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ALLEVIATING NITROGEN LIMITATION IN MEDITERRANEAN MAQUIS VEGETATION LEADS TO ECOLOGICAL DEGRADATION

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N ENRICHMENT DEGRADES ECOLOGICAL PARTNERSHIPS AND ITS FUNCTIONS

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ABSTRACT

Soils are being degraded at an alarming rate and thereby also crucial ecosystem goods and services. Nitrogen (N) enrichment is a major driver of this degradation. While the negative impacts of N enrichment on vegetation are well known globally, those on various ecological interactions, and on ecosystem functioning, remain largely unknown. Since Mediterranean ecosystems are N-limited, they are good model systems for evaluating how N enrichment impacts not only vegetation, but also ecological partnerships and ecosystem functioning. Using a 7-yr N-manipulation (dose and form) field experiment running in a Mediterranean Basin maquis located in a region with naturally low ambient N deposition (<4 kg N ha^{-1} yr^{-1}), we assessed the impacts of the N additions on: i) the dominant plant species (photosynthetic N use efficiency – PNUE); ii) plant-soil ecological partnerships with ectomycorrhiza and N-fixing bacteria; and iii) ecosystem degradation (plant-soil cover, biological mineral weathering and soil N fixation). N additions significantly disrupted plant-soil cover, plant-soil biotic interactions, and ecosystem functioning compared to ambient N deposition conditions. However, the higher the ammonium dose (alone or with nitrate), the more drastic these disruptions were. We report a critical threshold at 20-40 kg ammonium ha^{-1} yr^{-1} whereby severe ecosystem degradation can be expected. These observations are critical to help explain the mechanisms behind ecosystem degradation, to describe the collective loss of organisms and multifunction in the landscape, and to predict potential fragmentation of Mediterranean maquis under conditions of unrelieved N-enrichment.

Keywords: Ammonium; Ecosystem degradation; Ecosystem functioning; Mediterranean; Plant–soil ecological partnerships

INTRODUCTION

Despite being vital for the sustainable provisioning of ecosystem services in terrestrial ecosystems, soils are being degraded at an alarming rate (Corvalan *et al.*, 2005). Consequently, managing soil functioning and related services, especially by improving soil resilience to global environmental change, has become a priority. However, soil ecology remains a 'black box' in many regions globally; most literature comes mainly from northern hemisphere ecosystems, and is often limited to basic parameters and local-scale variations in microbial and nutrient pools, while the regulatory mechanisms behind such variations remain largely unknown (Ekblad *et al.*, 2013). Since nitrogen (N) enrichment is a major driver of ecological degradation (Sutton *et al.*, 2011), elucidating the mechanisms behind landscape degradation *via* N-enrichment could help our understanding of its true impact on a variety of organisms and ecosystem multifunction. Studying ecosystems with intrinsically lower nutrient pools would further enable better detection of the effect of N-enrichment on local plant and soil regulatory mechanisms. Mediterranean ecosystems are nutrient-poor (Cruz *et al.*, 2008; Cowling *et al.*, 1996) yet harbour one of the richest flora in the world. These ecosystems are threatened worldwide by increasing N deposition (Phoenix *et al.*, 2006), and are therefore good models for studying N-driven changes.

Of the five world regions that harbour Mediterranean-type climate and vegetation, California and the Mediterranean Basin are most threatened by N enrichment (Ochoa-Hueso *et al.*, 2011). However, between these two regions, the impacts of N enrichment in the Mediterranean Basin remain poorly studied (Ochoa-Hueso *et al.*, 2011) although N deposition in the Basin is expected to increase three-fold by 2050 (Galloway *et al.*, 2004). Despite the high conservation interest within the Mediterranean Basin region (e.g. ~20% of the terrestrial area is conserved in Portugal - http://www.icnf.pt/portal/ap), increasingly multipurpose landscapes make these ecosystems vulnerable to N enrichment of urban-

industrial (both mostly emitting NO_x) and agricultural (mostly emitting NH_y) origins (Dias *et al.*, 2014; Ochoa-Hueso *et al.*, 2011; Sutton *et al.*, 2011).

On-going increases in N deposition within the Mediterranean Basin have already changed patterns of N availability (Dias *et al.*, 2012; 2011a), allowing new plant species to appear and forcing others to disappear (Dias *et al.*, 2014). But apart from vegetation changes, plant-soil ecological partnerships in Mediterranean ecosystems also need attention as they provide plants with nutrients and water in these nutrient-limited environments (van der Heijden *et al.*, 2008). In N-limited Mediterranean ecosystems with low soil organic matter (Cruz *et al.*, 2008), plants constitute a 'pipeline' of plant-derived carbon (up to 20% of photosynthates), which sustains a hotspot of microbes inhabiting roots and the rhizosphere. Especially in these ecosystems, specific plant-associated microbes grant plants the access to nutrients that would otherwise be inaccessible. N-fixing bacteria (symbiotic and free living) that fix atmospheric N_2, and ectomycorrhiza that weather minerals to access limiting nutrients (Wallander, 2000; Balogh-Brunstad *et al.*, 2008; Thorley *et al.*, 2015) are examples of such microbes. Any factor that affects (directly and/or indirectly) the 'pipeline' of plant-derived carbon will affect plant-microbe associations, plant and soil microbial communities (van der Heijden *et al.*, 2016), and thus ecosystem processes and functions (Finlay, 2008). Examples of losses in services to plants include N and phosphorus acquisition, also *via* microbial mineral weathering, and resistance to drought and pathogens (Finlay, 2008; Thorley *et al.*, 2015; Balogh-Brunstad *et al.*, 2008; Dias *et al.*, 2015a). Despite this, the impact of N enrichment on plant-soil ecological partnerships within Mediterranean Basin ecosystems are scarce and are limited to short-term observations (Ochoa-Hueso & Manrique, 2013; 2014).

Based on the responsiveness of Mediterranean maquis to ammonium (NH_4^+) (Dias *et al.*, 2014), and the low NH_4^+ tolerance of the dominant perennial plant species *Cistus ladanifer* L. (Dias *et al.*, 2011b; 2015b), we hypothesize that apart from the vegetation, various ecological

interactions (e.g. plant physiological ecology and plant-soil ecological partnerships) and even ecosystem functioning will degrade in a concerted way. Furthermore, we predict the existence of an N threshold from which point more rapid ecological disruption and ecosystem degradation can be expected. As population dynamics of perennial plants respond to environmental variation over long timescales, we tested these hypotheses during the seventh spring of a N-manipulation (dose and form) experiment in a Mediterranean maquis in Portugal. We evaluated changes in plant-soil cover by tracking the changes in bare soil and *C. ladanifer* cover over the 7-year period; determined *C. ladanifer* ecophysiological responses (leaf traits, N pools and photosynthetic N-use efficiency – PNUE); and measured plant-soil ecological partnerships with ectomycorrhiza and N-fixing bacteria. Further, the impacts of the N additions on the following ecosystem functions were also quantified: i) mineral weathering, using leaf strontium (Sr) concentration as a surrogate (Wallander, 2000); and ii) soil N fixation, using the acetylene reduction assay (Ochoa-Hueso & Manrique, 2013). Determining the ecological impacts of N enrichment on plant physiological responses could explain plant health and persistence in Mediterranean Basin landscapes, and studying the interactions between organisms would more realistically describe potential ecosystem multifunction loss.

