

# Biogeochemical indicators of elevated nitrogen deposition in semiarid Mediterranean ecosystems

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**Abstract** Nitrogen (N) deposition has doubled the natural N inputs received by ecosystems through biological N fixation and is currently a global problem that is affecting the Mediterranean regions. We evaluated the existing relationships between increased atmospheric N deposition and biogeochemical indicators related to soil chemical factors and cryptogam species across semiarid central, southern, and eastern Spain. The cryptogam species studied were the biocrust-forming species *Pleurochaete squarrosa* (moss) and *Cladonia foliacea* (lichen).

**Highlights** • N deposition promotes soil acidification in kermes oak shrublands

• N deposition contributes to increase soil C storage in pine forests  
• PME/NR enzyme ratios measured in mosses can be a good bioindicator of N deposition

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Sampling sites were chosen in *Quercus coccifera* (kermes oak) shrublands and *Pinus halepensis* (Aleppo pine) forests to cover a range of inorganic N deposition representative of the levels found in the Iberian Peninsula (between 4.4 and 8.1 kg N ha<sup>-1</sup> year<sup>-1</sup>). We extended the ambient N deposition gradient by including experimental plots to which N had been added for 3 years at rates of 10, 20, and 50 kg N ha<sup>-1</sup> year<sup>-1</sup>. Overall, N deposition (extant plus simulated) increased soil inorganic N availability and caused soil acidification. Nitrogen deposition increased

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phosphomonoesterase (PME) enzyme activity and PME/nitrate reductase (NR) ratio in both species, whereas the NR activity was reduced only in the moss. Responses of PME and NR activities were attributed to an induced N to phosphorus imbalance and to N saturation, respectively. When only considering the ambient N deposition, soil organic C and N contents were positively related to N deposition, a response driven by pine forests. The PME/NR ratios of the moss were better predictors of N deposition rates than PME or NR activities alone in shrublands, whereas no correlation between N deposition and the lichen physiology was observed. We conclude that integrative physiological measurements, such as PME/NR ratios, measured on sensitive species such as *P. squarrosa*, can provide useful data for national-scale biomonitoring programs, whereas soil acidification and soil C and N storage could be useful as additional corroborating ecosystem indicators of chronic N pollution.

**Keywords** Acidification · Biocrusts · Bioindicators · C and N storage · Cryptogams · N deposition · Nitrate reductase · Phosphomonoesterase · Semiarid Mediterranean

## Introduction

Increased nitrogen (N) deposition, derived from human activities such as fossil fuel combustion and crop and livestock production, is a global threat to terrestrial ecosystems worldwide (Gruber and Galloway 2008), including those from the Mediterranean region (Ochoa-Hueso et al. 2011a). Dry deposition (either as gases or as particles) is the dominant form of N deposition in semiarid ecosystems of the Mediterranean Basin (Ochoa-Hueso et al. 2011a), where field and gradient studies on the effects of increased N deposition on natural and seminatural ecosystems are scarce (Ochoa-Hueso et al. 2011a). Reported effects of increased N deposition on terrestrial ecosystems include altered soil nutrient cycling and ecosystem functioning and reduced native biodiversity (Bobbink et al. 2010). Nitrogen deposition effects on soils include acidification, eutrophication, base cation depletion, increased aluminum toxicity, and enhanced nitrate ( $\text{NO}_3^-$ ) leaching (Horswill et al. 2008). Nitrogen deposition can also alter soil N and carbon (C) storage and thus significantly contribute to either mitigate or reinforce climate change effects

(Reich et al. 2006). The impacts of N deposition on local plant communities include the replacement of specialist species adapted to low-nutrient environments by nitrophilous species able to thrive under high-N conditions (Stevens et al. 2004).

In the semiarid portions of the Mediterranean region, plant communities commonly include the presence of a well-developed and late-successional biological soil crust (hereafter biocrusts), where they can represent up to 90 % of the total soil cover (Maestre et al. 2011). Biocrusts are defined as a consortium of different organism types such as algae, protozoa, fungi, mosses, and lichens found in the uppermost millimeters of the soil surface (Belnap et al. 2008). These biocrusts are highly sensitive to global change drivers such as climate change (Escolar et al. 2012; Reed et al. 2012), and given their high exposition to nutrient inputs from the atmosphere and their adaptation to low-nutrient environments, they are also expected to be highly sensitive to increased atmospheric N deposition (Belnap et al. 2008; Ochoa-Hueso et al. 2013a). Late-successional biocrusts in the semiarid Mediterranean region of Europe are usually dominated by species such as the moss *Pleurochaete squarrosa* (Brid.) Lindb. and the foliose lichen *Cladonia foliacea* (Huds.) Willd. (Maestre et al. 2011). *Cladonia foliacea* is considered as a moderately pollution-tolerant species (Ochoa-Hueso and Manrique 2011; Ochoa-Hueso et al. 2013a), whereas *P. squarrosa* seems to be widespread regardless of pollution levels (Ochoa-Hueso and Manrique 2013). It has been demonstrated that these two species can respond to increased N availability in physiological terms under controlled greenhouse and field experimental conditions, which suggests their potential suitability as bioindicators of increased N deposition (Ochoa-Hueso et al. 2013a; Ochoa-Hueso and Manrique 2013). However, no previous study has evaluated their suitability as bioindicators along N deposition gradients in the Mediterranean Basin under natural, non-manipulated conditions. Certain enzymatic activities such as the phosphomonoesterase (PME) and nitrate reductase (NR), considered as indicators of an induced N to phosphorus (P) imbalance and of N saturation, respectively, are among the most widely measured physiological biomarkers of N deposition on plants, mosses, and lichens (Arróniz-Crespo et al. 2008; Hogan et al. 2010a, b) and thus can be good candidates for biomonitoring studies on these two species. Other indicators using cryptogams have already been proposed for the Mediterranean regions (e.g., functional diversity

of epiphytic lichens (Pinho et al. 2012)). However, parameters such as enzyme activities measured on widespread and tolerant cryptogam species are likely to respond faster to an increase/cessation in the exposure to pollutants. Other potential advantages of this type of indicators are that they are easy to measure and many samples from different locations can be taken to the lab and analyzed in a short period of time.

