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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

Anthropogenic N deposition poses a threat to European Mediterranean ecosystems. We combined data from an extant N deposition gradient (4.3–7.3 kg N ha^{-1} yr^{-1}) from semiarid areas of Spain and a field experiment in central Spain to evaluate N deposition effects on soil fertility, function and cyanobacteria community. Soil organic N did not increase along the extant gradient. Nitrogen fixation decreased along existing and experimental N deposition gradients, a result possibly related to compositional shifts in soil cyanobacteria community. Net ammonification and nitrification (which dominated N-mineralization) were reduced and increased, respectively, by N fertilization, suggesting alterations in the N cycle. Soil organic C content, C:N ratios and the activity of β-glucosidase decreased along the extant gradient in most locations. 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 when experiencing increased N deposition.

1. Introduction

Increased nitrogen (N) deposition into terrestrial ecosystems is an outcome of human activities that is promoting major alterations of the global N and C cycles (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 (Galloway et al., 2004). Mediterranean ecosystems are now considered to be particularly vulnerable to increased anthropogenic N inputs (Ochoa-Hueso et al., 2011a), but studies dealing with N deposition impacts on natural and semi-natural ecosystems across extant anthropogenic N pollution gradients in southern Europe are lacking. Particular considerations for Mediterranean ecosystems include a seasonal summer-drought period and dry deposition as the main source of anthropogenic N (between 30 and 70% of total N deposition), which also result in high amounts of nitrate (NO_3^- – N) leaching after the onset of the first autumnal rains (Ochoa-Hueso et al., 2011a). Critical load determinations for N effects on particular ecosystems within this region are also still to be defined (Bobbink et al., 2010) but are potentially complicated in that deposited N may exert different influences on the receptors (soils, plants, and biological soil crusts-BSCs). These BSCs represent a characteristic and intimate association of cyanobacteria, algae, fungi, bryophytes and lichens found at the uppermost millimetres of the soil surface in drylands (including semiarid Mediterranean ecosystems) that play an important role in the N cycle by fixing N (Belnap et al., 2008), affecting N mineralization (Castillo-Monroy et al., 2010) and curtailing N leaching (Hawkes, 2003). Determining critical N deposition loads on specific habitats must combine manipulation experiments 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). Many manipulation experiments have been conducted to study potential impacts of N addition on ecosystems such as temperate grasslands (e.g., Morecroft et al., 1994; Horswill et al., 2008), Mediterranean shrublands (e.g., Vourlitis et al., 2009; Dias et al., 2011), deserts (e.g., Allen et al., 2009; Schneider and Allen, 2012), and the boreal tundra

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(e.g., Nemergut et al., 2008; DeLuca et al., 2007). However, the approach has some drawbacks, such as the limited number of years that it is practical to maintain such experiments (Duprè et al., 2010). To address a longer temporal and larger spatial scale, it is especially useful to combine experiments with surveys across extant N deposition gradients.

Enzyme activities of soil microorganisms have been used as indicators of the effects of N deposition in many of the manipulation and gradient experiments conducted so far 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 soil 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 soil 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 productivity and stability of the terrestrial ecosystems suffering chronic N pollution (Caldwell, 2005). In this context, it has been proposed that increased soil N availability could result in greater below- and above-ground C sequestration in terrestrial ecosystems, which could contribute to mitigating the negative effects of increased CO_2 emissions to the atmosphere and thus climate change (Reich et al., 2006; Ramirez et al., 2012; Liu and Greaver, 2010). 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 also been repeatedly reported in terrestrial ecosystems (Nadelhoffer et al., 1999). In a meta-analysis, Liu and Greaver (2010) demonstrated that N addition can increase short term belowground C storage in the organic layer but failed to predict long term responses since there was no significant change in the mineral soil C content. Waldrop et al. (2004) also argued that high concentrations of inorganic N could accelerate the degradation of easily decomposable litter, but slow down the decomposition of recalcitrant litter with large amounts of lignin and that 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 contribution of arbuscular mycorrhizal fungi to 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; Liu and Greaver, 2010). Finally, soil organic and inorganic N contents are also potentially affected by increased N deposition. However, the transient nature of inorganic N in soil and the plethora of environmental factors that determine soil organic N content usually make them poor predictors of increased N deposition when compared with community structure and ecosystem functioning indicators (Stevens et al., 2009).

In this study, we combined a manipulation N fertilization experiment conducted in a semiarid Mediterranean shrubland with observations on low-productivity semiarid Mediterranean shrublands, tussock grasslands and woodlands along an extant N deposition gradient in central, eastern and southern Spain to evaluate the effects of atmospheric N deposition on (1) soil N and C fertility and (2) soil functioning. We also sought to address (3) if simulated N deposition effects on microbial function under experimental field conditions can replicate the impacts of extant N deposition gradients and (4) if the effects on N fixation rates could be related to alterations in N-fixing community structure. Finally, (5) we sought to determine if simulated N deposition can alter N cycling processes (i.e., net mineralization, nitrification and ammonification rates) and (6) if these responses depend on seasonal variations.