MATERIALS AND METHODS

Study site

The study site (38°29'N - 9°1'W) is located in a Natura 2000 site (PTCON0010 Arrábida/Espichel) in Serra da Arrábida (Portugal), within the sub-humid thermomediterranean bioclimatic domain (http://www.globalbioclimatics.org/form/tb_med.htm). According to records (1981-2010, from the Instituto Português do Mar e da Atmosfera – Setúbal meteorological station), mean

annual precipitation is 735 mm, mean maximum temperature, 30.1°C (August); highest maximum temperature, 43.5ºC (July); mean minimum temperature, 4.8°C (January); and lowest minimum temperature, -4.8ºC (January). The skeletal soil (topsoil is c.a. 15 cm) is classified as calcic rhodo-chromic luvisols and calcareous chromic cambisols (Dias *et al.*, 2014), being mainly composed of silt (50%), while sand and clay contents are 32% and 18% (silt-sand-loam texture). The experiment is being carried out on a southeast-facing slope (5%) at 130 m altitude, which is protected from public access and has not been managed in recent decades. Dense Mediterranean maquis vegetation (Eunis class F5.2 – Mediterranean maquis) dominates the site comprising mainly shrubs with some small trees, annuals and geophytes. The plant community developed after a fire in summer 2003, four years before the first N addition of this experiment. *Cistus ladanifer* L., a Cistaceae, is the dominant plant species under ambient N deposition. Other abundant plant species include *Erica scoparia* L. (Ericaceae), *Calluna vulgaris* (L.) Hull (Ericaceae), *Genista triacanthos* Brot. (Fabaceae) and *Ulex densus* Welw. ex Webb (Fabaceae). Herbaceous species comprise ~10% of the total plant cover (Dias *et al.*, 2011a).

N-manipulation experimental design

Since the beginning of the experiment, the estimated background N deposition was < 4 kg N ha^{-1} yr^{-1}, and in 2013 it dropped to 2.8 kg ha^{-1} yr^{-1} (1.6 kg NO_x + 1.2 kg NH_y) according to the model used by the European Monitoring and Evaluation Programme (grid location: x = 53 and y = 4 - http://www.emep.int/mscw/index_mscw.html). Our experimental N doses simulated 'worst case' scenarios of N enrichment, but were lower than the values reported for highly N polluted areas in Mediterranean-type ecosystems (Dias *et al.*, 2014). The N forms applied mimicked the most likely N pollution scenarios within the Mediterranean Basin: agricultural sources that emit mostly NH_y; or agricultural and

urban/industrial sources that emit both NH_y and NO_x (Sutton *et al.*, 2011). The N was applied in three equal applications along the year: spring, summer and middle autumn/winter. Control plots received no added N, while there were three N treatments: **40A** received 40 kg NH_4^+-N ha^{-1} yr^{-1} as a 1:1 mixture of NH_4Cl and $(NH_4)_2SO_4$; **40AN** received 20 kg NH_4^+-N ha^{-1} yr^{-1} and 20 kg NO_3^--N ha^{-1} yr^{-1} as NH_4NO_3; and **80AN** received 40 kg NH_4^+-N ha^{-1} yr^{-1} and 40 kg NO_3^--N ha^{-1} yr^{-1} as NH_4NO_3. Thus 40A and 40AN provided the same N dose, while 40A and 80AN provided the same NH_4^+ dose. Each treatment, including the control, was replicated three times (3 plots of 400 m^2 each). All measurements, analyses and sample collection were performed within the central 100 m^2 square to restrict boundary effects and dilution processes. The experimental plots were randomly distributed in three rows across the slope, except the controls, which were placed in the uppermost row to prevent N 'contamination' through runoff from the N-plots. Beginning in January 2007, the dry N salts were added homogenously, by hand, sprinkled over the soil surface.

Risk of soil erosion

The risk of soil erosion was assessed as the changes over time on bare soil between 2007 (the first spring of N additions) and 2013 (the seventh spring of N additions). Since the perennial shrub *C. ladanifer* with 25-45% cover dominates under ambient N deposition, and also strongly responds to the applied N treatments (Dias *et al.*, 2014), its changes in cover over time were also evaluated. *Cistus ladanifer* cover and bare soil were assessed within one 5x5 m square per experimental plot (within the internal 100 m^2) in June 2007 and 2013.

Soil and plant sampling

Soil inorganic N pools were measured to monitor if N, and NH_4^+ in particular, accumulated in the soil to levels that could induce NH_4^+ toxicity (Dias *et al.*, 2015b). Soil was sampled in

May (immediately before the spring N addition), June (6 weeks after the N addition) and September 2013. Soil was sampled from the four corners and the centre of the internal 100 m^2 square of each plot. Soil samples (2 cm diameter and 15 cm depth) were removed, sieved (2 mm) and stored at -20ºC until analysis. Individual soil samples (five per plot) were used to determine the concentrations of nitrate ($N\text{-}NO_3^-$), ammonium ($N\text{-}NH_4^+$) and inorganic N, while bulk samples (equal mixtures of the five soil samples from each experimental plot) were used to determine concentrations of nutrients, trace elements, and relative abundance and activity of N-fixing bacteria.

The shoots (leaf traits, nutrients and PNUE) and roots (ectomycorrhiza) used for *C. ladanifer* ecophysiological measurements and biotic interactions were sampled between 17 and 22 June 2013. From each plot, two *C. ladanifer* plants were randomly chosen from the internal sampling square and one shoot per individual plant was collected between 6:00 and 8:00 AM. Since it was impossible to carry the equipment for gas exchange measurements within the dense vegetation, photosynthetic parameters were determined on detached shoots: selected shoots were carefully cut under water to prevent xylem cavitation and stomatal closure (de Dato *et al.*, 2013) and handled at Faculdade de Ciencias, Universidade de Lisboa (40 km distance from the study site). The high stomatal conductance recorded in laboratory (0.25 ± 0.01 mmol H_2O m^{-2} s^{-1}) ensured that leaf physiological activity was not impaired by shoot cutting. To ensure that all leaves were at the same developmental and activity stage, measurements were performed on fully expanded leaves from the third and fourth youngest pairs of *C. ladanifer* leaves (Dias *et al.*, 2011b). From each analysed leaf pair, one leaf was used for morphological and chemical determinations (concentrations of nutrients and trace elements) while the other was used for CO_2 gas-exchanges. *In vivo* leaf physiological measurements were performed on the day of sampling. The other leaf pair was frozen upon arrival for determination of ammonium and chlorophyll concentrations.

Simultaneously, samples from the rhizospheres of the two *C. ladanifer* plants per plot were collected. Sampling squares of 0.16 m^2 and 10-15 cm deep were established around the plants. Roots were kept in the soil at 4ºC until analysis.