The main objectives of this study were (1) to investigate the impacts of increased N deposition in semiarid Mediterranean ecosystems in Spain based on different biogeochemical variables related to both soil chemistry and the physiology of two cryptogam species (*P. squarrosa* and *C. foliacea*) and (2) to identify those biogeochemical variables that are best predictors of N deposition rates in kermes oak (*Quercus coccifera* L.) shrublands and Aleppo pine (*Pinus halepensis* Mill.) forests, two of the major vegetation types in the Mediterranean Basin (Blondel and Aronson 1999). We hypothesized that (1) the N deposition gradient would be positively and negatively related to soil inorganic N availability and soil pH, respectively (Horswill et al. 2008). We also hypothesized (2) a positive relationship between N deposition and PME activity and a negative relationship between NR activity and N deposition in both the moss and lichen species (Arróniz-Crespo et al. 2008). (3) The clearer cryptogam response was predicted to be found when measuring the PME activity in the moss species. We hypothesized that (4) the responses evaluated would differ between ecosystem types and (5) that the inclusion of N-fertilized experimental plots, thus artificially extending the length of the N gradient, would contribute to clarify results in the case of the physiological responses, but not in the case of variables whose response usually need several years to become evident, such as soil organic C and N contents (Vourtilis et al. 2009).

## Material and methods

### Study area

In June 2010, 28 sites were surveyed along a N deposition gradient spanning central, southern, and eastern portions of the semiarid Mediterranean Spain. Sampling sites were chosen to represent either *Q. coccifera* shrublands ( $N=18$ ) or *P. halepensis* forests ( $N=10$ ) and to cover a range of modeled inorganic N deposition between 4.4 and 8.1 kg N ha<sup>-1</sup> year<sup>-1</sup>. These

values can be considered as moderately low in the European context (Duprè et al. 2010) but well adjusted to the current levels of N deposition found in the Iberian Peninsula (Ochoa-Hueso et al. 2011a; Garcia-Gomez et al. 2014). The selection of the sites was based on our previous knowledge of locations with well-developed biocrusts, which conditioned the number of sites for each ecosystem type. The sampling was conducted at the end of the spring growing season to maximize the potential to find ecosystem responses to increased N deposition. During the sampling period, we specifically avoided wet weather conditions to maximize comparability between sites. In addition to these 28 locations, we sampled plots from an ongoing field N fertilization experiment located in central Spain (Aranjuez, Table S1) in which simulated N deposition loads are being applied as NH<sub>4</sub>NO<sub>3</sub> since October 2007 to a *Q. coccifera* shrubland at rates of 0, 10, 20, and 50 kg N ha<sup>-1</sup> year<sup>-1</sup> (see Ochoa-Hueso and Manrique (2013) and Ochoa-Hueso et al. (2013b; c) for further details on the experimental design and local climatic and soil conditions). The total N deposition loads that these plots are receiving were calculated by summing the simulated N deposition load to the N deposition background of the experimental site (6.8 kg N ha<sup>-1</sup> year<sup>-1</sup>, Table S1). Sampling sites were always located between 500 and 1,000 m a.s.l. and with the annual rainfall ranging between 370 and 570 mm; therefore, dry inputs (either as gases or as particles) are supposed to represent a major fraction of total N deposition. 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; Garcia-Gomez et al. 2014).

### Soil sampling and analyses

Ten soil samples (0–4-cm depth) were collected in the interspaces between trees and shrubs at all the surveyed sites using a graduated garden trowel (extant N deposition gradient) and a soil corer (N fertilization experiment). Soil samples were then transported to the laboratory, sieved through a 2-mm mesh and air-dried for 1 month. Soil NO<sub>3</sub><sup>-</sup>-N and ammonium (NH<sub>4</sub><sup>+</sup>-N) were extracted by orbitally shaking 10 g of soil in 50 ml of 0.1 M CaCl<sub>2</sub> solution for 30 min at 160 rpm and colorimetrically evaluated (AutoAnalyzer 3, High Resolution Digital Colorimeter, SEAL). Extractable P was

calculated following the Burriel and Hernando (1950) method. Organic N was evaluated after Kjeldahl acid digestion with  $\text{SeSO}_4\text{-K}_2\text{SO}_4$  as a catalyst. Organic matter and C contents were determined by the dichromate oxidation method (Walkley and Black 1934). Base cations (Na, K, Mg, and Ca) were extracted with 1 M  $\text{CH}_3\text{COONH}_4$  at pH 7 (2.5 g of soil in 25 ml). The extractant was then orbitally shaken with the soil for 30 min, filtered, and analyzed by ICP-OES (Perkin-Elmer 4300 DV). Non-base cations (Fe, Mn, Zn, Cu, and Al) were extracted with Lakanen solution (0.5 M  $\text{CH}_3\text{COONH}_4$ , 0.5 M  $\text{CH}_3\text{COOH}$ , and 0.02 M EDTA) at pH 4.5: we shook 2.5 g of soil in 25 ml for 60 min, and the extractant was then filtered and analyzed by ICP-OES (Perkin-Elmer 4300 DV).

#### Terricolous cryptogam sampling and analyses

In the case of *P. squarrosa*, green parts of ten subsamples per site were bulked in a composite sample, whereas in the case of *C. foliacea*, fragments of different thalli from ten subsamples per site were also bulked in a composite sample. The lichen was only sampled when present, i.e., in 19 out of the 28 sampling sites (Table S1). The activity of PME was determined by measuring the release of *p*-nitrophenol (*p*-NP) from *p*-nitrophenyl phosphate (*p*-NPP). Two ml of 0.1 M maleate buffer at pH 6.5 and 0.5 ml of substrate were added to approximately 50 mg of dry tissue and incubated at 30 °C for 90 min. The reaction was stopped by cooling rapidly to 4 °C for 15 min.  $\text{CaCl}_2$  of 0.5 M and 2 ml of 0.5 M NaOH were added and the amount of *p*-NP determined using a spectrophotometer (Shimadzu UV-1603) at 398 nm. Constitutive NR activities were evaluated on acclimated mosses and lichens (~110 mg) following the procedures described in Arróniz-Crespo et al. (2008).

#### Statistical analyses

Stepwise multiple linear regression analyses were used to relate the cryptogam and soil indicators evaluated to modeled N deposition loads (untransformed and  $\log_{10}$ -transformed) and climatic variables (mean annual maximum daily temperature and rainfall). In the case of the cryptogam indicators, we also included selected soil fertility variables (organic matter and base and non-base cations) in the regression analyses given the potential of

biocrust-forming species to extract nutrients directly from the soil (Hauck 2010). We conducted these analyses with all samples, including ( $N=32$ ) and excluding ( $N=28$ ) the N-fertilized plots, and also distinguishing between ecosystem type, i.e., kermes oak shrublands versus pine forests. All statistical analyses were done with SPSS17.0 (SPSS Inc., Chicago, IL, USA) and statistical significance was established at  $P \leq 0.05$ .