2. Material and methods

2.1. Extant N deposition gradient

A total of 19 study sites were surveyed along the N deposition gradient in central, southern and eastern semiarid Mediterranean Spain (Fig. S1 and Table S1). Sampling sites comprised low-productivity semiarid shrublands, tussock grasslands and woodlands located below 1000 m a.s.l., with annual rainfall ranging between 300 and 650 mm, and with a well-developed and late-successional BSC. Soils were derived from calcareous or gypsum parent material (Table S1). Nitrogen deposition estimates at the study sites were obtained from the CHIMERE model applied for 2009 and for a domain covering the Iberian Peninsula and the Balearic Islands at a 0.1° horizontal resolution (Vivanco et al., 2009).

Ten soil sub-samples (0–1 cm depth) were collected at all sites as described in Bowker et al. (2011). This sampling depth was selected because most of the soil organisms forming part of the soil crusts are concentrated in the first centimetre. Sampling was conducted from May–September 2008 and from May–August 2009. Total BSC cover was also recorded at each site as described in Bowker et al. (2011). Soil samples from the extant N deposition gradient were transported to the laboratory, where they were sieved (2 mm mesh) and air-dried for two months. Respiration rates were determined in the laboratory by NaOH absorption followed by titration with HCl with soils incubated in a dark chamber at 80% field capacity and at 22 °C for 4–5 days (Froment et al., 1972). Soil organic C was estimated using the Yeomans and Bremmer (1989) method. Soil organic N was measured using a SKALAR San^{++} Analyser (Skalar, Breda, The Netherlands) after digestion in sulphuric acid. Soil urease, phosphatase and β-glucosidase activities were determined as described in Tabatabai (1982), whereas soil nitrogenase activity was determined by acetylene reduction (Maestre et al., 2012).

2.2. Field N fertilization experiment

The field experiment was located within the "El Regajal-Mar de Ontígola" Nature Reserve, near Aranjuez (central Spain; 40° 00′ N, 3° 36′ W; 580 m a.s.l.; Fig. S1; Table S1). The climate is semiarid Mediterranean, with cold-wet winters and hot-dry summers. The mean annual rainfall is ~425 mm, with a characteristic summer-drought. The vegetation is characterized by a well-preserved shrubland, 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., 2011b). Legumes are rare and thus any N fixation reflects the activity of free-living N fixers (cyanobacteria, cyanolichens and, possibly, heterotrophic microorganisms), improving soil fertility. Mean cover of cyanolichens at the study site is also low (Ochoa-Hueso et al., 2011b), and thus most of the fixed N likely comes from free-living microorganisms. Soils are quaternary limestone and texture ranges from loamy to sandy-loam. A total of 24 2.5 m × 2.5 m plots were set up in October 2007 in a randomized block design. Six blocks, each one consisting of a control (0 kg N ha^{-1} yr^{-1}) and three N-fertilized plots (with 10, 20 or 50 kg N ha^{-1} yr^{-1}), were randomly chosen in apparently homogeneous and separated sites within the shrubland. The background N deposition of the study area is 6.4 kg N ha^{-1} yr^{-1} (Table S1). 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 ammonium nitrate (NH_4NO_3) dose (0, 0.019, 0.037 and 0.093 M, respectively) were monthly added to each plot (see Ochoa-Hueso and Manrique, 2011 for more details).

Seasonal soil surveys were conducted from autumn 2008 to summer 2009. Eight cores (2 cm width) were seasonally removed from the first 0–4 cm of soil within the 25 cm internal perimeter band of each plot (we used this band to minimize disturbances). Sampling procedures and depth differed between the manipulation and gradient studies because of practical constraints but were consistent within each experimental approach. Thus comparisons between approaches must take this into account. Soil samples were bulked in air-tight plastic bags 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 as described above. This season was selected because it coincides with peak biological activity at the study site. Seasonal potential N mineralization was calculated in the soils from the field experiment after 28-day laboratory incubations as extensively described in Vourlitis et al. (2009).

We also evaluated the effects of the experimental N additions on the diversity of crust-forming cyanobacteria communities 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 this case) 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 CYA781r (Nübel et al., 1997). Given that the pair of primers used can also amplify 16S rRNA from plastids, PCR products could also include 16S rRNA fragments from other oxygenic phototrophic

microorganisms. Each 25 μl volume of PCR mix [75 mM Tris pH 9.0/50 mM KCl/20 mM $(NH_4)_2SO_4$] 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 5×Taq Master PCR enhancer (Prime) and ca 10–50 ng genomic DNA. Annealing conditions were 60 °C. Acrylamide gels (6%) with a 30–60% urea-formamide denaturing gradient were prepared following the manufacturer's instructions. Lanes were loaded with 22 μl 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 analysed against the GenBank database and deposited in GenBank.

