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Nitrogen deposition alters nitrogen cycling and reduces soil carbon content in low-productivity semiarid Mediterranean ecosystems

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Abstract

Nitrogen (N) deposition is a threat to European Mediterranean ecosystems, but the evidence of real ecological impacts is still scarce. We combined data from a real N deposition gradient (4.3–7.3 kg N ha⁻¹ yr⁻¹) from semiarid portions of Spain with data from a field experiment in central Spain to evaluate N deposition effects on soil fertility, function and cyanobacteria community structure. Soil organic N did not increase along the extant deposition gradient, whereas C:N ratios decreased in most locations. Nitrogen fixation decreased along existing and experimental N deposition gradients, a result possibly related to compositional shifts in soil cyanobacteria community. Nitrogen mineralization rates were reduced by N fertilization, suggesting ecosystem N saturation. Soil organic C content and the activity of β -glucosidase decreased along the extant gradient. Our results suggest that semiarid soils in low-productivity sites are unable to store additional N inputs, and that are also unable to mitigate increasing C emissions to the atmosphere when experiencing increased N deposition.

Keywords

C stocks; enzyme activities; extant gradient; fertilization experiment; N cycling; soil cyanobacteria community

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Introduction

Increased nitrogen (N) deposition into terrestrial ecosystems is an outcome of human activities that is promoting major alterations of the global N cycle (Gruber and Galloway, 2008; Sala et al., 2000). These anthropogenic N inputs are predicted to increase in future scenarios in most of the biodiversity hot-spots, including the Mediterranean Basin (Phoenix et al., 2006). Mediterranean ecosystems are considered as particularly vulnerable to increased anthropogenic N inputs (Bobbink et al., 2010; Ochoa-Hueso et al., 2011), but studies dealing with N deposition impacts on natural and semi-natural ecosystems across real anthropogenic gradients in Europe are lacking. Particular considerations for Mediterranean ecosystems include a seasonal summer-drought period and dry deposition as a main source of anthropogenic N, representing 30-70% of total N deposition (Ochoa-Hueso et al., 2011). Critical load determinations for N effects on particular ecosystems within this region are also still to be defined (Bobbink et al., 2010; Ochoa-Hueso et al., 2011), and are potentially complicated in that deposited N may exert different influences on soils, plants, and biological soil crusts (BSCs; a soil surface community active in N-cycling). Determining critical N loads on specific habitats should combine manipulative experiments with N applications mimicking the frequency and temporal nature of the dry and wet N deposition at levels low enough to include the range of known N deposition, with observations on biodiversity, ecosystem processes and indicator species along anthropogenic N deposition gradients (e.g., Bobbink et al., 2010; Ochoa-Hueso et al., 2011).

Many manipulative experiments have been conducted to study potential impacts of N addition on ecosystems such as temperate grasslands (Morecroft et al., 1994), Mediterranean shrublands (Vourlitis et al., 2009), deserts (Allen et al., 2009), and the boreal tundra (Nemergut et al., 2008). However, this approach has some drawbacks, such as the limited number of years that it is practical to maintain such experiments (Dupre et al., 2010). To address a longer temporal scale and larger spatial scale, it is especially useful to combine experiments with surveys across real N deposition gradients. Such gradients have already been used outside Mediterranean Europe to provide evidence on the effects of human activities on natural and semi-natural ecosystems. For example, N deposition gradients correlate with declining plant species richness in the UK (Stevens et al., 2004) and continental Europe (Stevens et al., 2010), declining ectomycorrhizal fungal sporocarp diversity in Alaska (Lilleskov et al., 2001) or increased grass invasion in Californian deserts (Rao et al., 2010).