#### Results

##### Nitrogen deposition gradient and soil chemistry

The extant N deposition gradient length covered in this study was of  $3.7 \text{ kg N ha}^{-1} \text{ year}^{-1}$ , and based on the model, most of the N is in the oxidized form ( $1.9 \pm 0.1$  SE times higher). In support of our first hypothesis, stepwise regressions with 32 samples (i.e., including N-fertilized plots) showed positive linear relationships between N deposition and soil  $\text{NO}_3^-$ -N and total inorganic N and negative relationships between N deposition and soil pH (Table 1 and Fig. S1). Stepwise linear regressions excluding the N-fertilized plots revealed a positive correlation between N deposition and soil C content ( $N=28$ ) (Table 1 and Fig. 1). Stepwise linear regressions separately conducted for each ecosystem type showed that N deposition correlated positively with organic C and N contents only in the case of pine forests (Table 2 and Fig. 1). There was also an inverse relationship between N deposition and soil pH in kermes oak shrublands ( $N=18$ ). Some other nutrients were best explained by temperature (Tables 1 and 2). As the general rule,  $\text{NO}_3^-$ -N dominated over  $\text{NH}_4^+$ -N in soil (4.2 times higher on average) probably due to high  $\text{NH}_3$  volatilization and  $\text{NH}_4^+$ -N nitrification rates, both common and dominant processes in basic soils (Ochoa-Hueso et al. 2013b).

##### *Pleurochaete squarrosa*

Stepwise regressions with 32 samples revealed expected linear relationships between N deposition and PME activity (positive), NR activity (negative), and PME/NR ratio (positive) (Table 1 and Fig. S2), which totally supports our second hypothesis. In kermes oak shrublands ( $N=18$ ), PME and PME/NR responded positively to increased N deposition, whereas NR activity remained unexplained (Table 2 and Fig. 2). Phosphomonoesterase/NR ratio in kermes oak shrublands ( $N=18$ ) was the only

**Table 1** Significant relationships between soil and cryptogam indicators and environmental variables (including N deposition) after stepwise linear regression analyses for all the locations studied including the N-fertilized plots

		Total (N=32)				
		Variable	Sign	d.f.	R <sup>2</sup>	P
Soil	Mean (SE)					
NH <sub>4</sub> <sup>+</sup> -N (mg kg soil <sup>-1</sup> )	0.42 (0.12)	SOM	+	1, 30	0.17	0.02
NO <sub>3</sub> <sup>-</sup> -N (mg kg soil <sup>-1</sup> )	1.75 (0.27)	<b>N deposition</b>	+	1, 30	0.33	<0.01
N <sub>i</sub> (mg kg soil <sup>-1</sup> )	2.16 (0.30)	<b>N deposition</b>	+	1, 30	0.34	<0.01
pH	8.18 (0.03)	<b>N deposition-log</b>	-	2, 31	0.43	<0.01
		Temperature	-			
N <sub>org</sub> (%)	0.22 (0.01)	n/s				
SOM (%)	4.77 (0.36)	n/s				
C/N	12.41 (0.43)	n/s				
Mg (mg kg soil <sup>-1</sup> )	141.34 (15.16)	n/s				
Ca (mg kg soil <sup>-1</sup> )	3667.88 (349.48)	n/s				
Fe (mg kg soil <sup>-1</sup> )	40.51 (4.81)	Temperature	-	1, 31	0.26	<0.01
Mn (mg kg soil <sup>-1</sup> )	49.48 (6.70)	n/s				
Cu (mg kg soil <sup>-1</sup> )	1.87 (0.10)	n/s				
P <sub>2</sub> O <sub>5</sub> -P (mg kg soil <sup>-1</sup> )	<0.5 (0.00)	n/s				
<i>Pleurochaete squarrosa</i>						
PME (μmolp-NP h <sup>-1</sup> g <sup>-1</sup> DW)	137.85 (10.08)	<b>N deposition-log</b>	+	2, 31	0.60	<0.01
		SOM	-			
PME/NR (LOG)	2.25 (0.09)	<b>N deposition-log</b>	+	2, 31	0.64	<0.01
		SOM	-			
NR (μmol NO <sub>2</sub> h <sup>-1</sup> g <sup>-1</sup> DW)	0.87 (0.07)	<b>N deposition-log</b>	-	1, 31	0.40	<0.01
<i>Cladonia foliacea</i>						
PME (μmolp-NP h <sup>-1</sup> g <sup>-1</sup> DW)	119.04 (7.66)	<b>N deposition-log</b>	+	1, 22	0.50	<0.01
		<b>N deposition</b>	+	1, 22	0.22	0.02
NR (μmol NO <sub>2</sub> h <sup>-1</sup> g <sup>-1</sup> DW)	0.21 (0.02)	Rainfall	-	2, 22	0.39	<0.01
		SOM	+			

Nitrogen deposition impacts are shown in bold

n/s none significant model found, d.f. degrees of freedom, SOM soil organic matter, PME phosphomonoesterase, NR nitrate reductase

variable that explained the modeled N loads, whereas in the case of PME, non-base cations and rainfall played a significant role in the response (Table 2 and Fig. 2).

