hole Research Center, MA (USA). Once there, they were brought to 65% of their water holding capacity and held for 3 days before beginning the labelling experiment.

One sub-sample of each replicate was extracted using 0.5 M K_2SO_4 (5:1 extractant to soil ratio) to determine background concentrations of extractable N. Extracts were shaken for 1 h and then vacuum filtered. The NO_3^- and NH_4^+ concentrations in soil extracts were determined by colorimetry using flow injection analysis (FIA; Quikchem-Method-10-107-04-1-L 1999 and Quikchem-Method-10-107-06-2-A 1997, respectively) in a LACHAT "Quik Chem FIA+ 8000 Series" autoanalyzer (Lachat Instruments, Milwaukee, USA). For TDN, an in-line persulfate digestion followed by FIA was performed in the same autoanalyzer (QuikChem-Method 10-107-04-3-P, 2000). The DON concentration was calculated by difference: $DON = TDN - NO_3^- - NH_4^+$.

Four sub-samples of about 50 g of dry-mass equivalent from each replicate were placed in 1 l

glass mason jars. Two jars of soil from each replicate were sterilized by autoclaving twice (121°C for 0.5 h) while the other two remained with live soil. Sterilized samples were allowed to cool at room temperature under a sterile transfer hood, where all the sterile work was performed.

All soil sterilization techniques modify the soil, potentially causing unintended artifacts (Wolf and Skipper 1994). Mercuric chloride ($HgCl_2$) is a sterilant that produces very few changes in soil chemical and physical properties, but the addition of this reagent could interfere with the proposed mechanism, the 'ferrous wheel hypothesis', as this is a process that depends on redox chemistry. There are also doubts about how quickly $HgCl_2$ sterilizes the sample (Morier et al. 2008). Although autoclaving can change concentrations of extractable Fe and dissolved organic matter, it is an efficient method to eliminate viable microorganisms and their exoenzymes while other soil properties, such as exchange capacity, surface area and pH, are less affected (Wolf and Skipper 1994). The effectiveness of sterilization was tested by placing 1 g of sterilized soil from each replicate into a set of tubes (2 soil types × 3 replicates = 6 tubes) of sterile nutrient broth (Fisher Scientific, Cat#S716942A) using sterile transfer methods. Another 1 g sample of live soil from each replicate was placed in another set of six tubes of sterile nutrient broth. All tubes were incubated at room temperature in the dark for 7 days. The broth remained clear and no growth was observed in the tubes that received twice-autoclaved soil, whereas the broth became cloudy in all of the tubes that received live soil.

$^{15}NO_3^-$ amendement

A 5 ml aliquot of a sterilized aqueous solution of 0.68 g $K^{15}NO_3\,l^{-1}$, with 99% ^{15}N atom enrichment (the equivalent to approximately 10 μg N g^{-1} dry soil) was added to each jar and mixed thoroughly. This step was carried out under aseptic conditions for the sterilized soil samples. Immediately after addition of the label, jars were covered with an airtight lid and incubated for 24 h at room temperature.

After the incubation period, one sterilized and one live soil sample from each group of four replicate jars were extracted using 0.5 M K_2SO_4, while the other pair of jars were extracted using DI H_2O (5:1 extractant to soil ratio). Jars were shaken, left for sedimentation for 1 h, and vacuum filtered using glass microfiber filters (Whatman GF/F) previously rinsed with DI H_2O. Both, H_2O and K_2SO_4 extracts were brought to pH 2 with concentrated H_2SO_4 and stored at 4°C to preserve them until further analysis.

All soil extracts were analyzed for DIN, TDN, and DON, as described above. The NO_3^- concentrations in DI H_2O extracts were also measured by ion chromatography (IC) using a DIONEX system with IonPac AG14 guard column and AS14 analytical column (Dionex Corporation, document # 031199, "IonPac AS14 Manual"). Soluble iron was also measured in both salt and water extracts by the phenanthroline method following boiling in acid and hydroxylamine (APHA 1989).