Soil and leaf chemistry and leaf traits

Soil extracts were prepared as described in Dias *et al.*, (2012) and analysed colorimetrically (spectrophotometer, Tecan Spectra Rainbow A-5082) for NH_4^+ (Cruz & Martins-Loução, 2000) and NO_3^- (Hood-Nowotny *et al.*, 2010). Soil inorganic N was determined as the sum of the water-extracted N-NH_4^+ and N-NO_3^-, and was expressed as μg N per g of dry soil.

After CO_2 gas-exchanges measurements, leaf area was determined with image analysis software (Skyleaf, Skye Instruments, Llandrindod Wells, Powys, UK). Leaves were dried at 70°C until constant weight to determine leaf: i) biomass and leaf mass per area (LMA); ii) nitrogen (N) concentration using an elemental analyser (Carlo Erba model 1108EA, Milan, IT); and iii) ionome (i.e., the mineral nutrient and trace element composition) using Inductively Coupled Plasma - Optical Emission Spectroscopy (ICP-OES – Spectro Ciros CCD, Spectro, Germany). The LMA and N obtained on the opposite leaf were used as estimations of the values of the measured leaf. Leaf NH_4^+ concentration was quantified as described in Pintó-Marijuan *et al*. (2013), while chlorophylls were determined as described in Ritchie (2006). Concentrations of both leaf NH_4^+ and chlorophylls were then expressed per g of dry leaf.

***Cistus ladanifer* photosynthetic N use efficiency (PNUE)**

Net photosynthetic CO_2 assimilation rate (A) versus intercellular CO_2 concentration (C_i) response (A–C_i curves) were measured in *C. ladanifer* leaves using a Licor 6400 Portable Photosynthesis System (LI-COR Biosciences Inc., Lincoln, NE, USA) in combination with a

conifer chamber (6400-22 Opaque Conifer Chamber) equipped with a Red, Green, Blue (RGB) light source (6400-18). Measurements were performed at 25°C, photosynthetic photon flux density (PPFD) of 1500 µmol photons m^{-2} s^{-1} and 1.3 kPa vapour pressure deficit. The ambient CO_2 concentration (C_a) was initially set at 400 ppm until photosynthesis reached a stable value, then decreased stepwise to 300, 250, 150 and 50 ppm and increased to 400, 550, 650, 750, and 1000 ppm (Long & Bernacchi, 2003). The apparent maximum rate of carboxylation by Rubisco (V_{cmax}) and the maximum rate of electron transport (J_{max}) were calculated from the CO_2 response curves according to Long & Bernacchi (2003), using the Farquhar model of leaf photosynthesis (Farquhar *et al*., 1980).

Mesophyll conductance (g_m) was calculated following the variable J method (Harley *et al*., 1992):

$$g_m = \frac{A}{C_i - \frac{\Gamma^* \cdot \left[J_f + 8 \cdot (A + R_d)\right]}{J_f - 4 \cdot (A + R_d)}}$$

where A and C_i are the net photosynthetic CO_2 assimilation rate and the intercellular CO_2 concentration when C_a= 400 ppm, respectively; J_f is the photosynthetic electron transport rate calculated on the basis of chlorophyll *a* fluorescence measured as described in Maxwell & Johnson (2000); R_d the rate of non-photorespiratory CO_2 evolution in the light; and Γ* the CO_2 compensation point in the absence of mitochondrial respiration in the light.

R_d and Γ* were estimated using a subsample of six leaves of *C. ladanifer* (one or two from each treatment) following the Laisk method (Brooks & Farquhar, 1985): the photosynthetic response to CO_2 at low C_a values (150, 100, 50 and 30 ppm) was determined for three levels of subsaturating PPFD (300, 200 and 100 µmol photons m^{-2} s^{-1}), then for each PPFD level the photosynthesis response to CO_2 was fitted with a linear regression line, allowing the determination of Γ* and R_d as the x and y coordinates of the intersection point of the three lines, respectively. Since R_d (expressed on a leaf area basis) was significantly related to LMA

(R_d = 0.0064 X LMA + 0.8686, R^2=0.81, p=0.03), this relationship was used to estimate the R_d of the leaves used for mesophyll conductance determination on the basis of their LMA. The Γ* value used in mesophyll conductance calculation was instead the average of the values obtained through the Laisk method (38.4±1.9 μmol mol^{-1}).

Combining leaf CO_2 gas exchange parameters, leaf biomass, and N concentrations, the following PNUE parameters were derived: net photosynthetic CO_2 assimilation rate at 400 ppm CO_2 per N (A_{400}/N), apparent maximum rate of carboxylation by Rubisco per N (V_{cmax}/N), mesophyll conductance per N (g_m/N) and maximum rate of electron transport per N (J_{max}/N).

Plant-soil ecological partnerships with ectomycorrhiza and soil N-fixing bacteria

Fine roots (<2 mm) were retrieved from the soil using a sieve under a stream of cold water. The final separation and counting of roots between dead, mycorrhized and non-mycorrhized (data not shown) was conducted under the stereomicroscope. Mycorrhized root tips were classified into morphotypes based on morphological characters and exploration types (Agerer, 2001), with the number of each morphotype recorded separately for each sample (i.e., the root system of each *C. ladanifer* plant). A sample of five ectomycorrhized tips from each morphotype was stored at -20ºC until analysis. DNA from each morphotype was extracted using the GeneMATRIX Plant & Fungi DNA Purification Kit (EURx, Poland). DNA amplification and molecular identification of mycorrhiza was achieved by sequencing the PCR amplified internal transcribed spacer (ITS) of n-rDNA, as described by Leski *et al.* (2010). Ectomycorrhiza included in the contact type had a smooth mantle in close contact with surrounding substrates while the few emanating hyphae were in close contact with dead roots. Those classified as short distance type had a voluminous envelope of emanating hyphae, but no rhizomorphs, while those of the medium distance type formed rhizomorphs

(Agerer, 2001). Leaf strontium (Sr) was used as a proxy of ectomycorrhizal mineral weathering, as described by Wallander (2000), because: i) ectomycorrhiza acquire micronutrients and trace metals (Wallander, 2000); ii) the soils at our study site have gypsum and calcite minerals that most likely contain Sr (António Mateus, personal communication); and iii) Sr and calcium compete for plant uptake (Kabata-Pendias, 2011) but since the concentration of calcium in the soil and in the plant did not change (data not shown) it was Sr availability that changed.

Bulk soil samples were used for DNA extraction and analysed by large-scale pyrosequencing. Soil DNA was extracted using the PowerSoil DNA Isolation Kit (Mo Bio, USA). DNA amplification and identification of bacteria was based on the PCR amplified 16SrRNA gene sequence. Analysis of pyrosequenced raw sequences and determination of the taxonomic identity of pyrosequenced operational taxonomic units (OTUs) was done as described in Martínez-García *et al.* (2015). Identification of the OTUs up to at least the genus level combined with references of N fixation by those bacteria enabled the quantification of the relative abundance of OTUs from N-fixing bacteria. Soil N fixation was estimated using the acetylene reduction assay as described in Ochoa-Hueso & Manrique (2013).