2.3. Statistical analyses

2.3.1. Extant N deposition gradient

A Principal Component Analysis (PCA) with a varimax rotation was conducted with modelled N deposition loads, total BSC cover, soil organic N and C contents, nitrogenase and β-glucosidase activities (two important soil enzymes related to the N and C cycles) and climatic variables (temperature and rainfall amount) compiled from each location surveyed (hereafter, PCA_ECO) to identify major components of environmental variability between sites. To detect common patterns of enzyme response along the extant N deposition gradient, we also conducted a PCA with soil enzyme activities (hereafter, PCA_ACT). Stepwise multiple linear regression analyses were used to relate soil measurements (organic C, organic 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 two revealed the existence of a major group of 14 sites (Fig. 1; see below in the Results section for further details), 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 distance units from the origin of coordinates (Fig. 1).

2.3.2. Field N fertilization experiment

Three-way analyses of variance (ANOVA) were used to test the effects of N fertilization treatments, sampling season and experimental block on net N mineralization, net nitrification and net ammonification in the field experiment. Nitrogen fertilization and sampling season were considered as fixed factors whereas experimental blocks were considered as a random factor. Analyses were also separately conducted for each season. Two-way ANOVA tests were used for the effects of N addition and experimental block 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).

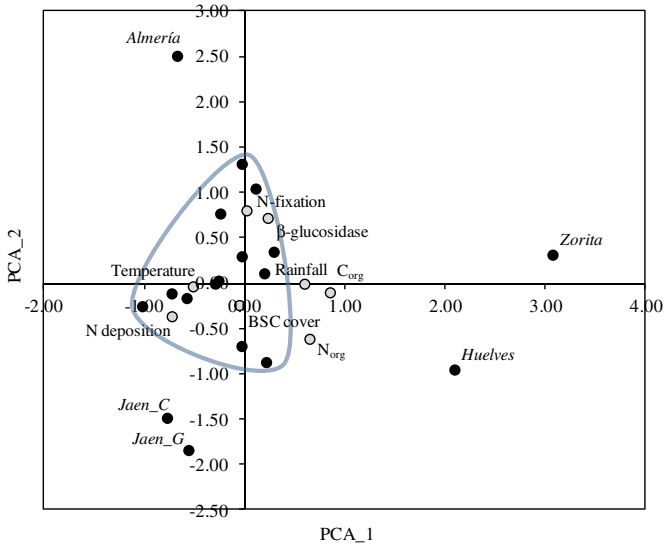

Fig. 1. Scatter plot (first and second PCA_ECO components) showing the main group of locations ($N = 14$) and five locations excluded from further stepwise regressions. Black dots are locations and light grey dots are eigenvalues for each variable. Eigenvalues are presented un-rotated for visual clarity (see Supplementary material for rotated eigenvalues).

3. Results

3.1. Extant N deposition gradient

The extant N deposition gradient received between 4.3 and 7.3 kg N ha^{-1} yr^{-1}. Four principal components explained 78.8% of the total variance in N deposition, total BSC cover and ecosystem function, climatic and fertility variables (Table S2). Two groups of locations were identified when components one and two were plotted against each other (Fig. 1). The locations outside of the major group (five locations) had higher rainfall (+13.3%) and temperature (+7.26%) and also higher soil organic N (+93.4%) and organic 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 soil nitrogenase and β-glucosidase activities, whereas the second component was related to soil phosphatase and urease activities.

Stepwise regressions with 19 sites showed a negative linear relationship between modelled N deposition and soil organic C (Fig. 2; Table 1a), but none of the other measurements analysed were related to the N deposition gradient (Table 1a). Stepwise regressions with 14 locations revealed that soil nitrogenase and β-glucosidase activities and soil organic C content were best explained by geographical variables (i.e., altitude). Additional univariate regression analyses with 14 locations revealed inverse relationships between estimated N deposition and soil N fixation (logarithmic; Fig. 3a) and β-glucosidase (Fig. 3b) activities. A negative relationship was also found between N deposition and the first component of the 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). Soil organic C (Fig. 3c) and soil C:N ratio (logarithmic; Fig. 3d) also decreased with increasing N deposition loads.