^{15}N pool enrichment determination

Figure 1 shows a summary of the procedure that was followed in this experiment to determine the ^{15}N enrichment of the soil extractable NH_4^+, NO_3^- and DON pools. A diffusion method was used to trap inorganic-N on acidified filter disks to prepare them for isotope ratio mass spectrometry (Stark and Hart 1996). This procedure consists of the following steps: NO_3^- is reduced to NH_4^+ with Devarda's alloy, ammonia (NH_3) is volatilized from the solution made basic by adding MgO and, finally, NH_3 vapor is captured on acidified glass fiber filters. An aliquot of each K_2SO_4 soil extract was diffused sequentially to obtain NH_4^+ and then NO_3^- on acidified filter disks. Blank and ^{15}N standards were also diffused and aliquots of ^{15}N standards were pipetted directly onto filter papers in order to correct for possible sources of contamination and ^{15}N dilution during diffusions (Stark and Hart 1996). Filters from all the diffusions were sent to the stable isotope facility at the University of California, Davis, for ^{15}N analyses.

Another aliquot of each K_2SO_4 extract was digested following a modification of the persulfate oxidation method (D'Elia et al. 1976) to oxidize all the nitrogenous compounds in the soil extract to NO_3^-. Depending on the TDN concentration of the extracts, a volume of the digested extract was taken to provide a range of 80–300 μg N and diluted with DI H_2O in Duran flasks to a total volume of 40 ml. A 5 ml aliquot of a persulfate solution (25 g of twice recrystallized $K_2S_2O_8$ in 500 ml of DI H_2O) and 1.5 ml of 1 N NaOH were added. The flasks were

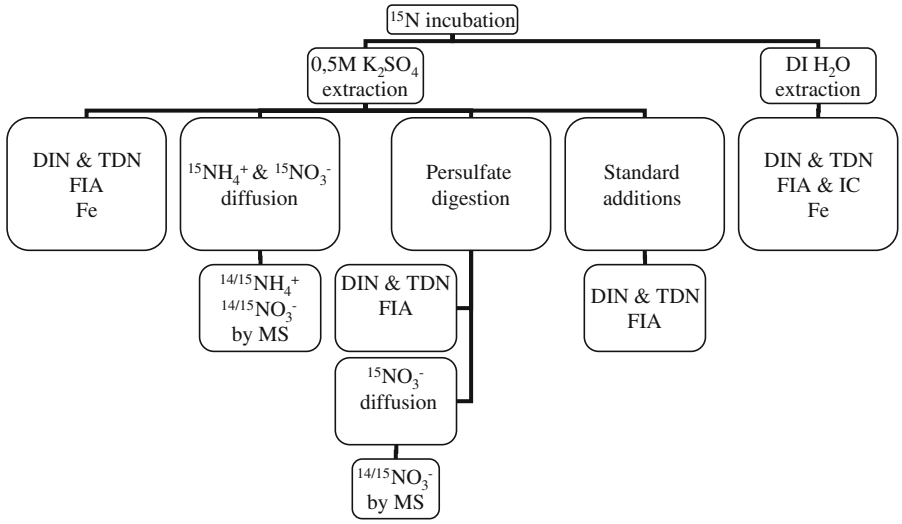

Fig. 1 Procedure for the determination of the ^{15}N enrichment of the soil extractable ammonium (NH_4^+), nitrate (NO_3^-) and dissolved organic nitrogen (DON) pools. Other abbreviations include dissolved inorganic nitrogen (DIN), total dissolved nitrogen (TDN), flow injection analysis (FIA), iron (Fe), ion chromatography (IC), and mass spectrometry (MS)

then closed for the digestion in the autoclave (121°C for 1 h). After the persulfate digestion, solutions were diffused to acidified disks as described above to determine the ^{15}N enrichment of the TDN pool by mass spectrometry.