### *Cladonia foliacea*

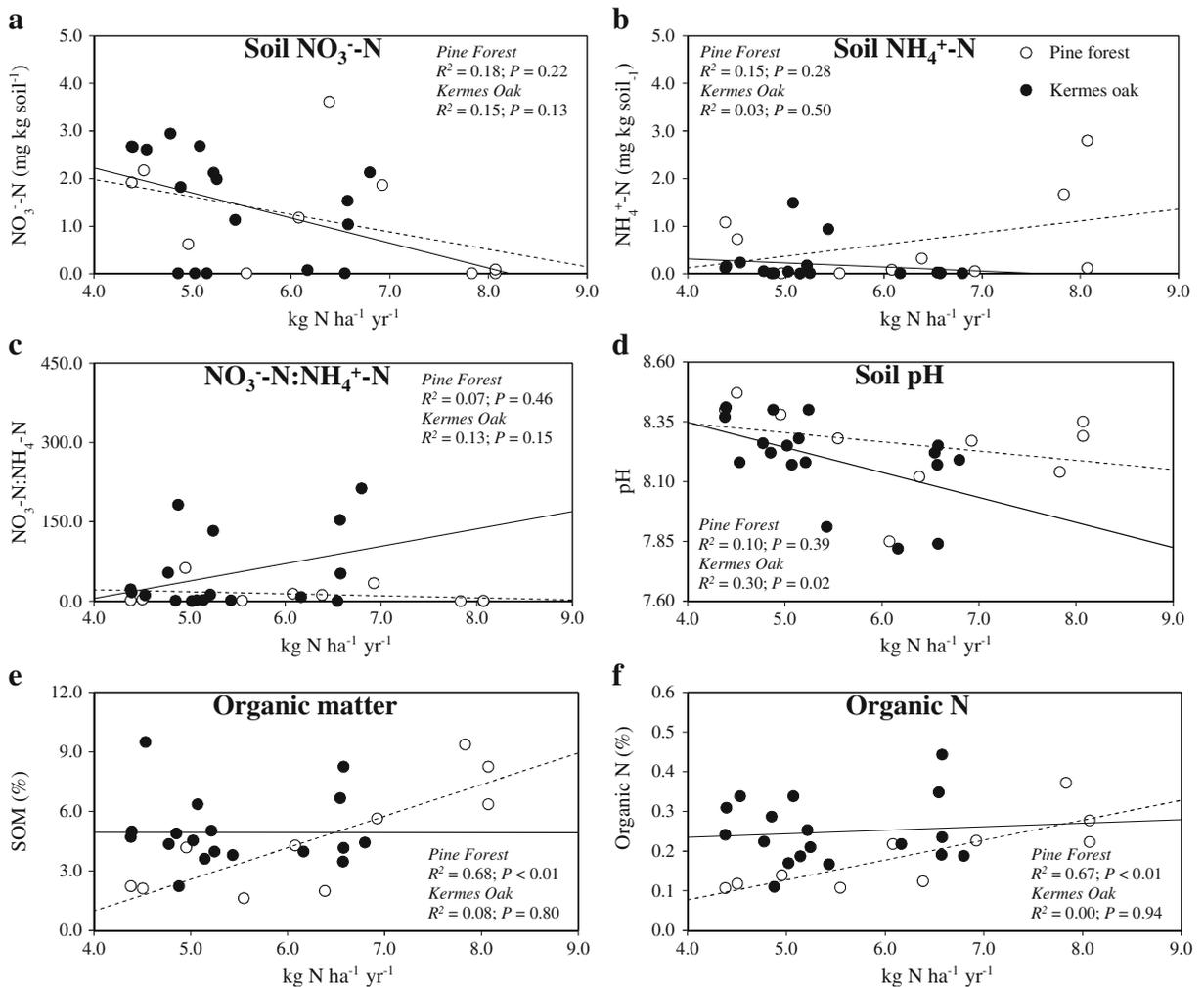
Soil N content was the only measured environmental factor (including N deposition) that differed between the sites with and without *C. foliacea*, being significantly higher in locations where the species was present (P=0.02). Stepwise regressions with 23 samples revealed positive linear relationships between N deposition and PME activity and PME/NR ratios (Table 1 and Fig. S2).

Stepwise regressions excluding the N-fertilized plots revealed that *C. foliacea* physiology was regulated more by soil fertility (positively) and rainfall (negatively) than by N deposition in the range between 4.4 and 8.1 kg N ha<sup>-1</sup> year<sup>-1</sup> (Table 2).

### Discussion

#### Soil chemistry

The N deposition gradient studied induced changes in soil chemistry. For example, N deposition increased soil



**Fig. 1** Relationships between increased N deposition and soil nitrate (a), ammonium (b), nitrate to ammonium ratio (c), pH (d), organic matter (e), and organic N (f). Regression lines are

shown regardless of the significance of the regression analysis and excluding the N-fertilized plots (see Table 2). Solid lines represent kermes oak shrublands; dashed lines represent Aleppo pine forests

inorganic N availability along the evaluated gradient, a response commonly observed in other studies (Hu et al. 2010; Rowe et al. 2012). However, this response was ecosystem- and N fertilization-dependent, as it was only evident when the N-fertilized plots were included in the statistical analyses, which could possibly indicate a transition from a closed to an open system once the critical N load has been passed. Given the transient nature and mobility of  $\text{NO}_3^-$ -N in soils (O'Sullivan et al. 2011), a lack of a clear pattern of inorganic N availability in soils can also be found (Stevens et al. 2009, 2011). In this sense, inorganic N concentration in soils is considered as a bad predictor of increased N deposition because inorganic N levels are dependent on

the soil organic matter mineralization and nitrification rates, and also on plant and microbial uptake (Stevens et al. 2009, 2011; Rowe et al. 2012). Supporting this statement,  $\text{NH}_4^+$ -N availability in soils of the extant N deposition gradient studied was highly dependent on the soil organic matter content (Table 1). In agreement with results reported by Stevens et al. (2011), climatic and geographical variables can be as or even more important predictors of ecologically relevant soil properties such as pH, organic N, and nutrient (e.g., Ca and Fe in this study) availability than increased N deposition.

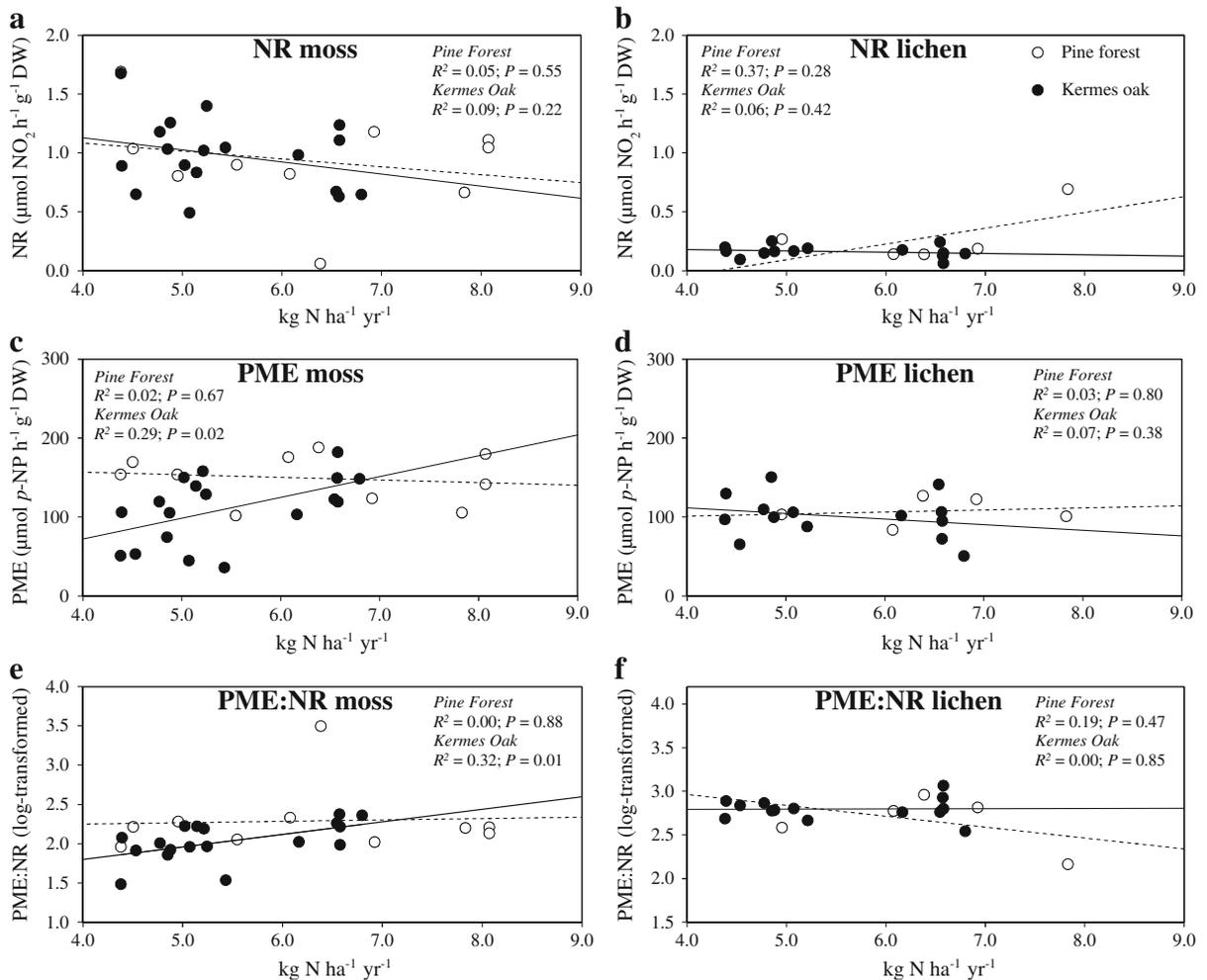
Nitrogen deposition increased soil organic C and N contents along the studied gradient, which is either in agreement (Reich et al. 2006) and/or in disagreement