3.2. Field N fertilization experiment

Soil β-glucosidase (N fertilization: df = 3, 15; $F = 0.22$; $P = 0.88$; block effect: df = 5, 15; $F = 12.04$; $P < 0.01$) and phosphatase (N fertilization: df = 3, 15; $F = 1.85$; $P = 0.18$; block effect: df = 5, 15; $F = 21.14$; $P < 0.01$) activities were not significantly affected by the

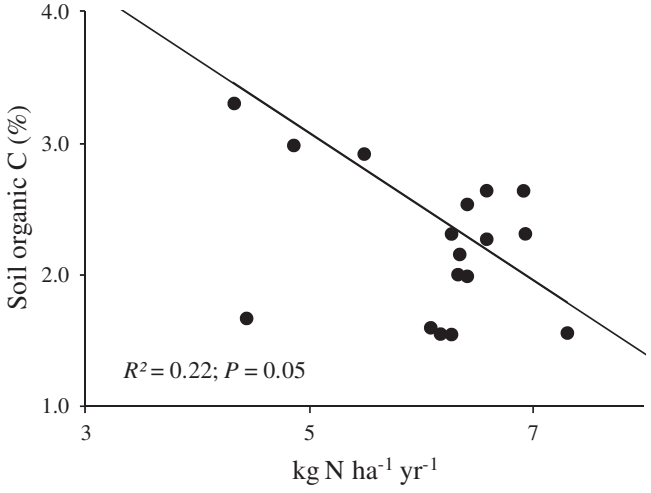

Fig. 2. Relationship between the extant N deposition gradient and soil organic C content (%) ($N = 19$).

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. NS indicates that none variable was selected in the model.

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

N addition treatments after 1.5 years of experimental duration (Fig. 4a and b). In contrast, the N-fixation activity was strongly reduced by simulated N deposition (N fertilization: df = 3, 15; $F = 4.28$; $P = 0.02$; block effect: df = 5, 15; $F = 1.28$; $P = 0.32$; Fig. 4c). The richness of oxygenic phototrophic microbial OTUs significantly increased with N fertilization (N fertilization: df = 3, 15; $F = 5.92$; $P < 0.01$; block effect: df = 5, 15; $F = 1.20$; $P = 0.35$; Fig. 5a). The analysis of DGGE profiles revealed a common predominant OTU (arrow in Fig. S2) in all the plots, which corresponds 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 OTUs richness were also negatively related (Fig. 5b).

Net N mineralization reflected net nitrification rates (Table 2; Fig. 6). The peak of N mineralization and nitrification was in winter, and the minimum values were observed during spring (Fig. 6). In contrast, net ammonification was higher in spring and lower and negative in the rest of seasons (Fig. 6). Overall, potential N mineralization was not affected by N fertilization (Table 2; Fig. 6). In contrast, net ammonification and net nitrification were significantly reduced and increased, respectively, by simulated N deposition (Table 2; Fig. 6). Independently analysed by season, net nitrification significantly increased in spring 2009, whereas net ammonification decreased in winter 2008 and summer 2009 (Table 2; Fig. 6).

4. Discussion

Modelled values of N deposition at the sampling sites were moderately low in the European context, where atmospheric N deposition ranges between ~2 and 44 kg N ha^{-1} yr^{-1} (Stevens et al., 2010), and well adjusted to the current levels of N deposition found in the Iberian Peninsula (usually <10 kg N ha^{-1} yr^{-1}; Ochoa-Hueso et al., 2011a). In addition, modelled N values were frequently below reported critical loads for N deposition effects in other European terrestrial ecosystems (normally between 10 and 15 kg N ha^{-1} yr^{-1}; Bobbink et al., 2010), although some