Calculations and statistics

Changes in *C. ladanifer* cover and in bare soil *(X)* between 2007 (the first spring of N additions – t1) and 2013 (the seventh spring of N additions – t7) were calculated as positive (an increase) or negative (a decrease) as follows (Dias *et al.*, 2014):

$$\text{Changes in } X\ (\%) = \frac{(X\ \text{t7} - X\ \text{t1})}{(X\ \text{t7} + X\ \text{t1})/2}\ \text{x}\ 100$$

Linear correlations between the changes in *C. ladanifer* cover and in bare soil and between ecological partnerships and the indicators of its functions (ectomycorrhiza and leaf Sr concentration and N-fixing bacteria and soil N fixation) were examined using Pearson's

correlations. The effect of the N additions on soil parameters and on ectomycorrhiza was tested separately using a two-way ANOVA, with treatment and time (for soil parameters) and treatment and morphotype (for ectomycorrhiza) as fixed factors. The effect of the N additions on plant and N-fixing bacteria parameters was tested separately using a one-way ANOVA, with treatment as fixed factor. Bonferroni post hoc multiple comparisons tested for differences (p<0.05) in soil, plant, ectomycorrhiza and N-fixing bacteria parameters between treatments. In all cases, preliminary analyses were performed to ensure that there was no violation of statistical assumptions (including the Levene's test to check for homogeneity of variances). SPSS (version 23.0, IBM, Inc.) was used for all these analyses.

To evaluate if the four treatments differentially impacted the all variables (plant-soil cover, *C. ladanifer* PNUE and plant-soil ecological partnerships) in some way, we used canonical analysis of principal coordinates (CAP) in PRIMER 6 (PRIMER-E, 2008). CAP analysis would effectively delineate any N-addition gradient in this multivariate dataset, despite other potentially important unmeasured factors (Anderson, 2008). This CAP analysis was performed using a similarity/dissimilarity matrix based on Euclidean distances. All CAP ordinations (*trace statistic* and *first squared canonical axis*) were tested for significance using a permutation test with 9999 permutations. CAP analyses also detected whether each observed site placement in ordination space is by chance alone through cross validation by 'leave-one-out' allocations (Anderson & Willis, 2003). Leave-one-out analysis then renders a misclassification error for each treatment category, where a low misclassification error in ordination space confirms the treatment is driving the observed unique groupings of measured response variables.

To test the effect strength of N-treatments in driving distinct groupings among response variables in multivariate space, and to also infer which N-treatments are positively and negatively related to the selected plant and ecosystem variables, we conducted a redundancy

analysis (RDA). The significance of the overall RDA ordination and the *first canonical axis* were calculated using 9999 permutations. Forward selection of variables was then used (as the main RDA tests were significant) to statistically rank the importance of each N treatments individually. It is important to note that the 40A category was automatically excluded from the analysis since three dummy variables are enough to code a treatment block with four categories (Lepš *et al.*, 2003). However, the position that 40A occupies in ordination space remains explanatory and useful to include in the final plot to compare to other treatments. All response variables were centered and standardized because of varying measurement units in the response data. Finally, to cross-validate whether we have indeed identified the most important explanatory gradients in our multivariate dataset, we compared the axes of an unconstrained principal component analysis (PCA) with those axes predicted by the broken stick model (Legendre & Legendre, 2012; ter Braak & Šmilauer, 2015). All these statistics were calculated in CANOCO 5 (ter Braak & Šmilauer, 2012).

RESULTS

Changes in plant-soil cover after 7 years

Under no added N (control plots) and addition of 20 kg NH_4^+ ha^{-1} yr^{-1} (40AN) bare soil significantly diminished and *C. ladanifer* cover expanded after the fire disturbance. In turn, under addition of 40 kg NH_4^+ ha^{-1} yr^{-1} (40A and 80AN) bare soil expanded and *C. ladanifer* cover diminished (Fig. 1). Ambient N deposition and up to 20 kg NH_4^+ ha^{-1} yr^{-1} (together with nitrate) clearly promoted plant-soil cover, while exposure to 40 NH_4^+ ha^{-1} yr^{-1} (alone or with nitrate) markedly increased bare soil and thus erosion potential.

The negative impacts of adding 40 kg NH_4^+ ha^{-1} yr^{-1} (40A and 80AN) on plant-soil cover were not related with the soil inorganic N pools (NH_4^+, NO_3^- and inorganic N), which remained lower than 20 µg N g^{-1} dry soil (from May to September) despite the amount of N

added over the 7 years of experiment (Figure 2). Most surprisingly, the N-fertilized plots never displayed higher N availabilities than the control plots at any sampling time.

Plant ecophysiology and photosynthetic N-use efficiency (PNUE)

Cistus ladanifer leaf traits (biomass, area, leaf mass area) and concentrations of NH_4^+ and chlorophylls (Table 1) showed no impact of the N additions, not even when expressed in relation to leaf N (data not shown). The N additions did however increase leaf N concentration by 20-40% in relation to the control. Even though there were no differences in the concentrations of the analysed macro- and micronutrients in the soil (data not shown), the N additions resulted in readjustments of specific nutrients (Table 1): leaves of plants receiving 40 kg NH_4^+ ha^{-1} yr^{-1} (40A and 80AN) had higher concentrations of potassium (K) but lower of zinc (Zn) and magnesium (Mg) than the leaves of plants receiving no N (control) and 20 kg NH_4^+ ha^{-1} yr^{-1} (40AN).

Adding 40 kg NH_4^+ ha^{-1} yr^{-1} (40A and 80AN) had a negative impact on *C. ladanifer* PNUE as the leaves of plants receiving 40 kg NH_4^+ ha^{-1} yr^{-1} (40A and 80AN) showed a reduction of 25-30% on photosynthetic CO_2 assimilation rate at 400 ppm (A_{400}/N), of ~27% on the apparent maximum rate of carboxylation by Rubisco (V_{cmax}/N), of ~30% on mesophyll conductance (g_m/N) and of ~35% maximum rate of electron transport (J_{max}/N) than the leaves of plants receiving no N (Fig. 3). By contrast, adding 20 kg NH_4^+ ha^{-1} yr^{-1} (40AN) had no impact on PNUE in comparison to the control.