**Table 2** Significant relationships between soil and cryptogam indicators and environmental variables (including N deposition) after stepwise linear regression analyses for all the locations studied, kermes oak shrublands, and Aleppo pine forests excluding the N-fertilized plots

	Total (N=28)				Kermes oak shrublands (N=18)				Pine forests (N=10)						
	Variable	Sign	d.f.	R <sup>2</sup>	P	Variable	Sign	d.f.	R <sup>2</sup>	P	Variable	Sign	d.f.	R <sup>2</sup>	P
<b>Soil</b>															
NH <sub>4</sub> <sup>+</sup> -N	SOM	+	1, 26	0.18	0.03	n/s					n/s				
NO <sub>3</sub> <sup>-</sup> -N	<b>N deposition-log</b>	-	1, 26	0.18	0.03	n/s					n/s				
N <sub>i</sub>	n/s					n/s					n/s				
pH	Temperature	-	1, 27	0.17	0.03	<b>N deposition-log</b>	1, 17	-	0.26	0.03	n/s				
N <sub>org</sub>	n/s					n/s					<b>N deposition</b>	2, 9	+	0.88	<0.01
SOM	<b>N deposition</b>	+	1, 27	0.18	0.03	n/s					Temperature	+			
C/N	n/s					n/s					<b>N deposition</b>	1, 9	+	0.68	<0.01
Mg	n/s					n/s					n/s				
Ca	n/s					n/s					Temperature	1, 9	+	0.45	0.05
Fe	Temperature	-	1, 27	0.25	<0.01	n/s					n/s				
Mn	n/s					n/s					n/s				
Cu	n/s					n/s					n/s				
P <sub>2</sub> O <sub>5</sub> -P	n/s					n/s					n/s				
<i>Pleurochaete squarrosa</i>															
PME	n/s					Non-bases	3, 17	-	0.65	<0.01	n/s				
						<b>N deposition-log</b>		+							
						Rainfall		+							
PME/NR (LOG <sub>10</sub> )	n/s					<b>N deposition</b>	1, 17	+	0.36	0.01	n/s				
NR	n/s					n/s					n/s				
<i>Cladonia foliacea</i>															
PME	n/s					n/s					n/s				
PME/NR (LOG <sub>10</sub> )	n/s					Bases	1, 13	+	0.43	0.01	SOM	1, 4	-	0.80	0.04
NR	Rainfall	-	2, 18	0.44	<0.01	n/s					SOM	1, 4	+	0.80	0.04
	SOM	+				n/s									

Nitrogen deposition impacts are shown in bold

n/s none significant model found, d.f. degrees of freedom, SOM soil organic matter, PME phosphomonooesterase NR nitrate reductase



**Fig. 2** Relationships between increased N deposition and nitrate reductase (**a, b**), phosphomonoesterase (**c, d**), and phosphomonoesterase to nitrate reductase ratio (**e, f**) for the moss (*P. squarrosa*) and the lichen (*C. foliacea*) species. Regression lines are shown

regardless of the significance of the regression analysis and excluding the N-fertilized plots (see Table 2). Solid lines represent kermes oak shrublands; dashed lines represent Aleppo pine forests

(Ochoa-Hueso et al. 2013b) with other studies evaluating the effects of N deposition on the ability of terrestrial ecosystems to store extra C and N in soils. Increased N deposition typically increases the decomposition rates of labile C fractions while reducing the decomposition of more recalcitrant C fractions (Neff et al. 2002; Reid et al. 2012). In this direction, Waldrop et al. (2004) suggested that terrestrial ecosystems with low litter quality should gain soil C, whereas those with high litter quality should lose soil C in response to increased N deposition. In addition, Averill et al. (2014) showed that soil C storage is greater in ecosystems dominated by ectomycorrhizal fungi (e.g., pine forests) compared to those dominated

by arbuscular mycorrhizal fungi (e.g., kermes oak shrublands). Given that, in this study, the positive effects of N deposition on soil C and N accumulation were determined by the response in pine forests, it is arguable that this response is related to the high dominance of recalcitrant litter C in pine forests, to the N limitation commonly found in these ecosystems, and also to the ability of ectomycorrhizal fungi to acquire N and therefore compete for it with free-living decomposers (Averill et al. 2014; Reid et al. 2012; Waldrop et al. 2004). On the other hand, the lack of response in the case of kermes oak shrublands when independently analyzed in terms of soil C and N accumulation could