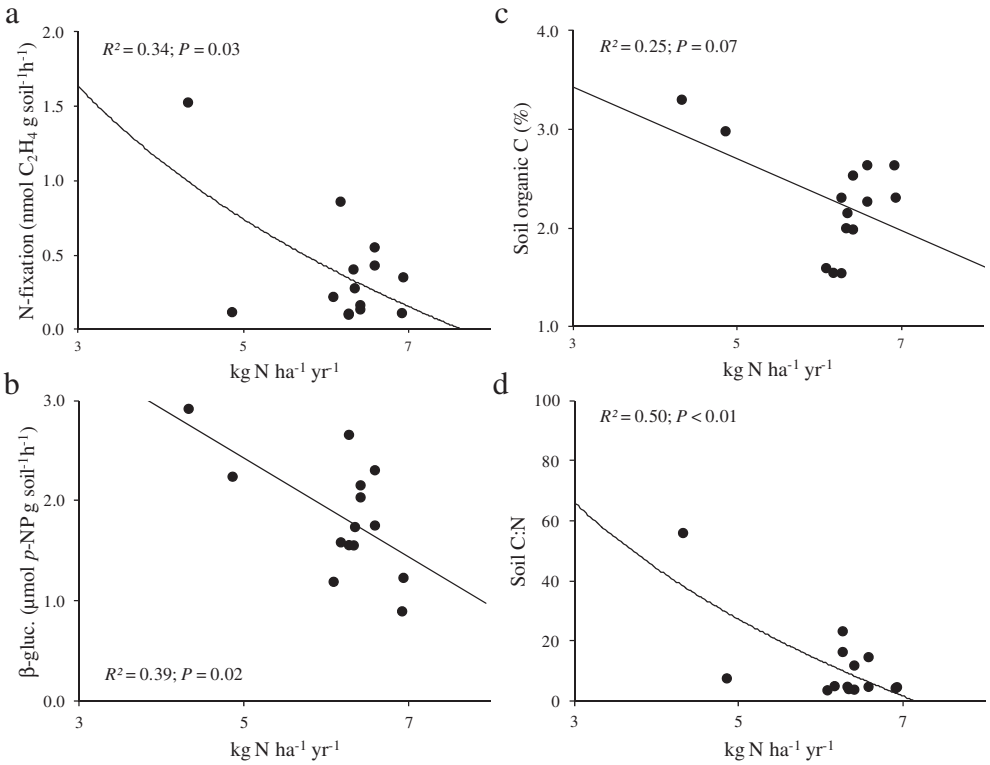

Fig. 3. Relationship between the extant N deposition gradient and soil (a) N-fixation, (b) β-glucosidase activity, (c) organic C and (d) C:N ratio. Graphs are presented with $N = 14$ (see "Methods section" for details on location groups).

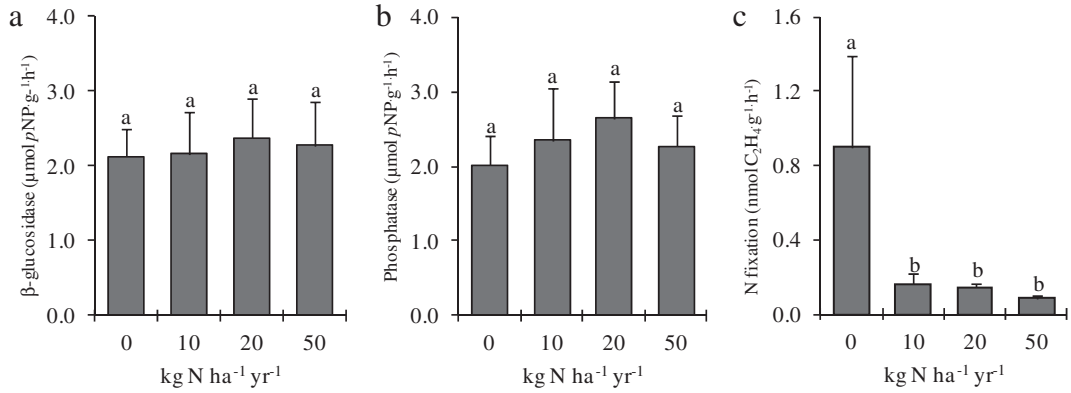

Fig. 4. Simulated N deposition effects on soil (a) β-glucosidase, (b) phosphatase and (c) nitrogenase activities measured in spring 2009. Different lower-case letter above SE bars ($N = 6$) denotes significant differences between treatments after LSD post-hoc tests. Soil nitrogenase data are presented un-transformed but analyses were conducted on log-transformed data.

Mediterranean ecosystems of California show critical N loads even below our lowest N deposition value (3 kg N ha^{-1} yr^{-1} for epiphytic lichens; Fenn et al., 2010).

4.1. Nitrogen cycling

Soil organic N content did not increase along the extant N deposition gradient studied, suggesting that low-productivity semiarid Mediterranean ecosystems where late-successional BSCs are usually well developed do not store extra atmospherically-derived N in soils. Interestingly, soil organic N content remained unexplained when climatic predictors were 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, soil C:N ratios were negatively correlated with modelled N deposition in most of the sites surveyed, a response driven by a reduction in soil organic C content (see below for discussion of responses related to the C cycling). The lack of a significant relationship between N deposition and soil organic N content has also been documented in other similar studies and the consensus is that it is a poor predictor of increased N deposition (Stevens et al., 2009).