Plant-soil ecological partnerships and its functions

Adding 40 kg NH_4^+ ha^{-1} yr^{-1} (40A and 80AN) had a negative impact on plant-soil ecological partnerships and its functions (Fig. 4). Only the plants receiving 40AN had more root tips than those from the control (Fig. S1). However, the largest contribution to this difference

came from dead root tips. Furthermore, only the addition of 80AN resulted in a decline of root tips colonized by ectomycorrhiza in relation to the control. Molecular identification of the ectomycorrhiza colonizing *C. ladanifer* root tips allowed grouping them according to the exploration types described by Agerer (2001): i) contact morphotype: *Russula* sp, *Tomentella* sp and *Tuber* sp; ii) short distance morphotype: *Cenococcum geophilum* and *Hebeloma cistophilum*; and iii) medium distance morphotype: *Entoloma* sp. Under ambient N deposition (control), root tips were mostly colonized by contact and short distance morphotypes, while upon adding N, colonization by short distance morphotypes dropped drastically especially upon addition of NO_3^- (Fig. 4). In the root tips of the plants receiving 80AN no medium distance morphotype was identified. Since colonization by contact morphotypes declined ~25% in plants receiving 40 kg NH_4^+ ha^{-1} yr^{-1} (40A and 80AN), as did leaf Sr (> 50% reduction), these two variables were highly correlated (r=0.787, *p*=0.002). But leaf Sr was not correlated with short- or medium-distance morphotypes, or the sum of the three morphotypes (data not shown).

Molecular identification of the soil N-fixing bacteria detected OTUs of the following genera: *Azospirillum*, *Bradyrhizobium*, ß *Burkholderia*, *Devosia*, *Methylobacterium*, *Mesorhizobium*, *Rhizobium* and *Rhizomicrobium*. Soils which received 40 kg NH_4^+ ha^{-1} yr^{-1} (40A and 80AN) had a reduction of 40% in abundances of N-fixing bacteria and of 50% in N fixation rates compared to soils, which received no N or 20 kg NH_4^+ ha^{-1} yr^{-1} (40AN – Fig. 4). The abundance of N-fixing bacteria and N fixation rates were correlated (r=0.580, *p*=0.048).

Collective impacts of the N additions on the ecosystem

Adding N over 7 years in different doses and forms uniquely influenced a wide variety of plant and ecosystem functions compared to the control (Fig. 5). Indeed, when constrained, the N treatments strongly influenced the selected ecosystem variables (Test on All Axes: pseudo-

F=10.9; p<0.001; Fig. 6). Forward selection showed all categories of the N-treatment to be significant in ordination space using 9999 permutations. However, there was a strong univariate response (Test on First Axis: pseudo-F=12.5; p=0.003), a result further supported in that only the first eigenvalue of the unconstrained PCA was found to be larger than what was predicted by the broken stick model (data not shown) (Legendre & Legendre, 2012). Since Axis 1 explained 61% of the variation, the NH_4^+ dose again appeared as a particularly significant explanatory gradient influencing all the measured response variables. This result further emphasizes an NH_4^+ threshold (20-40 kg ha^{-1} yr^{-1}) that more markedly disrupts multiple ecosystem functions than do lower NH_4^+. High NH_4^+ is associated with lower plant-soil cover, lower *C. ladanifer* PNUE, less plant-soil ecological partnerships with ectomycorrhizal and N-fixing bacteria, and consequently lower mineral weathering and lower N fixation.

DISCUSSION

Studying the only field experiment in the Mediterranean Basin that manipulates both N form and dose at a biotope-scale, we provide strong evidence that N-enrichment goes far beyond the impacts on plants alone, as alleviating N limitation in this competitive context (nutrient-poor and species-rich environment) also degrades plant-soil ecological partnerships and ecosystem functioning. Overall, levels of NH_4^+ were the key driving force in Mediterranean ecosystem functioning (Dias *et al.*, 2014), where the strongest and most rapid ecosystem degradations were observed at higher NH_4^+ doses. We suggest an NH_4^+ threshold below 20-40 kg ha^{-1} yr^{-1} to at least maintain more locally characteristic plant and soil functioning. Finally, the strong correlation between the changes in bare soil and in *C. ladanifer* cover suggests we can use *C. ladanifer* and its ecological partnerships as surrogates for the impacts of N-enrichment on these ecosystems.

N enrichment enhanced the risk of soil erosion

Alleviating N limitation enhanced the risk of soil erosion *via* deterioration of the plant-soil cover (Fig. 1). The dominant plant *C. ladanifer*, was unable to profit from the higher NH_4^+ dose (40A and 80AN) to increase its cover. In fact, it caused higher *C. ladanifer* mortality. Moreover, the NH_4^+-benefited species (those whose presence or cover were promoted by the higher NH_4^+ doses – e.g. *Asphodelus ramosus*, *Brachypodium phoenicoides*, *Dactylis glomerata*) were small short-lived plants, providing a smaller contribution to plant-soil cover (Dias *et al.*, 2014). This ultimately resulted in the overall bare soil expansion.

Although NH_4^+ toxicity might have played an important role in the loss of plant-soil cover (Cruz *et al.*, 2008; Dias *et al.*, 2011b; 2015b), typical 'NH_4^+ toxicity symptoms', such as leaf NH_4^+ accumulation (Puritch & Barker, 1967; Pintó-Marijuan *et al.*, 2013; Dias *et al.*, 2015b), reduction of leaf area (Walch-Liu *et al.*, 2000) and chlorophyll loss (Puritch & Barker, 1967) were not observed in *C. ladanifer* plants (Table 1). NH_4^+ also did not accumulate in the soil (Fig. 2). The absence of any accumulation of inorganic N in the soil from Spring until Summer does not discard changes in the cycling of NH_4^+ and/or NO_3^- which could not be detected by quantifying the soil inorganic N pools alone. However, it raises the possibility that the added N was lost from the ecosystem. Even though we have no data on N losses, previous studies have shown that most of the added N was retained, cycling through the biotic compartment (Dias *et al.*, 2012), changing the plant community (Dias *et al.*, 2014) and increasing plant N concentration (Table 1). Nonetheless, higher NH_4^+ doses would directly increase the risk of soil erosion *via* decreasing plant-soil cover, while lower NH_4^+ doses may indirectly enhance the risk of soil erosion *via* increased productivity (Dias *et al.*, 2014) that increases biofuel during wildfires.

N enrichment affects plant photosynthetic N-use efficiency (PNUE)

NH_4^+ may affect photosynthesis and PNUE by interfering with the plant cation-anion balance (Esteban *et al.*, 2016). The observed deterioration of PNUE on 40A and 80AN plants (based on A_{400}/N and V_{cmax}/N, both related with CO_2 fixation by Rubisco – Fig. 3) may be due to the decrease in leaf Mg concentration (Table 1). Leaf Mg concentration of control plants was around the lower limit for optimal plant growth (1.5-3.5 mg g^{-1} – Shaul, 2002). In turn, plants that grew in the 40A and 80AN plots had Mg concentrations >40% lower than the control, which might limit the activities of Rubisco and other key photosynthetic enzymes (Spreitzer & Salvucci, 2002), and even protein synthesis (Sperrazza & Spremulli, 1983; Shaul, 2002). This is in agreement with the simultaneous decline in J_{max}/N (Fig. 3), which determines the rate of RuBP regeneration. Furthermore, 40A and 80AN had a strong negative impact on J_{max} expressed per leaf area, but this was not so clear for A_{400} or V_{cmax} (Table S1). Altogether these results support the hypothesis that Mg deficiency affected enzymatic activity in 40A and 80AN plants.