be related to the importance of other factors limiting plant growth over N, such as P availability, to the higher quality of the kermes oak litter, and also to the inability of arbuscular mycorrhizal to compete with free-living decomposers for the extra N (Averill et al. 2014; Sardans and Peñuelas 2004). Other ecological factors potentially determining the differential response of pine forests and kermes oak shrublands to increased N deposition can be tentatively related to the amount of litter production (not evaluated in this study) which is, in turn, related to the net productivity and structure of the ecosystem. In this sense, N deposition, particularly in its reduced form, can contribute to decrease annual plant productivity in kermes oak shrublands because of the negative effects caused by increased  $\text{NH}_4^+$ -N availability in ecosystems where  $\text{NO}_3^-$ -N is usually the dominant inorganic N form and thus reduce the amount of easily decomposable litter inputs to soil (Dias et al. 2014). Interestingly, the inclusion of the N-fertilized plots in the analyses did not contribute to clarify the results found in kermes oak shrublands in terms of soil C and N storage, which is most likely related to the temporal scales in which these responses usually take place; i.e., whereas inorganic N levels in soils are highly transient, increasing right after N applications, changes in the soil organic matter (including soil C and N) can take several years before they can be detected (Vourlitis and Fernández 2012). In another N deposition gradient study conducted along low-productivity, low-litter-input semiarid Mediterranean sites (including rosemary shrublands, kermes oak shrublands, and tussock grasslands) of the Iberian Peninsula, Ochoa-Hueso et al. (2013b) found a negative relationship between N deposition and soil organic C content and C/N ratio, a response dependent on relevant climatic variables such as rainfall and temperature. These evidence suggests a gradient of responses in terms of soil C sequestration to increased N deposition, ranging from negative in low-productivity, low-litter-input semiarid shrublands (Ochoa-Hueso et al. 2013b) to neutral (in low- to medium-litter-input kermes oak shrublands; this study) and positive in high (but low-quality) litter input ecosystems (pine forests; this study). More research should be conducted along different semiarid Mediterranean regions of the world to further test this hypothesis and the mechanisms underlying it.

Nitrogen deposition decreased soil pH across the semiarid Mediterranean ecosystems studied, a response primarily driven by kermes oak shrublands. This was an expected response, as it has already been widely

described that N deposition causes soil acidification in terrestrial ecosystems as a consequence of altered mineralization and increased N uptake and  $\text{NO}_3^-$ -N leaching rates (Horswill et al. 2008; Vourlitis and Fernández 2012). The lack of a significant relationship in Aleppo pine forests was due to one particular location (El Plano), whose pH was lower than expected. This particularly low value can be tentatively attributed to several causes, including different stand ages (not evaluated) and, given the proximity of the site to a recreational area, the importance of other human impacts. When this location was removed from the analysis, we found a strong negative correlation between soil pH and N deposition, particularly for reduced N ( $R^2=0.61$ ;  $P=0.01$ ). It has also been described that N deposition typically increases soil base cation depletion as a consequence of base cations being leached with soil  $\text{NO}_3^-$ -N as counter-ions (Horswill et al. 2008; Di and Cameron 2004), but this was not the case in our gradient study, which is probably related to the basic nature of soils and their natural high buffering capacity, and also to the variability associated with the composition of the underlying bedrock (Horswill et al. 2008).

Cryptogam physiology: a highly informative indicator of N deposition

Nitrogen deposition has been shown to relate well with certain enzyme activities in terricolous mosses and lichens from temperate environments along extant and simulated N deposition gradients (Arróniz-Crespo et al. 2008; Hogan et al. 2010a, b; Pearce et al. 2003). In Mediterranean ecosystems, Ochoa-Hueso et al. (2013a) and Ochoa-Hueso and Manrique (2013) studied the effects of simulated N deposition on the physiological response of *C. foliacea* and *P. squarrosa*, respectively, under field experimental conditions. Phosphomonoesterase activity increased with increasing N deposition loads in *P. squarrosa* irrespective of the N dose supplied (0, 10, 20, and 50 kg N ha<sup>-1</sup> year<sup>-1</sup> as  $\text{NH}_4\text{NO}_3$ ) (Ochoa-Hueso and Manrique 2013), whereas the response found in *C. foliacea* was a critical load-like response, i.e., showing increased PME activity with loads of up to 20 kg N ha<sup>-1</sup> year<sup>-1</sup> and then decreasing PME activity because of the negative effects attributed to the excessive N (Ochoa-Hueso et al. 2013a). *Cladonia foliacea* response was also dependent on the availability of certain micronutrients in soil and on soil pH (Ochoa-Hueso et al. 2013a). In agreement

with these results, micronutrient availability has also been shown to be related to *C. foliacea* physiology along the N gradient analyzed here (Tables 1 and 2) and in a semiarid shrubland in central Spain (Ochoa-Hueso et al. 2011b). These results are consistent with the ability of certain terricolous lichens to obtain nutrients directly from the soil (Hauck 2010).

In this study, PME activity increased in both *P. squarrosa* and *C. foliacea*, this consistent pattern being driven by the response in kermes oak shrublands. However, when the N-fertilized plots were excluded from the analyses, PME response to N deposition in *C. foliacea* was not significant, suggesting that *C. foliacea* is of less help as a bioindicator species along short, low N deposition gradients (Ochoa-Hueso and Manrique 2013). The induction of PME activity in *P. squarrosa* and *C. foliacea* was interpreted as a physiological response to an induced nutrient imbalance; i.e., as species become N-saturated, P availability becomes the limiting factor of growth in relation to N (Hogan et al. 2010a, b; Arróniz-Crespo et al. 2008). In this sense, semiarid Mediterranean ecosystems from the Iberian Peninsula are usually recognized as highly P-over N-limited (Ochoa-Hueso et al. 2011a; Hobbs and Richardson 1995). Nitrate reductase activity responses in this study were also in agreement with our initial hypotheses but were less clear than those responses shown by PME, suggesting that PME is a better indicator of increased N deposition than NR along short, low N deposition gradients (Arróniz-Crespo et al. 2008). In agreement with PME responses, NR responses found in *P. squarrosa* were also clearer than those in *C. foliacea*, which also confirms that *P. squarrosa* is a better bioindicator species than *C. foliacea*.

Finally, some authors have suggested the use of integrative physiological measurements (for example, enzyme activity ratios) to study the response of terrestrial ecosystems to increased N availability and nutrient decoupling (Zeglin et al. 2007; 2009). In our study, we analyzed the relationship between N deposition and PME/NR ratios ( $\text{Log}_{10}$ -transformed) as an indicator of an induced P over N limitation. Phosphomonoesterase/NR ratios proved to be better predictors of increased N deposition rates than PME and/or NR activities alone in kermes oak shrublands, even along short, low N deposition gradients, which suggests the applicability of enzyme activity ratios in biomonitoring studies involving atmospheric N deposition and/or nutrient loading into terrestrial ecosystems (Zeglin et al. 2007; 2009).

These results are also consistent with the idea that nutrient cycles in terrestrial ecosystems are being decoupled by the global environmental change (Peñuelas et al. 2012; Delgado-Baquerizo et al. 2013).