The ^{15}N enrichment in DON was calculated by substracting $^{15}NO_3^-$ and $^{15}NH_4^+$ from TD^{15}N in the salt extracts. This standard estimation has been criticized for possibly overestimating DON because of incomplete quantification of the NO_3^- pool in the presence of iron (Colman et al. 2007). We addressed this concern in three ways. First, we used the water extracts to measure NO_3^- concentrations by both FIA and IC methods—while the former could be affected by Fe interference, the latter uses a completely different detection system that should not be affected by the presence of Fe. Second, we measured soluble Fe to determine if the concentrations were high enough to be worrisome. Third, we conducted NO_3^- standard additions to both salt and water extracts of both live and sterilized soils to measure the recovery of known amounts of added NO_3^- by the FIA method (Davidson et al. 2008). The extracts were analyzed for NO_3^- by the same FIA methodology described above, both before and after adding an aliquot of NO_3^- standard measured to raise its concentration by 2 mg N l^{-1}.

Statistical analysis

A two-way analysis of the variance (ANOVA) was performed to test for the effects of site and sterilization on the ^{15}N recovery as NO_3^-, NH_4^+, and DON. For variables that did not meet ANOVA requirements of normality (Kolmogorov–Smirnov test) and/or homogeneity of the variances (Cochran C, Hartley, Bartlett test), $\ln(x + 1)$ transformations were applied. In the case of $^{15}NH_4^+$, assumption of homogeneity of variances was still not met after data-log transformation, so a rank transformation of the data followed by a two-way ANOVA was performed (Potvin and Roff 1993).

Results

Fate of inorganic ^{15}N added to soil

On average, 85% of the $^{15}NO_3^-$ added was recovered in the same form (Table 2) without significant differences between sterilization treatments or soils ($P > 0.05$). One of the three replicate samples of the live Y soil had low ^{15}N recovery (57% as $^{15}NO_3^-$ and 65% as TD^{15}N), which brought down mean recoveries and increased standard errors for that soil and treatment, but the differences among soils and sterilization treatments (and their interaction) were not statistically significant.

None of the ^{15}N was recovered as NH_4^+ in the sterile samples, whereas all of the live soil samples showed a small amount (mean = 0.06 μg ^{15}N g^{-1} dry soil) of recovery as $^{15}NH_4^+$. The ANOVA of rank-transformed data (see "Methods") indicated that recovery was significantly higher in live than sterilized soils ($P < 0.01$) and higher in Y soils compared

Table 2 ^{15}N recovered as NO_3^-, NH_4^+, TDN and DON (μg ^{15}N g^{-1} dry soil) in live and sterilized soil samples (means ± one standard error) amended with $^{15}NO_3^-$ at the rate of 10 μg ^{15}N per gram soil dry mass

Treatment	Soil	Amended NO_3^-	Recovered NO_3^-	NH4	TDN	DON
Live	Yunquera	10.66 ± 0.22	7.87 ± 1.00	0.10 ± 0.07	8.82 ± 1.00	0.85 ± 0.36
	Bermeja	10.48 ± 0.06	9.83 ± 0.02	0.02 ± 0.00	10.49 ± 0.43	0.64 ± 0.45
Sterilized	Yunquera	10.59 ± 0.10	9.08 ± 0.10	0.00 ± 0.00	10.53 ± 0.15	1.45 ± 0.05
	Bermeja	10.65 ± 0.19	9.33 ± 0.25	0.00 ± 0.00	10.47 ± 0.12	1.13 ± 0.27

Two-way ANOVAs showed no significant effect ($P > 0.05$) of soil type, sterilization, or their interaction on the recovery of NO_3^-, TDN and DON. An ANOVA of rank-transformed data indicated that recovery of NH_4^+ was significantly higher in live than sterilized soils ($P < 0.01$) and higher in Y soils compared to B soils ($P < 0.01$)

to B soils ($P < 0.01$). The Y soil replicate that had lowest $TD^{15}N$ and $^{15}NO_3^-$ recovery also had the highest $^{15}NH_4^+$ (0.24 μg ^{15}N g^{-1} dry soil), which is consistent with this sample having the greatest biological immobilization, primarily to an insoluble N pool that we did not recover, and partly to a remineralized $^{15}NH_4^+$ pool. These data indicate that a small amount of biological immobilization of $^{15}NO_3^-$ and remineralization as $^{15}NH_4^+$ occurred in the live soils, particularly in the more N-limited Y soil.