The higher NH_4^+ dose decreased g_m per unit of leaf N (Fig. 3) and of leaf area (Table S1), perhaps due to the impacts of NH_4^+ on the leaf diffusional pathway (e.g. intercellular air spaces, cell size, cell walls – Flexas *et al.*, 2012). The analysed leaves were developing at the time of the N addition (or shortly after) and it is during development that leaf anatomy and structure change in response to stress factors (Tosens *et al.*, 2012). Therefore, the NH_4^+-driven differences in g_m of the fully developed leaves may represent a 'footprint' of higher NH_4^+ availabilities at the time of leaf development, similarly to observations in *Quercus suber* (Pintó-Marijuan *et al.*, 2013).

N enrichment affects plant-soil partnerships

A decline in microbial function may result from a decline in its activity and/or a decline in the microbial community performing the given function. In our study, plant-soil ecological partnerships were significantly degraded by the continued addition of the high NH_4^+ dose (40A and 80AN), with a decline in interactions with contact ectomycorrhizal morphotypes and soil N-fixing bacteria (Fig. 4). These results are in agreement with previous N deposition studies (Treseder, 2004; Berthrong *et al.*, 2014). Besides the photosynthetic decline (Fig. 3 and Table S1), the observed Mg deficiency and low Zn (Zn deficiency is only considered for concentrations < 15-20 µg g^{-1} – Sinclair & Kraemer, 2012) in 40A and 80AN plants (Table 1) might have affected the movement of carbohydrates to roots and the rhizophere (Shaul, 2002) thus affecting plant-microbe associations and its functions (N fixation and mineral weathering, based on leaf Sr concentration – Wallander, 2000). N enrichment could also have affected these plant-soil ecological partnerships through changes in soil pH, competition for N among microbes, or in soil macronutrients (e.g. calcium, Mg). Since the N additions did not affect soil pH (Dias *et al.*, 2014) or soil macronutrients, both a decline in carbohydrate transport to roots and the rhizosphere and changes in competition for N among microbes are likely involved in disrupting plant-soil ecological partnerships. As both ectomycorrhiza and, in this case, the detected N-fixing bacteria are not obligatory symbionts, the decline in their interactions with *C. ladanifer* plants does not allow us to conclude whether or not these ecological partnerships became redundant to the partners or if there was an NH_4^+-driven decline in the microbes.

The use of leaf Sr concentration as a surrogate of ectomycorrhizal mineral weathering was corroborated by the observed decline in ectomycorrhizal colonization, especially by contact morphotypes. It is interesting that not all the ectomycorrhizal morphotypes responded similarly to the NH_4^+ dose, which might be related to the functional specificities of each

morphotype. For example, some contact morphotype species (e.g. *Russula* sp), but not the other morphotypes, produce extracellular phenoloxidases that degrade lignin to access nutrients (Agerer, 2001). The increased plant productivity (Dias *et al.*, 2014), and consequent demand for nutrients in plants exposed to the lower NH_4^+ dose (40AN) may explain why these plants continued to harbour contact morphotypes and N-fixing bacteria in the same range as the control.

Implications of alleviating N limitation in ecosystems

Adding N, even the lower NH_4^+ dose (40AN), had an impact on ecosystem functioning (Fig. 5), suggesting that alleviating N limitation in these typically nutrient-poor Mediterranean environments would induce shifts away from more characteristic ecosystem functioning. However, since the plots receiving the lower NH_4^+ dose were functioning more similar to control conditions, it is clear that the higher the NH_4^+, the more drastic and rapid ecosystem degradation would be (Fig. 6). Collectively, higher NH_4^+ was associated with higher risk of soil erosion (lower plant-soil cover), lower plant PNUE, less plant-soil ecological partnerships with ectomycorrhizal and N-fixing bacteria, and consequently lower mineral weathering and lower N fixation. This means that Mediterranean maquis receiving NH_4^+ inputs > 20 kg ha^{-1} yr^{-1} are at greatest risk of severe ecological degradation. This NH_4^+ threshold should guide policy and restoration efforts in these already highly fragmented environments, and help prioritize areas for restoration in time of uncertainty and limited funding to respond to all affected areas.

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SUPPORTING INFORMATION

Table S1 – Impact of the N additions on *Cistus ladanifer* leaf CO_2 gas-exchanges expressed per leaf area unit.

Fig S1 – Impact of the N additions on the *C. ladanifer* root tips.

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Table 1 – Impact of the N additions on *Cistus ladanifer* leaf traits, leaf N pools and concentrations of macro- and micronutrients.

	Control	40A	40AN	80AN
Leaf traits				
Biomass (mg leaf^{-1})	75 ± 15	70 ± 16	85 ± 20	76 ± 20
Area (cm^2 leaf^{-1})	3.6 ± 0.7	3.3 ± 0.7	3.7 ± 0.5	3.5 ± 0.5
LMA (g m^{-2})	216 ± 14	214 ± 17	230 ± 27	220 ± 22
Leaf N pools				
[NH_4^+] (μmol g^{-1})	2.3 ± 0.4	2.6 ± 0.3	2.4 ± 0.2	3.8 ± 0.3
[Chl_a+Chl_b] (μg g^{-1})	104 ± 13	104 ± 16	121 ± 27	89 ± 10
[N] (μg g^{-1})	10.2 ± 0.3 ^{b}	13.1 ± 0.2 ^{a}	12.3 ± 0.8 ^{a}	14.2 ± 0.2 ^{a}
[Macronutrients] (mg g^{-1})	26.1 ± 0.5	28.3 ± 1.1	27.2 ± 0.6	28.5 ± 0.3
[K] (mg g^{-1})	4.9 ± 0.1 ^{b}	6.0 ± 0.1 ^{a}	4.9 ± 0.1 ^{b}	5.8 ± 0.1 ^{a}
[Mg] (mg g^{-1})	1.4 ± 0.0 ^{a}	0.8 ± 0.0 ^{c}	1.2 ± 0.1 ^{b}	1.0 ± 0.0 ^{bc}
[Micronutrients] (μg g^{-1})	1013 ± 46	808 ± 189	923 ± 29	939 ± 100
[Zn] (μg g^{-1})	86 ± 4 ^{a}	56 ± 4 ^{c}	76 ± 4 ^{ab}	63 ± 3 ^{bc}

Leaf traits included biomass, area and leaf mass per area – LMA; N pools included concentrations of NH_4^+, total chlorophylls and total N; concentrations of macronutrients included the sum of CaKMgNPS, and of micronutrients included that of BCuFeMnMoNiZn. Different letters show significance at the 5% level. Values are the mean ± 1SE (n = 3).