In many of the experiments conducted so far, extra-cellular enzyme activities of soil micro-organisms are used as indicators of the effects of N deposition because they should reflect the metabolic requirements of soil communities in relation to available nutrients (Zeglin et al., 2007). Some of the most widely studied enzymes are urease and nitrogenase for the N cycle, phosphatases for the P cycle, and β -glucosidase for the C cycle (Ramirez et al., 2012; Zeglin et al., 2007). Changes in the activity of these enzymes may reflect alterations in the soil community and/or physiological plasticity of their constituent species, and it is known that the ability of microbial communities to modulate these activities is a key driver of the productivity and stability of terrestrial ecosystems suffering chronic N pollution (Caldwell, 2005). In this context, it has been proposed that increased soil N availability could result in

greater soil and above-ground C sequestration in terrestrial ecosystems, which could contribute to mitigate the negative climatic effects of increased CO₂ emissions to the atmosphere (Reich et al., 2006; Ramirez et al., 2012). However, neutral or even negative effects of N deposition on C accumulation (as above-ground plant biomass or as part of soil organic matter) have been repeatedly reported in terrestrial ecosystems (Nadelhoffer et al., 1999; Waldrop et al., 2004). Waldrop *et al.* (2004) argued that high concentrations of inorganic N could accelerate the degradation of easily decomposable litter, but also slow down the decomposition of recalcitrant litter with large amounts of lignin; these disparate responses could determine differences in the ability of different ecosystems to store C. In both cases, responses can be attributed to the stimulation or repression of different sets of enzymes. It has also been suggested that N deposition might increase the turnover rates of fungal tissue and, therefore, negate the positive effects that arbuscular mycorrhizal fungi usually have in terms of C sequestration (Treseder and Allen, 2000), whereas the consistent negative impacts of N addition on soil microbial biomass has usually been related to reduced soil C emissions to the atmosphere (Treseder, 2008).

In this study, we combined a manipulative experiment conducted in a semiarid shrubland with observations conducted on shrublands along a productivity and N deposition gradient in Spain to address the following questions:

1. Is atmospheric N deposition already affecting soil fertility, function and N-fixing community structure?
2. If so; are these impacts related to the activity of soil enzymes and to soil respiration?
3. Is the ability of soils to act as a C sink being modified by N deposition?
4. Can simulated N deposition in experimental field conditions replicate impacts of real N deposition on microbial function (i.e., activity of extra-cellular enzymes related to the main nutrient cycles - C, N and P)?
5. Can simulated N deposition alter cyanobacteria community structure and N cycling (net mineralization) processes?
6. Finally, can seasonal variation influence N deposition impacts on N cycling processes, as demonstrated for other ecosystems (e.g, O'Sullivan et al., 2011)?

Material and methods

Study area

A total of 19 study sites were surveyed along the N deposition gradient found in central, southern and eastern semiarid Mediterranean Spain (Figure S1). Sampling sites were always low-productivity semiarid shrublands located below 1000 m a.s.l., with annual rainfall ranging between 300-650 mm, and with a well-developed biological soil crusts (BSCs). These crusts are an intimate association of cyanobacteria, algae, fungi, bryophytes and lichens found at the uppermost millimeters of the surface, which also play an important role in the N cycle in semiarid Mediterranean ecosystems by fixing N (Belnap et al., 2008), affecting N mineralization (Castillo-Monroy et al., 2010) and curtailing N leaching

(Hawkes, 2003). Soils were either calcareous or gypsum (Table S1). Nitrogen deposition estimates at the study sites were obtained from the CHIMERE model (Vivanco et al., 2009).

The field experiment was located within the “El Regajal-Mar de Ontígola” Nature Reserve, near Aranjuez (Madrid province, in central Spain; 40° 00' N, 3° 36' W; Figure S1; Table S1). The mean altitude of the site is 500-600 m asl. The climate of this region is semiarid Mediterranean, with cold-wet winters and hot-dry summers. The mean annual rainfall is ~ 425 mm, with a characteristic summer-drought period. The vegetation is characterized by a well-preserved thicket, with kermes-oak (*Quercus coccifera* L.) and rosemary (*Rosmarinus officinalis* L.) as dominant shrub species. Soils are frequently covered by a well-developed soil crust (Ochoa-Hueso et al., 2011; de Viedma et al., 1985). Legumes are practically absent and thus N fixation is necessarily due to the activity of free-living cyanobacteria and cyanolichens, which contribute to increase soil fertility. Mean cover of cyanolichens at the study site is low (Ochoa-Hueso et al., 2011), and thus most of the atmospherically fixed N should come from free-living cyanobacteria. Soils at the study site are quaternary limestone and texture ranges from loamy to sandy-loam.