The ^{15}N recovered as DON ranged from 0.6 to 1.5 μg ^{15}N g^{-1} dry soil (Table 2). There were no significant differences between sites nor between treatments (sterile vs. live soils) in the ^{15}N recovered as DON ($P > 0.05$), indicating that this immobilization of $^{15}NO_3^-$ into $DO^{15}N$ must have been abiotic and of similar amount in both soils. The recovery of ^{15}N as DON appears to be somewhat higher in the sterilized samples, which could be related to release of DOC by autoclaving, but the effect of sterilization on $DO^{15}N$ recovery was not statistically significant.

Nitrate quantification

Three steps were taken to ensure that the estimates of recovery of ^{15}N as DON were not due to an artifact of inaccurate NO_3^- quantification due to iron interference (Colman et al. 2007). First, NO_3^- concentrations in DI H_2O extracts were measured by both ion chromatography (IC) and flow injection analysis (FIA). The IC analysis should not be affected by the presence of iron. The comparison of the two independent analyses indicates good agreement (Fig. 2). The slope of 0.934 suggests that the FIA analysis could possibly have underestimated concentrations by 6.6%, although differences of <10% are generally considered within the precision of the instruments.

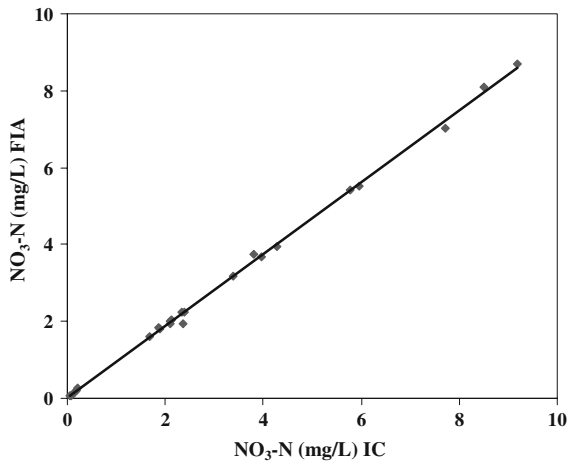

Fig. 2 Linear regression of N–NO_3 measurements performed by flow injection analysis (FIA) and ion chromatography (IC) of DI H_2O extracts: $y = 0.934x + 0.02$, $R^2 = 0.999$. The standard error of the estimate for the slope = 0.007

Second, we measured soluble iron in both water and salt extracts of both live and sterile soils. The soluble iron concentrations ranged from 0.4 to 1.2 mg l^{-1} (Table 3), which is more than 10 times lower than the concentrations reported by Colman et al. (2007) as being problematic with regard to iron interference of NO_3^- quantification by FIA. These results also demonstrate that, on average, the 0.5 M K_2SO_4 did not extract more soluble iron than did DI H_2O. Although the comparison between IC and FIA NO_3^- quantification can be conducted only in water extracts (the K_2SO_4 could interfere with IC analysis), the similar soluble Fe concentrations in both water and salt extracts indicate that Fe interference of NO_3^- quantification is also unlikely in the salt extracts.

The third and most unequivocal approach to rule out significant iron interference was to conduct standard

Table 3 Soluble iron concentrations of soil extracts in DI H_2O and in 0.5 M K_2SO_4 salt (means ± one standard error; $n = 3$)