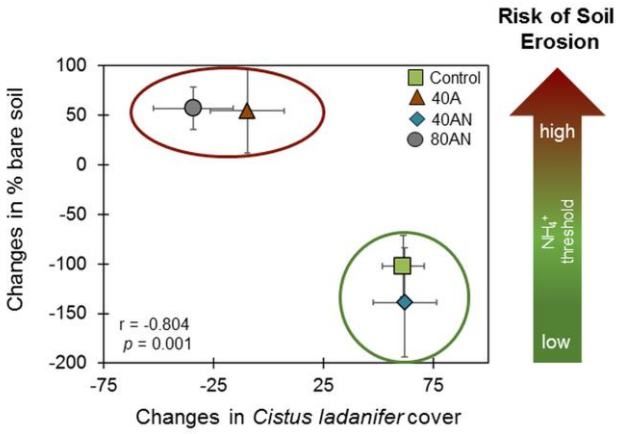

Figure 1 – The association between the changes in bare soil and *Cistus ladanifer* cover between 2007 (first spring of the experiment) and 2013 (seventh spring of the experiment), depicted as a function of the N additions and how they relate to soil erosion risk. Symbols are the mean ± 1SE (n = 3), but Pearson's correlation was calculated for the 12 experimental plots.

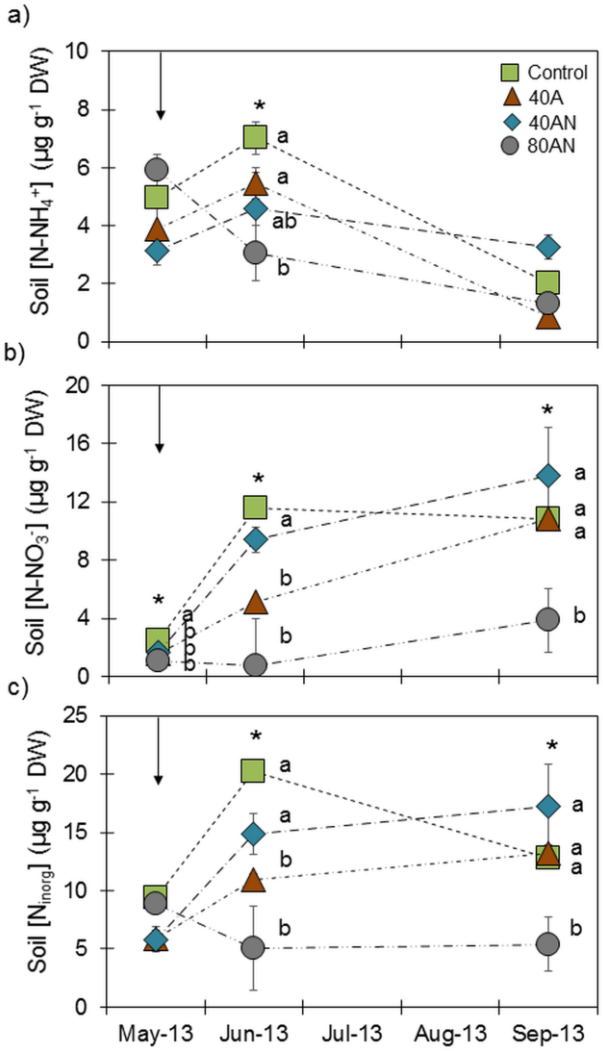

Figure 2 – Impact of the N additions on soil inorganic N pools: ammonium (a), nitrate (b) and inorganic N (c). May 2013 sampling coincided with the spring N addition (arrows), which was only applied after the soils were sampled. Asterisks (*) identify the sampling occasions for which there was a significant effect of the N additions. Different letters show significance at the 5% level. Symbols are the mean ± 1SE (n = 3).

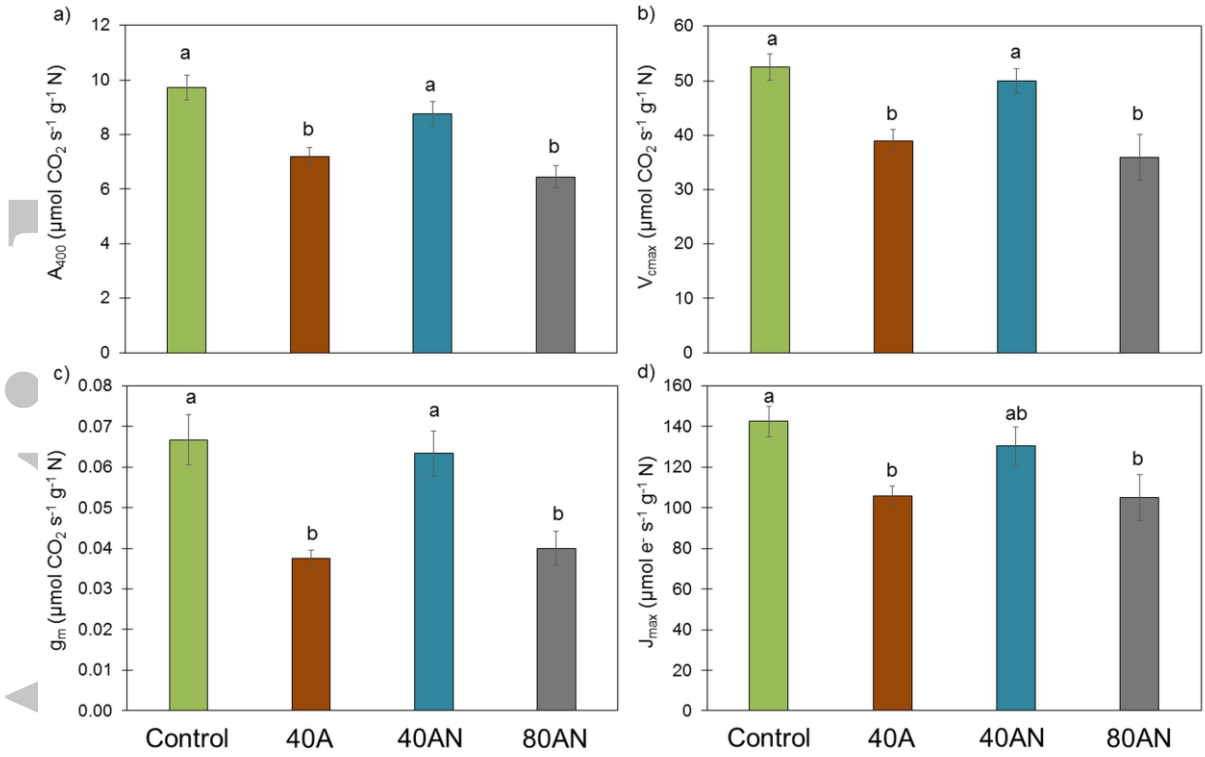

Figure 3 - Impact of the N additions on *Cistus ladanifer* photosynthetic N-use efficiency (PNUE): net photosynthetic CO_2 assimilation rate at 400 ppm CO_2 (A_{400}/N – a), apparent maximum rate of carboxylation by Rubisco (V_{cmax}/N – b), mesophyll conductance (g_m/N – c) and maximum rate of electron transport (J_{max}/N – d). Different letters show significance at the 5% level. Bars are mean ± 1SE (n = 3).