## Conclusions

Semiarid Mediterranean ecosystems in southern Europe are currently threatened by increased N deposition, as evidenced by some significant relationships between ecosystem biogeochemical indicators and modeled inorganic N deposition gradients. Some of these relationships (particularly increased C storage and altered enzyme stoichiometry) suggest that N can still be considered a limiting nutrient in certain semiarid Mediterranean ecosystems but also that they may be undergoing a transition from N- to P-limited systems. However, the ecosystems and species studied differed in their sensitivities to increased N deposition. In general terms, kermes oak shrublands were more prone to soil acidification, whereas Aleppo pine forests stored more C and N in soil in response to increased N deposition, suggesting their higher ability to mitigate the climate change. The terricolous moss *P. squarrosa* was also demonstrated to be a better potential indicator species than the lichen *C. foliacea*, whose physiology was more affected by other environmental factors. We conclude that integrative physiological measurements related to nutrient decoupling, such as PME/NR ratios, measured on widely distributed and sensitive cryptogam species such as the moss *P. squarrosa*, are likely to provide useful data for national-scale biomonitoring programs focusing on atmospheric N deposition. Finally, soil acidification and soil C and N storage could also be useful as additional corroborating ecosystem indicators of chronic N pollution, but the results provided by these measurements are less precise as they are ecosystem-dependent and highly affected by local climatic and edaphic conditions.

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## References

- Arróniz-Crespo, M., Leake, J. R., Horton, P., & Phoenix, G. K. (2008). Bryophyte physiological responses to, and recovery from, long-term nitrogen deposition and phosphorous fertilisation in acidic grassland. *New Phytologist*, *180*, 864–874.
- Averill, C., Turner, B. L., & Finzi, A. C. (2014). Mycorrhiza-mediated competition between plants and decomposers drives soil carbon storage. *Nature*, *505*, 543–545.
- Belnap, J., Phillips, S. L., Flint, S., Money, J., & Caldwell, M. (2008). Global change and biological soil crusts: effects of ultraviolet augmentation under altered precipitation regimes and nitrogen additions. *Global Change Biology*, *14*, 670–686.
- Blondel, J., & Aronson, J. (1999). *Biology and wildlife of the Mediterranean region*. Oxford: Oxford University Press. 328 pp.
- Bobbink, R., Hicks, K., Galloway, J., Spranger, T., Alkemade, R., Ashmore, M., et al. (2010). Global assessment of nitrogen deposition effects on terrestrial plant diversity: a synthesis. *Ecological Applications*, *20*, 30–59.
- Burriel, F., & Hernando, V. (1950). El fósforo en los suelos españoles V. Nuevo método para determinar el fósforo asimilable en los suelos. *Anales de Edafología y Agrobiología*, *9*, 611–622.
- Delgado-Baquerizo, M., Maestre, F. T., Gallardo, A., Bowker, M. A., Wallenstein, M. D., Quero, J. L., et al. (2013). Decoupling of soil nutrient cycles as a function of aridity in global drylands. *Nature*, *502*, 672–676.
- Di, H. J., & Cameron, K. C. (2004). Treating grazed pasture soil with a nitrification inhibitor, eco nTM, to decrease nitrate leaching in a deep Sandy soil under spray irrigation - a lysimeter study. *New Zealand Journal of Agricultural Research*, *47*, 351–361.
- Dias, T., Clemente, A., Martins-Loução, M. A., Sheppard, L., Bobbink, R., & Cruz, C. (2014). Ammonium as a driving force of plant diversity and ecosystem functioning: observations based on 5 Years' manipulation of N dose and form in a mediterranean ecosystem. *PLoS one*, e92517.
- Dupré, C., Stevens, C. J., Ranke, T., Bleekers, A., Peppeler-Lisbach, C., Gowing, D. J. G., et al. (2010). Changes in species richness and composition in European acidic grasslands over the past 70 years: the contribution of cumulative atmospheric nitrogen deposition. *Global Change Biology*, *16*, 344–357.
- Escolar, C., Martínez, I., Bowker, M. A., & Maestre, F. T. (2012). Warming reduces the growth and diversity of biological soil crusts in a semi-arid environment: implications for ecosystem structure and functioning. *Philosophical Transactions of the Royal Society B*, *367*, 3087–3099.
- García-Gómez, H., Garrido, J. L., Vivanco, M. G., Lassaletta, L., Rabago, I., Avila, A., et al. (2014). Nitrogen deposition in Spain: modeled patterns and threatened habitats within the Natura 2000 network. *Science of the Total Environment*, *485–486*, 450–460.
- Gruber, N., & Galloway, J. N. (2008). An Earth-system perspective of the global nitrogen cycle. *Nature*, *451*(7176), 293–296.
- Hauck, M. (2010). Ammonium and nitrate tolerance in lichens. *Environmental Pollution*, *158*, 1127–1133.
- Hobbs, R. J., & Richardson, D. M. (1995). *Mediterranean-type ecosystems: opportunities and constraints for studying the function of biodiversity*. In: Davis GW, Richardson D Mediterranean-type ecosystems. The function of biodiversity. Springer-Verlag, Berlin, Heidelberg.
- Hogan, E. J., Minnullina, G., Sheppard, L. J., Leith, I. D., & Crittenden, P. D. (2010a). Response of phosphomonoesterase activity in the lichen *Cladonia portentosa* to nitrogen and phosphorus enrichment in a field manipulation experiment. *New Phytologist*. doi:10.1111/j.1469-8137.2010.03221.x.
- Hogan, E. J., Minullina, G., Smith, R. I., & Crittenden, P. D. (2010b). Effects of nitrogen enrichment on phosphatase and nitrogen: phosphorus relationships in *Cladonia portentosa*. *New Phytologist*. doi:10.1111/j.1469-8137.2010.03222.x.
- Horswill, P., O'Sullivan, O., Phoenix, G. K., Lee, J. A., & Leake, J. R. (2008). Base cation depletion, eutrophication and acidification of species-rich grasslands in response to long-term simulated nitrogen deposition. *Environmental Pollution*, *155*, 336–349.
- Hu, Y.-L., Zeng, D.-H., Liu, Y.-X., Zhang, Y.-L., & Chen, Z.-H. (2010). Responses of soil chemical and biological properties to nitrogen addition in a Dahurian larch plantation in Northeast China. *Plant and Soil*. doi:10.1007/s11104-010-0321-6.
- Maestre, F. T., Bowker, M. A., Cantón, Y., Castillo-Monroy, A. P., Cortina, J., Escolar, C., et al. (2011). Ecology and functional roles of biological soil crusts in semi-arid ecosystems of Spain. *Journal of Arid Environments*, *75*, 1282–1291.
- Neff, J. C., Townsend, A. R., Gleixner, G., Lehman, S. J., Turnbull, J., & Bowman, W. D. (2002). Variable effects of nitrogen additions on the stability and turnover of soil carbon. *Nature*, *419*, 915–917.
- O'Sullivan, O. S., Horswill, P., Phoenix, G. K., Lee, J. A., & Leake, J. R. (2011). Recovery of soil nitrogen pools in species-rich grasslands after 12 years of simulated pollutant nitrogen deposition: a 6-year experimental analysis. *Global Change Biology*, *17*, 2615–2628.
- Ochoa-Hueso, R., & Manrique, E. (2011). Effects of nitrogen deposition and soil fertility on cover and physiology of *Cladonia foliacea* (Huds.) Willd. a lichen of biological soil crusts from Mediterranean Spain. *Environmental Pollution*, *159*, 449–457.
- Ochoa-Hueso, R., & Manrique, E. (2013). Effects of nitrogen deposition on growth and physiology of *Pleurochaete squarrosa* (Brid.) Lindb., a terricolous moss from Mediterranean ecosystems. *Water, Air, & Soil Pollution*, *224*, 1–14.
- Ochoa-Hueso, R., Allen, E. B., Branquinho, C., Cruz, C., Dias, T., Fenn, M. E., et al. (2011a). Nitrogen deposition effects on Mediterranean-type ecosystems: an ecological assessment. *Environmental Pollution*. doi:10.1016/j.envpol.2010.12.019.
- Ochoa-Hueso, R., Hernandez, R. R., Pueyo, J. J., & Manrique, E. (2011b). Spatial distribution and physiology of biological soil crusts from semi-arid central Spain are related to soil chemistry and shrub cover. *Soil Biology and Biochemistry*. doi:10.1016/j.soilbio.2011.05.010.
- Ochoa-Hueso, R., Mejías-Sanz, V., Pérez-Corona, M. E., & Manrique, E. (2013a). Nitrogen deposition effects on tissue chemistry and phosphatase activity in *Cladonia foliacea* (Huds.) Willd. a common terricolous lichen of semi-arid Mediterranean shrublands. *Journal of Arid Environments*. doi:10.1016/j.jaridenv.2012.07.007.