Experimental design

A total of 24 2.5 m × 2.5 m plots were set up in October 2007 in Aranjuez, according to a randomized block design. Six blocks, each one consisting of a control (0 kg N ha⁻¹ year⁻¹) and three fertilized plots (with 10, 20 or 50 kg N ha⁻¹ year⁻¹), were randomly chosen in apparently homogenous and separated sites within the shrubland thicket. The background N deposition of the study area is 6.4 kg N ha⁻¹ year⁻¹ (Table 1). The fertilization treatments reasonably fall within the predicted N deposition scenarios by the year 2050 for the Mediterranean Basin (Phoenix et al., 2006) or within the range of measurements reported in other Mediterranean areas (Fenn et al., 2003). 2 L of an aqueous solution with the corresponding NH₄NO₃ dose (0, 0.019, 0.037 and 0.093 M, respectively) were monthly added to each plot. Fertilizations were not made during the extreme summer-drought period (July-August) to keep the experiment as natural as possible, avoiding soil re-wetting processes (Austin et al., 2004) and simulating the peak of N availability associated with the onset of rains (Fenn et al., 2003). A triple dose was then applied in September.

Soil sampling and analyses

Soil samples (0-1 cm depth) were collected in all the surveyed sites along the natural N deposition gradient as described in Bowker *et al.* (2011). This sampling depth was selected because most of the soil organisms, including soil crusts, are concentrated in the first centimeter. Sampling was conducted from May - September 2008 and from May - August 2009. Total biological soil crust cover was also recorded at each site as described in Bowker *et al.* (2011). Soil samples from the natural N deposition gradient were transported to the laboratory, where they were sieved by 2 mm mesh and air-dried for two months. Respiration rates were determined by NaOH absorption followed by titration with HCl (Froment 1972). Organic carbon was estimated using the Walkley-Black method (Nelson and Sommers 1982). Total N was obtained using a San++ Analyzer (Skalar, Breda, The Netherlands) after digestion in sulfuric acid. Urease, phosphatase and β-glucosidase activities were determined

as described in Tabatai (1982). Nitrogenase activity was determined as described in Maestre *et al.* (2012).

Seasonal soil surveys were conducted in the manipulation experiment from the early autumn 2008 to the early summer 2009. Eight soil cores (2 cm width) were seasonally removed from the first 0–4 cm of soil in the 25 cm internal perimeter band of each plot (we used this band to minimize disturbances). Soil samples were bulked in air-tight plastic bags, brought to the lab, and air-dried for several days. Prior to laboratory analyses and incubations, samples were sieved through a 2-mm mesh. Nitrogenase, β -glucosidase and phosphatase activities were also measured in soils from the fertilization experiment in spring 2009. This season was selected because it coincides with the peak of biological activity at the study site. Potential N mineralization (evaluated as the sum of net nitrification and net ammonification) was seasonally calculated in the soils from the field experiment as described in Vourlitis *et al.* (2009).