Increased atmospherically-deposited N in soils frequently results in lower N fixation activity as a consequence of a functional response of soil microorganisms (DeLuca et al., 2007). Given that N fixation is a highly energetic process, alterations of soil inorganic N availability should be rapidly followed by changes in the N fixation rates (Vitousek and Field, 1999). This assertion is in agreement with our results from both the extant N deposition gradient study and the manipulation experiment. However, whether this type of response was related to increased levels of N availability in soil, as predicted, or to a potential soil acidification is not known, and so the mechanism behind this effect merits further attention. In any case, given that soil nitrogenase activity was significantly down-regulated by N deposition loads ranging from 4.3 to 56.4 (background + N fertilization) kg N ha^{-1} yr^{-1}, this enzyme seems particularly suitable for field studies conducted along wide N deposition gradients. The significant reduction of N fixation rates with simulated N deposition also parallels a significant increase in OTUs richness that can be attributed to a proliferation of facultative/non N_2-fixing nitrophytic cyanobacteria (or other oxygenic phototrophic microorganisms like green algae) able to thrive under high N levels. Similar responses, i.e., proliferation of photosynthetic soil microorganisms (including green algae and cyanobacteria), have already been shown for other ecosystem types after N enrichment (Poikolainen et al., 1998; Gilbert 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 the down-regulation of the activity of this species, and cautiously suggest that N-fixation responses along the N gradient could also be attributed to the synergistic effect of enzyme down-regulation and microbial community alteration. Contrary to our expectations (Enowashu et al., 2009), a decrease in the soil urease activity with increasing N deposition loads as a consequence of high inorganic N availability in soil was not found.

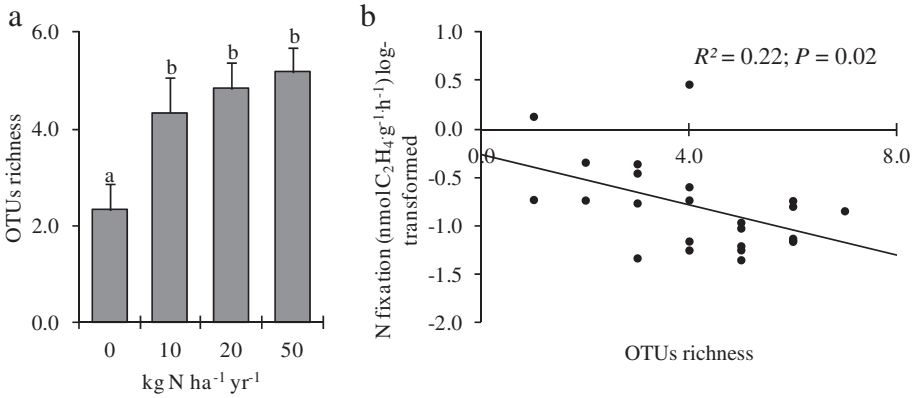

Fig. 5. Simulated N deposition effects on (a) OTUs richness and (b) relationship between OTUs richness and nitrogenase activity. Different lower-case letter above SE bars ($N = 6$) denotes significant differences between treatments after LSD post-hoc test.

Table 2
Nitrogen fertilization, sampling season and block effects on net ammonification, net nitrification and net N mineralization in the field experiment. Overall effects on net nitrification are reported after log-transformation of data. Values in bold indicate $P < 0.1$.

	Net ammonification			Net nitrification			Net mineralization		
	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Nitrogen – overall effect	3, 15	3.11	**0.06**	3, 15	3.25	**0.05**	3, 15	0.90	0.46
Season	3, 15	5.51	**<0.01**	3, 15	8.75	**<0.01**	3, 15	4.36	**0.02**
Block	5, 16	1.18	0.36	5, 16	15.32	**<0.01**	5, 16	6.93	**<0.01**
Nitrogen × season	9, 45	0.80	0.62	9, 45	0.81	0.61	9, 45	0.82	0.61
Nitrogen × block	15, 45	1.82	**0.06**	15, 45	0.73	0.74	15, 45	0.81	0.66
Block × season	15, 45	2.20	**0.02**	15, 45	2.11	**0.03**	15, 45	2.60	**<0.01**
Nitrogen – autumn 2008	3, 15	1.73	0.20	3, 15	0.33	0.81	3, 15	0.39	0.76
Block	5, 5	2.13	0.12	5, 5	5.03	**<0.01**	5, 5	4.97	**<0.01**
Nitrogen – winter 2008	3, 15	3.05	**0.06**	3, 15	1.14	0.37	3, 15	1.09	0.38
Block	5, 5	9.10	**< 0.01**	5, 5	9.21	**<0.01**	5, 5	9.09	**<0.01**
Nitrogen – spring 2009	3, 15	0.64	0.60	3, 15	3.93	**0.03**	3, 15	2.17	0.13
Block	5, 5	1.62	0.21	5, 5	12.69	**<0.01**	5, 5	7.01	**<0.01**
Nitrogen – summer 2009	3, 15	5.14	**0.01**	3, 15	0.47	0.71	3, 15	0.35	0.79
Block	5, 5	2.35	**0.09**	5, 5	3.35	**0.03**	5, 5	3.06	**0.04**