Pretreatment	Soil	Extract	Soluble Fe (mg l^{-1})
Live	Yunquera	Water	1.10 ± 0.51
		Salt	0.43 ± 0.03
	Bermeja	Water	1.06 ± 0.07
		Salt	0.74 ± 0.29
Sterilized	Yunquera	Water	0.55 ± 0.02
		Salt	0.71 ± 0.29
	Bermeja	Water	0.81 ± 0.16
		Salt	1.19 ± 0.19
Blank		Water	0.05 ± 0.00
		Salt	−0.01 ± 0.00

additions to the soil extracts (Davidson et al. 2008). An aliquot of approximately 0.3 ml of a NO_3^- standard was added to each K_2SO_4 soil extract to increase its concentration by approximately 2 mg N l^{-1}, and the NO_3^- concentrations were then re-analyzed by FIA. The exact amount of the standard added was determined gravimetrically. This provides a test of NO_3^- quantification with the same soluble iron concentrations as were present for the FIA analysis used to estimate DON from the difference between TDN and DIN. The aim of these standard additions was to assay the accuracy of NO_3^- quantification by FIA without changing the concentration of soluble iron in the soil extracts. Because of this, the accuracy of DON estimation as the difference between TDN and DIN in these soil extracts would also be tested. The recovery of added NO_3^- standard ranged from 97 to 103% (Table 4), indicating an unbiased analytical error of approximately ±3%. These data demonstrate that soluble iron was not present in sufficient quantities to affect the quantification of NO_3^- in these extracts.

Table 4 Recovery of standard additions of NO_3 to 0.5 M K_2SO_4 extracts, using standard flow injection analysis (means ± sd; $n = 3$)

Pretreatment	Soil	Percent recovery
Live	Yunquera	102.7 ± 0.6
	Bermeja	97.1 ± 2.5
Sterilized	Yunquera	99.8 ± 1.0
	Bermeja	96.7 ± 1.3
Blank		100.6 ± 1.6

Discussion

Methodological concerns

Because there has been doubt raised about inferring $DO^{15}N$ by subtracting $^{15}NO_3^-$ from $TD^{15}N$ (Colman et al. 2007), we took care to address this concern. The soluble iron concentrations were low in these extracts, suggesting that Fe interference of NO_3^- quantification would be unlikely. An independent analysis of water extracts, using ion chromatography (IC), indicated that NO_3^- quantification by flow injection analysis was within 7% of the IC results. Analyses of standard additions of NO_3^- to salt extracts revealed recovery of 100% (±3) of the added standards, with the 3% error term being random. In contrast, we estimate that 11–14% of the added $^{15}NO_3^-$ was recovered as $DO^{15}N$ in sterilized soils. Taken together, these results exclude the possibility that our calculation of recovery of the label as $DO^{15}N$ could be due to an artifact of incomplete NO_3^- quantification.

Fate of ^{15}N–NO_3 added to soil

A high percentage of the labeled N added was recovered as NO_3^-, indicating low rates of NO_3^- immobilization in these soils, which is consistent with our first hypothesis that these soils subjected to Mediterranean seasonality would exhibit lower rates of abiotic immobilization of NO_3^- compared to published results from more mesic forest sites. For example, 30–60% of the added $^{15}NO_3^-$ disappeared from the extractable inorganic-N pool of Harvard Forest soils within 15 min (Dail et al. 2001). Similar rapid losses of $^{15}NO_3^-$ were observed in soils from the Harvard Forest (Bernston and Aber 2000), in soils from tropical forests (Sotta et al. 2008), and in organic (62%) and mineral (43%) soils from a beech forest (Corre et al. 2003), while other studies have reported lower $^{15}NO_3^-$ recoveries (from non detectable to 10%) in tropical forests soils (Hall and Matson 2003; Corre et al. 2006). Colman et al. (2007) reported no evidence of abiotic immobilization of NO_3^- in a variety of mineral soils, although their laboratory incubations were carried out on well-mixed, sieved soils under aerobic conditions, which likely would have destroyed anaerobic microsites necessary for NO_3^- reduction (Davidson et al. 2008).

Although the rates of $^{15}NO_3^-$ immobilization were low compared to other studies, they were still

detectable. In most cases, recovery of nearly 100% of the added label as $TD^{15}N$ indicates that a significant fraction was recovered as $DO^{15}N$. The lack of significant effects between treatments (sterile vs. live soils) means that the transformation of the $^{15}NO_3^-$ into $DO^{15}N$ must have been abiotic. If it had been a biological process, $DO^{15}N$ would not have been detected in the sterilized soil extracts. As this retention rate was similar in the soils of both study sites, we conclude that the differences in N availability between the two assayed soils did not play an important role in NO_3^- abiotic retention in the Ah horizon under our experimental conditions.