We also evaluated the effects of the experimental N additions on the diversity of crust-forming cyanobacteria by using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments. Total genomic DNA was extracted from fresh soil samples (0.5 g) obtained from the different plots (0–1 mm soil depth) in spring 2009 using the PowerSoil™ DNA Isolation kit for subsequent PCR amplification of 16S rRNA genes. Cyanobacterial fragments of 16S rRNA genes suitable for DGGE analysis were amplified from total genomic DNA using the following primer pair: CYA359fGC and CY781r (Nubel *et al.*, 1997). Each 25 μ l volume of PCR mix [75 mM Tris pH 9.0/50 mM KCl/20 mM $(\text{NH}_4)_2\text{SO}_4$] contained 1 unit of Taq polymerase, 0.2 mM of each of the four dNTPs, 0.4 mM of each primer, 100 mg of bovine serum albumin, 1.5 mM of MgCl_2 , 5 ml de 5xTaq Master PCR enhancer (Prime) and ca 10–50 ng genomic DNA. Annealing conditions were 60°C. Acrylamide gels (6%) with a 30 to 60% urea-formamide denaturing gradient were prepared following the manufacturer's instructions. Lanes were loaded with 22 μ l of PCR product, run at a constant 200 V for 7 h at 60°C, and EtBr stained to visualize and photograph the resultant bands. The most predominant band was excised and incubated 1 h at 60°C in water before PCR amplification. The eluted DNA was reamplified as described above but using the primers devoid of the GC clamp. The PCR products were cleaned on a QIAGEN quick spin column. Both complementary strands were sequenced separately at MACROGEN (Seoul, South Korea). Sequences were BLAST analyzed against the GenBank database and deposited in GenBank.

Statistical analyses

A Principal Component Analysis (PCA) was conducted with N deposition, BSC cover, and soil C and N fertility, ecosystem function and climatic variables compiled from each location surveyed along the extant N deposition gradient (hereafter, PCA_ECO) to identify major components of variability among sites. To detect common patterns of enzyme response along this N gradient, we also conducted a PCA with soil enzyme activities (hereafter, PCA_ACT). Stepwise multiple linear regression analyses were used to relate soil measurements (C, N, individual soil enzymes, PCA_ACT components and soil respiration) as dependent variables to N deposition and climatic and geographical variables. PCA_ECO

components one and three revealed the existence of a major group of 14 sites (Fig. 2), and we also conducted further stepwise multiple linear regressions within this group. These 14 sites were consistently located at an Euclidean distance of less than 1.5 units from the origin.

Univariate repeated measures analyses of variance (RM-ANOVA) and covariance (RM-ANCOVA), with initial soil NO_3^- -N and NH_4^+ -N as covariates, were used to test the effects of N fertilization treatments and sampling season on net N mineralization, net nitrification and net ammonification in the field experiment. Univariate analyses were also separately conducted for each season. ANOVA tests were used for the effects of N addition on soil enzyme activities and OTUs (operational taxonomic units) richness. LSD post-hoc tests were used for multiple comparisons. All statistical analyses were done with SPSS17.0 (SPSS Inc., Chicago, IL, USA).

Results

Nitrogen deposition gradient

The N deposition gradient length covered in this study was of $3.0 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ (from 4.3 to $7.3 \text{ kg N ha}^{-1} \text{ yr}^{-1}$). Four principal components explained 78.8 % of the total variance in N deposition, soil crust cover, and ecosystem function, climatic and fertility variables (Table S2). Two groups of locations were identified when components one and three were plotted against each other (Fig. 1). The locations outside of the major group (five locations) were higher rainfall (+13.3%) and temperature sites (+7.26%) and with higher soil organic N (+93.4%) and C contents (+40.5%), whereas the locations in the major group were distributed along a wide N deposition-fertility-climatic-geographical gradient (Fig. 1). The locations outside of the major group were Almería, Huelves, Jaen (calcareous and gypsum) and Zorita (Fig. 1). The PCA_ACT analysis revealed two principal components, explaining 66.7% of the total variance (Table S3). The first component was mainly related to nitrogenase and β -glucosidase activities, whereas the second component was related to phosphatase and urease activities.

Stepwise regressions with 19 sites showed a negative direct relationship between N deposition and soil oxidizable C (Figure 2; Table 1a), but none of the other measurements analyzed were related to the N deposition gradient (Table 1a). Additional stepwise and univariate regression analyses conducted with 14 locations revealed inverse relationships between N deposition and N fixation (Figure 3a) and β -glucosidase (Figure 3b) activities. A negative relationship was also found between N deposition and the first component of PCA_ACT ($R^2 = 0.54$; $P < 0.01$), whereas the relationship between N deposition and the second component of the PCA_ACT was not significant (Table 1b). Oxidizable soil C (Fig. 3c) and soil C:N ratio decreased with increased N deposition loads (Fig. 3d).