- Ochoa-Hueso, R., Maestre, F. T., de los Ríos, A., Valea, S., Theobald, M. R., Vivanco, M. G., et al. (2013b). Nitrogen deposition alters nitrogen cycling and reduces soil carbon content in low-productivity semiarid Mediterranean ecosystems. *Environmental Pollution*, *179*, 185–193.
- Ochoa-Hueso, R., Pérez-Corona, M. E., & Manrique, E. (2013c). Impacts of simulated N deposition on plants and mycorrhizae from Spanish semiarid Mediterranean shrublands. *Ecosystems*, *16*(5), 838–851.
- Pearce, I. S. K., Woodin, S. J., & Van der Wal, R. (2003). Physiological and growth responses of the montane bryophyte *Racomitrium lanuginosum* to atmospheric nitrogen deposition. *New Phytologist*, *160*(1), 145–155.
- Peñuelas, J., Sardans, J., Rivas-ubach, A., & Janssens, I. A. (2012). The human-induced imbalance between C, N and P in Earth's life system. *Global Change Biology*, *18*, 3–6.
- Pinho, P., Bergamini, A., Carvalho, P., Branquinho, C., Stofer, S., Scheidegger, C., et al. (2012). Lichen functional groups as ecological indicators of the effects of land-use in Mediterranean ecosystems. *Ecological Indicators*, *15*, 36–42.
- Reed, S. C., Coe, K. K., Sparks, J. P., Housman, D. C., Zelikova, T. J., & Belnap, J. (2012). Changes to dryland rainfall result in rapid moss mortality and altered soil fertility. *Nature Climate Change*, *2*, 752–755.
- Reich, P. B., Hobbie, S. E., Lee, T., Ellsworth, D. S., West, J. B., Tilman, D., et al. (2006). Nitrogen limitation constrains sustainability of ecosystem response to CO<sub>2</sub>. *Nature*, *440*, 922–925.
- Reid, J. P., Adair, E. C., Hobbie, S. E., & Reich, P. B. (2012). Biodiversity, nitrogen deposition, and CO<sub>2</sub> affect grassland soil carbon cycling but not storage. *Ecosystems*, *15*, 580–590.
- Rowe, E. C., Emmett, B. A., Frogbrook, Z. L., Robinson, D. A., & Hughes, S. (2012). Nitrogen deposition and climate effects on soil nitrogen availability: influences of habitat type and soil characteristics. *Science of the Total Environment*, *434*, 62–70.
- Sardans, J., & Peñuelas, J. (2004). Increasing drought decreases phosphorus availability in an evergreen Mediterranean forest. *Plant and Soil*, *267*, 367–377.
- Stevens, C., Dise, N. B., Mountford, J. O., & Gowing, D. J. (2004). Impact of nitrogen deposition on the species richness of grasslands. *Science*, *303*, 1876–1879.
- Stevens, C. J., Dise, N. B., & Gowing, D. (2009). Regional trends in soil acidification and exchangeable metal concentrations in relation to acid deposition rates. *Environmental Pollution*, *157*, 313–319.
- Stevens, C. J., Duprè, C., Dorland, E., Gaudnik, C., Gowing, D. J. G., Bleeker, A., et al. (2011). The impact of nitrogen deposition on acid grasslands in the Atlantic region of Europe. *Environmental Pollution*, *159*, 2243–2250.
- Vivanco, M. G., Palomino, I., Vautard, R., Bessagnet, B., Martín, F., Menut, L., et al. (2009). Multi-year assessment of photochemical air quality simulation Spain. *Environmental Modelling and Software*, *24*, 63–73.
- Vourlitis, G. L., & Fernández, J. S. (2012). Changes in the soil, litter, and vegetation nitrogen and carbon concentrations of semiarid shrublands in response to chronic dry season nitrogen input. *Journal of Arid Environments*, *82*, 115–122.
- Vourlitis, G. L., Pasquini, S. C., & Mustard, R. (2009). Effects of dry-season N input on the productivity and N storage of Mediterranean-type shrublands. *Ecosystems*, *12*, 473–488.
- Waldrop, M. P., Zak, D. R., Sinsabaugh, R. L., Gallo, M., & Lauber, C. (2004). Nitrogen deposition impacts modifies soil carbon storage through changes in microbial enzymatic activity. *Ecological Applications*, *14*, 1172–1177.
- Walkley, A., & Black, A. I. (1934). An examination of the Degareff method for determining soil organic matter and a proposed modification of the chromic acid titration method. *Soil Science*, *37*, 29–38.
- Zeglin, L. H., Stursova, M., Sinsabaugh, R. L., & Collins, S. L. (2007). Microbial responses to nitrogen addition in three contrasting grassland ecosystems. *Oecologia*, *154*, 349–359.
- Zeglin, L. H., Sinsabaugh, R. L., Barrett, J. E., Gooseff, M. N., & Takacs-Vesbach, C. D. (2009). Activity in the McMurdo dry valleys: linked biotic processes, hydrology, and geochemistry in a cold desert ecosystem. *Ecosystems*, *12*, 562–573.