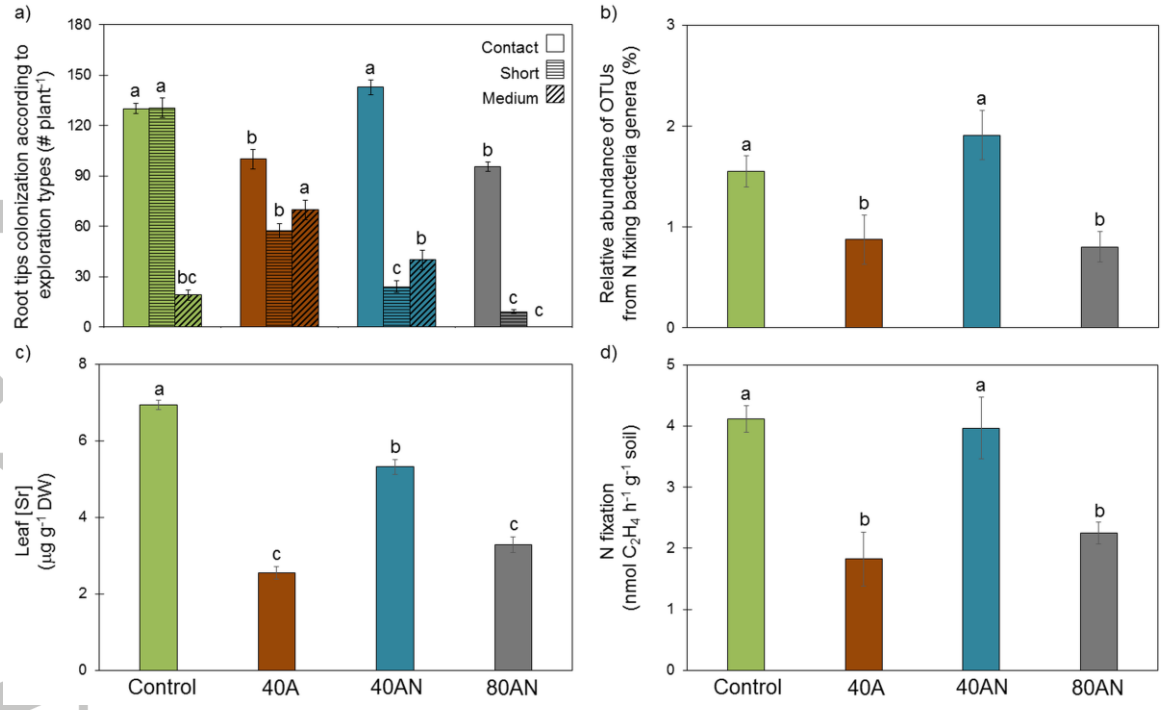

Figure 4 - Impact of the N additions on plant-soil ecological partnerships and their functions: ectomycorrhiza (according to exploration types: contact, short and medium distance explorers – a) and N-fixing bacteria (b) and respective functions (leaf strontium concentration as a surrogate for ectomycorrhizal mineral weathering – c; and acetylene reduction assay as a surrogate for soil N fixation – d). Different letters show significance at the 5% level. Bars are the mean ± 1SE (n = 3).

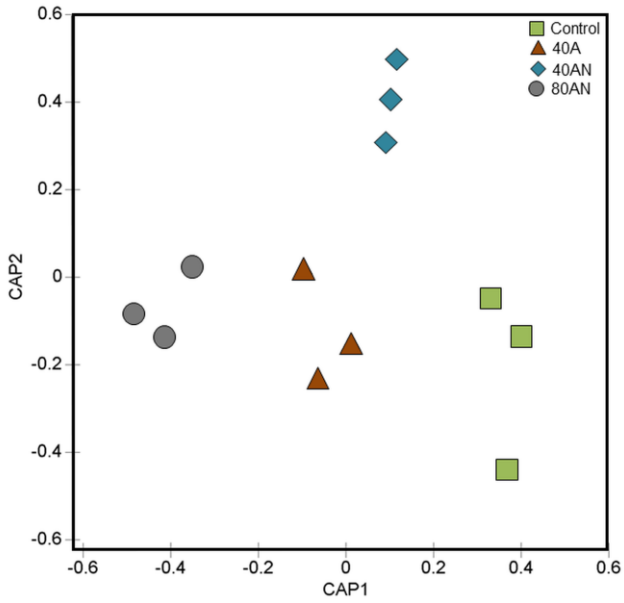

Figure 5 – Canonical analysis of principal coordinates (CAP) depicting how all the N treatments uniquely influence *C. ladanifer* PNUE, plant-soil ecological partnerships with ectomycorrhiza and N-fixing bacteria, and ecosystem functioning (plant-soil cover, mineral weathering and soil N fixation). The total misclassification error via 'leave-one-out' analysis was 8.33%, due to one 40AN plot responding similarly to the 40A plots. All other plots were 0% misclassified within their treatment groups. Both permutation tests (the trace statistic and the test of the first squared canonical axis) were significant (p<0.001).

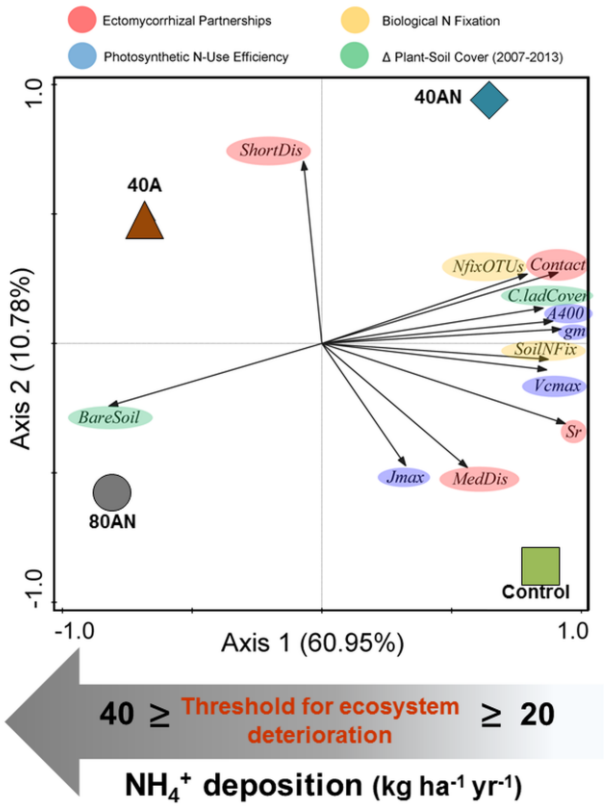

Figure 6 – Redundancy analysis (RDA) suggesting that only the highest NH_4^+ dose strongly (and collectively) disrupted *C. ladanifer* photosynthetic N use efficiency, plant-soil ecological partnerships (with ectomycorrhiza and N-fixing bacteria) and ecosystem functioning (plant-soil cover, mineral weathering and soil N fixation). Explanatory variables accounted for 80.3% of the variation (72.9% adjusted variation). Control: pseudo-F=4.7, p=0.005; 40AN: pseudo-F=12.2, p<0.001; 80AN: pseudo-F=3.8, p=0.001; and 40A: unnecessary to test due to linear combinations.