Field fertilization experiment

β -glucosidase and phosphatase activities were not affected by the N addition treatments after 1.5 years of continuous (Fig. 4ab; Table 2). In contrast, N-fixation was strongly reduced by simulated N deposition (Fig. 4c; Table 2). The richness of cyanobacterial OTUs significantly increased with N fertilization ($df = 3, 20$; $F = 5.63$; $P < 0.01$; Fig. 5a). The

analysis of DGGE profiles revealed a common predominant OTU (arrow in Fig. S2) in all the plots, which corresponded to a cyanobacteria closely related to *Phormidium autumnale* (99% similarity). This dominant OTU was accompanied of different weak bands (especially in N fertilized plots), but no clear pattern in relation to simulated N deposition could be established (Fig. S2). Nitrogen fixation activity and OTU richness were also negatively related (Figure 5b).

Net nitrification was the main process driving N mineralization and, thus, the impacts of simulated N deposition on N-cycling processes (i.e., net nitrification and net mineralization rates were almost equivalent; Table 2; Fig. 6). The peak of N mineralization was in winter, and the minimum values were observed during spring (Fig. 6). In contrast, net ammonification was highest in spring and lower and negative in the rest of seasons (Fig. 6). Potential N mineralization was reduced in the highest N fertilization treatment when soil NO_3^- -N and NH_4^+ -N were used as covariates (Table 2; Fig. 6). Net ammonification was also marginally affected by simulated N deposition, with a significant reduction of NH_4^+ -N accumulation in summer 2009 (Table 2; Fig. 6).

Discussion

Nitrogen cycling

Modeled values of N deposition at the sampling sites were relatively low, and were always below reported critical loads for European terrestrial ecosystems (between 10-15 kg N ha⁻¹ yr⁻¹; Dise and Wright, 1995). Mediterranean ecosystems of California have critical loads even below our lowest N deposition value, with some of them being negatively impacted with loads exceeding 3 kg N ha⁻¹ yr⁻¹ (Fenn et al., 2010).

Soil organic N content did not increase along the N deposition gradient studied, suggesting that low-productivity semiarid Mediterranean ecosystems do not store extra atmospherically-derived N. Interestingly, soil organic N content remained unexplained when climatic predictors were also taken into account. These results could be due to the confounding and compound effects of climate seasonality and stand development, amongst other factors. In contrast, C:N ratios were negatively correlated with N deposition in most of the sites surveyed, a response driven by the reduced soil C content. The lack of significant relationships between N deposition and soil N content has also been documented in other similar studies. For this reason, N content is frequently considered a poor predictor of increased N deposition when compared with community composition and/or ecosystem function (Stevens et al., 2009, 2011), including soil enzyme activities.

Increased atmospherically-deposited N in soils frequently results in lower N fixation activity as a consequence of a functional response of soil micro-organisms (DeLuca et al., 2007). Given that N fixation is a highly energetic process, alterations of soil N availability should be rapidly followed by changes in N fixation rates (Vitousek and Field, 1999). This assertion is in agreement with our results. Given that soil nitrogenase was significantly down-regulated by N deposition loads ranging from 4.3 to 56.4 (N fertilization + background) kg N ha⁻¹ yr⁻¹, this enzyme seems particularly advisable for field studies conducted along wide N deposition gradients. The reduction of N fixation rates with simulated N deposition

parallels a significant increase in OTUs richness that can be attributed to a proliferation of facultative/non N₂-fixing nitrophytic cyanobacteria able to thrive under high N levels. Similar responses have already been shown for other ecosystems after N enrichment (Poikolainen et al., 1998). Given that *P. autumnale*, a non-heterocystous facultative N-fixer, was the most abundant species across all the plots, we argue that N fixation response to increased N fertilization is most likely related to down-regulation of the activity of this species, and cautiously suggest that N-fixation responses along the gradient could also be attributed to the synergistic effect of enzyme down-regulation and cyanobacteria community alteration.