Nitrogen cycling processes can be promoted or down-regulated depending on the relative importance of N limitation and/or toxicity of soils, the dominant vegetation, and alterations in the soil pH (Kieft et al., 1998). In our experiment, N ammonification was down-regulated by simulated N deposition, suggesting that anthropogenic N may either impede organic N cycling (Kang and Lee, 2005) or stimulate NH_4^+ – N nitrification and thus NO_3^- – N accumulation in soils. The significant increase in net nitrification rates give support to the second of the invoked mechanisms and, as the dominant process in basic soils, net nitrification also drove the highest proportion of the net N mineralization response to simulated N deposition. However, net mineralization was not significantly affected by simulated N deposition given that the opposite effects of N deposition on net ammonification and net nitrification cancelled each other. Nitrogen cycling-related responses were also dependent on the sampling season and on the block effect, suggesting the relevance of taking into account both temporal and spatial scales on the response of heterogeneous Mediterranean ecosystems to increased N deposition. In this sense, Keeler et al. (2009) demonstrated that N addition on temperate forested and grassland sites can slow down soil organic matter decomposition, but with local differences in soil pH, moisture, organic C and microbial biomass explaining much of the within-site variation in enzyme activity. Our results of increased net nitrification rates parallel studies in a N-fertilized California chaparral (Vourlitis et al., 2009), and also parallel the reduction of net ammonification with N addition reported by Vourlitis et al. (2009) in a Californian coastal sage scrub, suggesting that these responses could be common across different Mediterranean ecosystem types and also across different Mediterranean regions. Finally, a meta-analysis study has demonstrated that soil organic matter decomposition can be inhibited by N fertilization when: (i) N fertilization rates are 2–20 times than the N deposition background, (ii) ambient N deposition exceeds 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 net ammonification rates can also be considered as a logical result after increasing by approximately eight times the N loads received by the experimental plots.

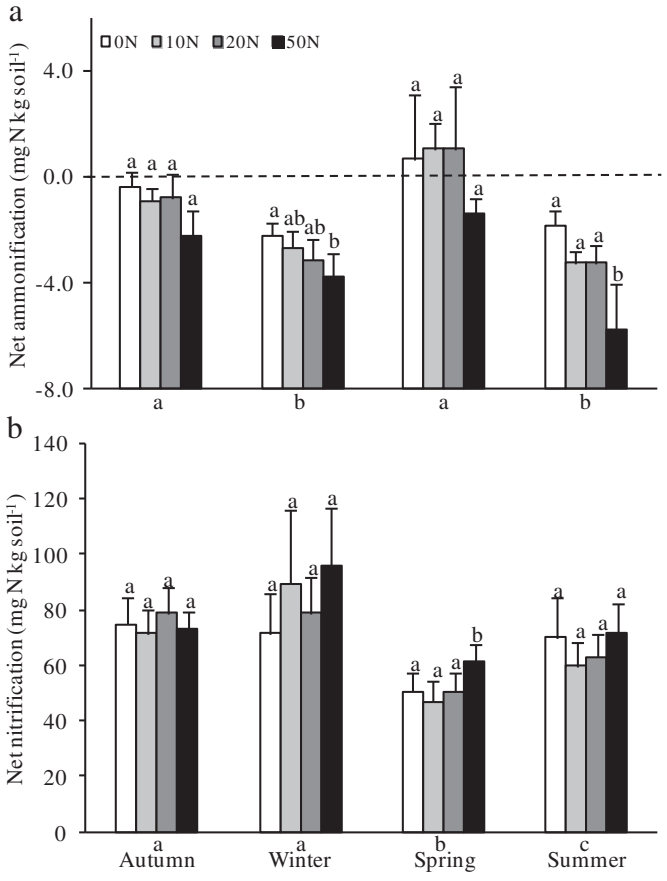

Fig. 6. Simulated N deposition effects on N cycling processes (net ammonification and net nitrification). Different lower-case letter above SE bars and between seasons denotes significant differences between N treatments ($N = 6$) and seasons ($N = 24$) after LSD post-hoc tests.

4.2. 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 (Reid et al., 2012; Ramirez et al., 2012; Liu and Greaver, 2010) and thus with soils functioning as net C sinks and/or sources (Reich et al., 2006). These effects have previously been described as ecosystem-dependent, and also related to the quality and quantity of the litter inputs and to the N saturation level of the studied ecosystems (Waldrop et al., 2004; Berg and Matzner, 1997). In our case study, soil organic C content decreased along the extant N deposition gradient, suggesting that low-productivity semiarid Mediterranean soils dominated by late-successional BSCs are not able to offset rising CO_2 emissions to the

atmosphere when experiencing increased N deposition loads (Vourlitis and Pasquini, 2008), although this effect may also depend on the local vegetation community present, local soil and climatic conditions, topography, etc.