A small amount of ^{15}N was recovered as NH_4^+ in the live soil samples, suggesting some biological immobilization and remineralization during the 24 h incubations. It is also possible that this transformation in the live soils could have been due to dissimilatory reduction of NO_3^- to NH_4^+. Although the differences were small, this biological immobilization was statistically significantly greater in the N-limited Y site, which partially supports our second hypothesis, that rates of NO_3^- immobilization in soils would be lower in the N-saturated site compared to the N-limited stand. However, the results do not support this hypothesis with respect to rates of abiotic immobilization of soil NO_3^-, which were not different between sites.

Factors affecting abiotic retention of NO_3^-

Other studies have reported higher percentages of abiotic retention of added NO_3^- in the O horizon (organic) of temperate forests soils compared to the A horizon (mineral), suggesting that this process may be most important in C-rich soils (Dail et al. 2001; Perakis and Hedin 2001; Davidson et al. 2003; Sotta et al. 2008). Our experiment was carried out using soil samples from an Ah horizon (organic-mineral horizon) due to the fact that a substantial O horizon does not exist in Pinsapo fir forest soil profiles. The lack of a C-rich horizon and the neutral soil pH, which are related to the seasonally dry climate, could explain the lower abiotic retention rates reported in this study.

Davidson et al. (2003) hypothesized that DOC drives NO_3^- abiotic incorporation into soil organic matter by the following steps: soil organic matter (SOM) reduces Fe(III) to Fe(II) in soil microsites; Fe(II) reduce NO_3^- to NO_2 in these anaerobic microsites; NO_2 reacts readily and abiotically with soil organic matter (Thorn and Mikita 2000; Dail et al. 2001). Abiotic NO_3^- retention may depend on the quality and quantity of SOM as well as the availability of reducing agents and the N status of the soil (Dail et al. 2001; Corre et al. 2007).

Other studies have demonstrated that high rates of chronic N deposition can reduce the capacity of the soil for NO_3^- immobilization. Bernston and Aber (2000) demonstrated that 9 years of high-N (150 kg N ha^{-1} $year^{-1}$) additions reduced soil capacity to immobilize NO_3^- in the Harvard Forest. Corre et al. (2007) found that abiotic NO_3^- retention into DON pool in organic and mineral soils from spruce (*Picea abies*) forests decreased across a N enrichment gradient (indicated by leaching:throughfall N ratios). Our results of no effect of N saturation status on soil abiotic NO_3^- immobilization are contrary to our original hypothesis, which was based on these previously published reports. An explanation for this may be that, despite differences in N availability between our study sites, N inputs are overall moderate (net throughfall <13 kg N ha^{-1} $year^{-1}$) compared to the range (26–32 kg N ha^{-1} $year^{-1}$) considered by Corre et al. (2007). While inputs have been sufficient relative to N retention capacity in Sierra Bermeja to provoke some N saturation symptoms (Table 1), they apparently have not yet significantly affected the modest abotic retention capacity reported in both sites, Sierra Bermeja and Yunquera in Sierra de las Nieves.

We had expected low rates of N retention in soils under these forests, due to the seasonally water-limited Mediterranean climate and the absence of a substantial O horizon, relative to other published estimates of temperate and tropical forests, and this expectation was confirmed. Nevertheless, our results indicate that abiotic NO_3^- incorporation is a plausible mechanism for retention of a small fraction of atmospheric-N deposition in *Abies pinsapo* fir forests soils. The mechanisms of abiotic immobilization of NO_3^- remain unconfirmed and controversial (Colman et al. 2007; Davidson et al. 2008), but a growing body of evidence suggests that the phenomenon is common, and that abiotic immobilization of nitrate occurs at high rates only when ideal conditions of plentiful available carbon, reduced minerals in anaerobic microsites, and adequate nitrate supply occur simultaneously.

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