Contrary to our expectations, urease activity did not respond to increased N deposition. However, a weak and negative relationship between urease and N deposition in the major group of sites suggests the importance of including a higher number of locations in further investigations to determine if the down-regulation of the urease activity with increased N deposition loads could be a relevant mechanism driving alterations in the N cycle.

Nitrogen cycling processes can be promoted or down-regulated depending on the relative importance of N limitation and/or toxicity of soils and depending on the dominant vegetation and plant cover (Kieft et al., 1998). In our experiment, N mineralization was down-regulated by simulated N deposition, suggesting that anthropogenic N may impede organic N cycling and lead to ecosystem N saturation (Kang and Lee, 2005). As the dominant process in basic soils, this response was driven by net nitrification. However, significant results were only found when accounting for between-plot heterogeneity in soil mineral N, both in oxidized and reduced forms, which is noteworthy. In addition, we also showed decreasing rates of NH₄⁺-N accumulation in soils after N fertilization, although this response was season-dependent and only clearly evident in the summer 2009 survey. Similar to our experiment, Keeler *et al.* (2009) demonstrated that N addition on temperate forested and grassland sites can slow down soil organic matter decomposition, with local differences in soil pH, moisture, soil C and microbial biomass explaining much of the among-site variation in enzyme activity. Our results of reduced N mineralization rates contrast with studies in a N-fertilized California chaparral (Vourlitis et al., 2009), but parallels the reduction of net ammonification with N addition reported by Vourlitis *et al.* (2009) in a Californian coastal sage scrub. Finally, a recent meta-analysis has demonstrated that decomposition is usually inhibited by N fertilization when: (i) N fertilization rates are 2-20 times than the N deposition background, (ii) ambient N deposition is higher than 5-10 kg N ha⁻¹ yr⁻¹ or (iii) litter quality is low (Knorr et al., 2005). Given that N deposition background at our study site is 6.4 kg N ha⁻¹ yr⁻¹, reduced N mineralization rates are a logical result after increasing by more than eight times the N loads received by the experimental plots.

Carbon cycling

The question of C storage ability of soils chronically affected by N pollution has also been widely debated, with studies on this topic reporting suppressed, enhanced, or unchanged C-storage ability in response to N deposition. Studies also report soils functioning ranging as net C sinks or sources under N fertilization (Reich et al., 2006). This effect has previously

been described as ecosystem-dependent, being also related to the N saturation level of the studied ecosystems (Waldrop et al., 2004). In our case study, soil C content decreased along the N deposition gradient, suggesting that low-productivity semiarid Mediterranean soils are not able to mitigate increasing C emissions to the atmosphere when experiencing increased N deposition loads (Vourlitis and Pasquini, 2008). Evidence from other type of ecosystems suggests that N deposition is also putting valuable C sinks at serious risk, such as *Sphagnum* peatlands (Bragazza et al., 2006).

In this study, the effects of N deposition on soil C pools cannot be directly attributed to increased soil respiration and altered soil C emissions. Therefore, our results implicate alternative mechanisms, including reduced ecosystem productivity after exceeding critical loads (Magill et al., 2000), although we did not test this hypothesis. Soil respiration was, in contrast, controlled by total BSC cover, as found in similar ecosystems (Castillo-Monroy et al., 2011). Considering other mechanisms, Sinsabaugh *et al.* (2005) demonstrated that decreased or increased soil C content in high-N fertilized plots varied with the type of forest studied; the type of response was attributed to an enzyme activity factor that included both oxidative and hydrolytic enzymes. Similarly, Carreiro *et al.* (2000) have suggested the importance of C-acquiring enzymes, such as the lignin-degrading phenol oxidase, in the context of increased atmospheric N pollution after evidence of either increased or decreased rates of litter decay by forest-floor microbial communities. In particular, phenol oxidase activity declined with N fertilization in high-lignin oak litter (Carreiro *et al.* 2000; Waldrop et al., 2004) and increased dissolved organic C leaching (Waldrop et al., 2006). The effects of increased N fertilization on ligninolytic enzymes can, thus, translate into either increased or reduced soil C pools.