In this study, the effects of N deposition on soil organic C pools cannot be directly attributed to increased soil respiration (see also Liu and Greaver, 2010), and thus to CO_2 losses to the atmosphere, which were strongly related to BSC cover (see also Castillo-Monroy et al., 2011). Therefore, our results implicate alternative mechanisms such as reduced ecosystem productivity (including increased BSC mortality and reduced litterfall inputs) after exceeding critical N loads (Magill et al., 2000) and/or increased plant litter decomposition rates (Berg and Matzner, 1997; Liu and Greaver, 2010), although we did not individually test any of these hypotheses. Considering mechanisms related to the organic matter decomposition, Sinsabaugh et al. (2005) demonstrated that decreased or increased soil organic C content in high-N fertilized plots varied with the type of forest studied and that the type of response was also related to the activity of 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 the 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 and Zak, 2006), whereas litter degradation usually increases with N fertilization in low-lignin and high-N litter (Berg and Matzner, 1997). These disparate effects of increased N fertilization on ligninolytic enzymes can, thus, translate into either increased or reduced soil C pools (Berg and Matzner, 1997).

The role of cellulolytic enzymes (our focus) as mediators of the impacts of N deposition on soil C dynamics is even less clear. Soil β-glucosidase is usually positively correlated with soil organic 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 organic C content associated with increased N deposition loads could imply lower β-glucosidase activity, unless an induced nutritional imbalance (C to N) alters this relationship (thus increasing β-glucosidase activity). 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: (1) the role of soil N toxicity (probably due to high levels of $NH_4^+ - N$ in soil) on soil microorganisms at high N loads (Alarcón-Gutierrez et al., 2008; Liu and Greaver, 2010; Cruz et al., 2006, 2008), (2) the potential repression of this enzyme by extra N (DeForest et al., 2004), and also (3) the importance of evaluating the activity of this enzyme in the context of soil organic C content and soil microbial biomass (Sinsabaugh et al., 2008). Supporting our findings, it has been demonstrated that variations in soil enzyme activities in the context of increased atmospheric N deposition are frequently related to changes in soil organic matter chemistry (Grandy et al., 2009).

Contrasting with the results from the extant gradient study, β-glucosidase activity was not altered in soils from the manipulation experiment. These results support the role of the unaltered soil organic C content (2.86% ± 0.22 SE in spring 2009) on the response of the cellulolytic enzyme activity to N fertilization (Grandy et al., 2009; Ochoa-Hueso et al., 2013). Other studies have also found disparate effects of N addition on soil 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 kg N ha^{-1} yr^{-1}. The evidence from Californian grasslands suggests that soil phosphatase and β-glucosidase activities are able to respond to increased N supply in other Mediterranean ecosystems, but also shows that these alterations can be of smaller magnitude than annual variations in their activity (Gutknecht et al., 2010), at least at the beginning of the experiment, when the experimental soils (highly spatially heterogeneous) have possibly not received N additions for long enough to show any evident effect.

4.3. Concluding remarks

Nitrogen deposition was negatively related to soil β-glucosidase and nitrogenase activities under extant conditions and this response was also dependent on superimposed environmental and geographical gradients. Given that soil phosphatase and urease activities were not related to the N gradient, future works evaluating the impacts of extant N pollution gradients using soils in semiarid Mediterranean ecosystems should mainly focus on soil nitrogenase (preferably) and β-glucosidase. Managers aiming at developing biomonitoring programs using soils as biomarkers should also be aware of the importance of having locations with disparate environmental and geographical conditions. Soil organic C content also decreased along the extant N gradient suggesting that semiarid soils in low-productivity sites are unable to mitigate increasing CO_2 emissions when experiencing increased N deposition. Based on the critical load definition (e.g., Pardo et al., 2011) and on the negative slopes of the regression lines found in the N gradient study it seems likely that the critical N loads for most of the soil indicators responding to atmospheric N pollution are at the lowest end of the gradient (i.e., 4.3 kg N ha^{-1} yr^{-1}). Critical N loads reported for more productive ecosystems are often higher, although in some Temperate and Boreal ecosystems critical N loads are also reported to be as low as 1–5 kg N ha^{-1} yr^{-1} (Bobbink et al., 2010; Pardo et al., 2011). In the N manipulation experiment, N fixation decreased with increasing N fertilization loads, which was related to compositional shifts in the soil microbial community. Net ammonification and nitrification were also reduced and increased, respectively, by N fertilization, suggesting alterations in the N cycle. Finally, given that locations with N deposition values above 4.3 kg ha^{-1} yr^{-1} are common across the central, eastern and southern Iberian peninsula (Ochoa-Hueso et al., 2011a), 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 European semiarid Mediterranean ecosystems.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2013.03.060.

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