The role of cellulolytic enzymes (our focus) as mediators of the impacts of N deposition on soil C dynamics is even less clear. β -glucosidase is usually positively correlated with soil C content and microbial biomass (Sinsabaugh et al., 2008; DeForest et al., 2004), although the activity of this enzyme can also be either directly (up or down-regulation) or indirectly (through altered soil C and microbial biomass) altered by N deposition. Thus, reduced soil C content associated with increased N deposition loads could imply lower β -glucosidase activity, unless an induced nutritional imbalance alters this relationship. Our results do not support the induced nutritional imbalance as the main driver of soil β -glucosidase activity in sites polluted with N, but suggests the role of soil N toxicity on soil microorganisms at high N loads (Alarcón-Gutiérrez et al., 2008), the potential repression of this enzyme by extra N (DeForest et al., 2004), and also the importance of evaluating the activity of this enzyme in the context of soil C content and soil microbial biomass (Sinsabaugh et al., 2008). Supporting our findings, Grandy *et al.* (2009) demonstrated that variations in enzyme activities in the context of increased atmospheric N deposition are frequently related with changes in soil organic matter chemistry and confounded by climate and altitude gradients (Britton and Fisher, 2010).

Contrasting with the results obtained in the gradient study, β -glucosidase activity was not altered in soils from the manipulation experiment. These results support the role of the unaltered soil C content on the response of the cellulolytic enzyme activity to N fertilization (Ochoa-Hueso and Manrique, unpublished data; Grandy et al., 2009). Other studies have

also found disparate effects of N addition on β -glucosidase activity. For example, DeForest *et al.* (2004) found reduced β -glucosidase activity in the mineral soil layer of Northern Hardwood Forests suffering chronic NO_3^- deposition, a response attributed to enzyme repression, whereas Chung *et al.* (2007) reported increased β -glucosidase activity in soils receiving $40 \text{ kg N ha}^{-1} \text{ yr}^{-1}$. The evidence from Californian grasslands suggests that phosphatase and β -glucosidase activities are able to respond to increased N supply in Mediterranean ecosystems, but also shows that these alterations can be of smaller magnitude than annual variations in their activity (Gutknecht *et al.*, 2010).

Concluding remarks

The impacts derived from N fertilization experiments or real N deposition gradients are often comparatively subtle and lie near the boundaries of statistical and methodological resolution, suggesting the importance of multivariate and covariance analyses and also of seasonal evaluations. We have shown that N deposition was negatively related to the activity of two soil enzymes (nitrogenase and β -glucosidase) under real conditions and that the response was also affected by preexisting and superimposed environmental gradients. Given that phosphatase and urease activities were not evidently related to the atmospheric N gradient, future works studying the impacts of N pollution or bio-monitoring programs using soils in European semiarid Mediterranean ecosystems should mainly focus on either nitrogenase or β -glucosidase or, even better, on both at the same time, whereas managers aiming to develop and establish bio-monitoring programs using soils as biomarkers should be aware of the potential presence and importance of having locations with disparate environmental conditions. Based on the critical load definition (a quantitative estimate of an exposure to one or more pollutants below which significant harmful effects on specified sensitive elements of the environment do not occur according to present knowledge), and on the negative slopes of regression lines found in the gradient study, it seems likely that most of the soil indicators responding to atmospheric N pollution have critical loads below the lowest end of the gradient ($4.3 \text{ kg N ha}^{-1} \text{ yr}^{-1}$). Finally, given that locations with N deposition values above $4.3 \text{ kg ha}^{-1} \text{ yr}^{-1}$ are common across central, eastern and southern Iberian peninsula (Ochoa-Hueso *et al.*, 2011), the implementation of political strategies aimed at reducing N emissions (both oxidized and reduced) should be a priority if we want to avoid negative impacts of N pollution on semiarid Mediterranean ecosystems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Scatter plot (first and third PCA_ECO components) showing the main group of locations ($N = 14$) and five locations excluded from further stepwise regressions, decision based on their higher altitude and lower mean N deposition values. Black dots are locations and light grey dots are eigenvalues for each variable.

Figure 2.
Relationship between the N deposition gradient and oxidizable soil C content (%) ($N = 19$).

Figure 3. Relationship between the N deposition gradient and (a) N-fixation, (b) glucosidase activity, (c) soil organic C and (d) soil C:N ratio. Graphs are presented with $N = 15$ (see “methods section” for details on location groups).

Figure 4. Simulated N deposition effects on (a) β-glucosidase, (b) phosphatase and (c) nitrogenase activities measured in spring 2009. Different lower-case letter above SE bars denotes significant differences between treatments after LSD post-hoc tests. $N = 24$.

Figure 5. Simulated N deposition effects on (a) OTUs richness and (b) relationship between OTUs richness and nitrogenase activity. Different lower-case letter above SE bars denotes significant differences between treatments after LSD post-hoc test. $N = 24$.

Figure 6.

Simulated N deposition effects on N cycling processes (net ammonification and net mineralization). Effects are shown both globally (inset graphs) and by season. Different lower-case letter above SE bars and between seasons denotes significant differences between treatments after LSD post-hoc tests. $N = 24$.

Table 1

Stepwise linear regression analyses with (a) all locations ($N = 19$) and (b) a subset of the sites surveyed ($N = 14$). See the Methods section for details.

a						
	Variable	R^2	P	Variable	R^2	P
Nitrogenase	-			-		
Phosphatase	Latitude (+)	0.34	0.01	-		
β -glucosidase	Longitude (-)	0.26	0.03	-		
Urease	-			-		
PCA_ACT_1	-			-		
PCA_ACT_2	Latitude (+)	0.25	0.03	-		
Carbon (%)	Total N deposition (-)	0.22	0.05	Latitude	0.41	0.02
Nitrogen (%)	-			-		
C:N	-			-		
Soil						
respiration	Total cover (+)	0.38	0.01	Longitude (-)	0.73	<0.01
b						
	Variable	R^2	P	Variable	R^2	P
Nitrogenase	Altitude (+)	0.34	0.03	-		
Phosphatase	Latitude (+)	0.43	0.01	Temperature (+)	0.63	<0.01
β -glucosidase	Altitude (+)	0.49	<0.01	Longitude (-)	0.75	<0.01
Urease	-			-		
PCA_ACT_1	Altitude (+)	0.62	<0.01			
PCA_ACT_2	-			-		
Carbon (%)	Altitude (+)	0.34	0.03	-		
Nitrogen (%)	-			-		
C:N	Total N deposition (-)	0.47	<0.01	-		
Soil						
respiration	-	0.48	<0.01	-		

Table 2

Nitrogen fertilization and sampling season effects on net ammonification, net N mineralization and enzyme activities in the field experiment. The effects of mineral N on net N mineralization as covariate are also shown.

	Net ammonification			Mineralization (log)			Phosphatase			β -glucosidase			Nitrogenase		
	df	F	P	df	F	P	df	F	P	df	F	P	df	F	P
Nitrogen	3, 20	2.50	0.09	3, 18	2.44	0.10									
Season	3, 18	12.88	<0.01	3, 16	3.10	0.06									
Nitrogen x season	9, 60	0.86	0.56	9, 54	0.75	0.66									
NO ₃ -N				1, 18	7.32	0.01									
NO ₃ -N x season				3, 16	0.87	0.48									
NH ₄ -N				1, 18	17.24	<0.01									
NH ₄ -N x season				3, 16	0.75	0.31									
Nitrogen - autumn 2008	3, 20	1.35	0.29	3, 20	0.20	0.90									
Nitrogen - winter 2008	3, 20	1.01	0.41	3, 20	0.35	0.79									
Nitrogen - spring 2009	3, 20	0.56	0.65	3, 20	0.93	0.45	3, 20	0.31	0.82	3, 20	0.06	0.98	3, 20	4.10	0.02
Nitrogen - summer 2009	3, 20	3.85	0.03	3, 20	0.15	0.93									