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N fertilization in a Mediterranean ecosystem alters N and P turnover in soil, roots and the ectomycorrhizal community

Florian Ulm*, Catarina Gouveia, Teresa Dias, Cristina Cruz

Faculdade de Ciencias da Universidade de Lisboa, Centre for Ecology, Evolution and Environmental Changes, Campo Grande, C2, PT-1749-016 Lisbon, Portugal

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ABSTRACT

Increased anthropogenic nitrogen (N) deposition is a major contributor to alteration of soil nutrient cycles particularly in nutrient poor ecosystems, such as the Mediterranean basin, where co-limitation of N and phosphorus (P) occurs and N addition might thus lead to an exacerbation of P limitation. Here, we measured the effect of medium term (6 years) N fertilization in different forms and doses (40 kg N ha^{-1} yr^{-1} as 1:1 NH_4Cl and $(NH_4)_2SO_4$; 40 and 80 kg N ha^{-1} yr^{-1} as NH_4NO_3) on nutrient stoichiometry, potential turnover rates and abundance of roots, ectomycorrhizal (ECM) root tips and adjacent soil in *Cistus ladanifer* L. In order to assess the impact of N addition at its most extreme point, we sampled roots and topsoil (10 cm) with and without plant influence in the summer months. We analysed N and P concentrations in soil and roots and determined the abundance of the most dominant mycorrhizal root tip morphotypes. We also assessed nutrient turnover in soil, roots and mycorrhizal root tips by measuring their N and C related enzyme activities (EAs) as well as acid phosphatase (AP) activity.

Results: showed decreased soil P_{inorg} and increased soil N:P_{inorg} in the treatment plots. Also, a decline in *Cenococcum geophilum* in N addition plots was found and a general reduction in ECM colonization in the treatment receiving ammonium without nitrate. We also detected a decrease of *C. geophilum* absolute EA and AP, as well as N related EA in the whole soil compartment. Furthermore, we observed lower root AP activity and found a loss of correlation between N related EA and AP activity in all treatments, while a high correlation between N related EA and C related EA persisted in all plots. EA was also generally negatively related with root P/soil P, which we used as a measure for plant P status.

The negative effect of ammonium on the ECM community of *C. ladanifer* and a putative loss of short distance exploration morphotypes, such as *C. geophilum*, together with decreased AP activity in the plant roots, might be connected with low P_{inorg} availability in soil with plant influence, thus being in line with the hypothesis of P depletion due to N addition. Furthermore, the decrease of N related EA in the soil compartment, as well as the decoupling of N and P cycles, might be signs of altered soil microbial communities. This decoupling, together with the strong dependence of EA on plant P status, could point to a shift from N and P scavenging ECM communities to more copiotrophic saprophytic fungi that rely on C and N acquisition from soil organic matter rather than plant C inputs. We posit that a decline in root colonization by ECM fungi and changes in N:P cycling could be detrimental to ecosystem development, as *C. ladanifer* is a common ECM species in early successional stages, providing a host for ECM fungi that also colonize late-successional plants.

1. Introduction

The increase in reactive nitrogen (N) availability due to anthropogenic activities has surpassed the input from natural biotic processes and is predicted to further increase twofold until 2050 (Peñuelas et al., 2012), which has poorly understood effects on the biosphere, particularly concerning belowground microbial responses (Ramirez et al., 2012). One proposed consequence of increased N availability could be an increased terrestrial phosphorus (P) limitation (Vitousek et al., 2010) as the rate of anthropogenic P fertilization is far smaller than that of N fertilization (Peñuelas et al., 2012). Microbial growth is highly dependent on

* Corresponding author.
E-mail address: ulm.florian@gmail.com (F. Ulm).

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both soil N and P availabilities, which together determine the rate of carbon (C) turnover in the soil (Cleveland and Liptzin, 2007). While nutrient turnover is essential for microbial life in the soil, it also drives primary production of biomass, as especially in nutrient-poor ecosystems plants heavily depend on microbial associations for nutrient acquisition (Van Der Heijden et al., 2008). Plants that do not form arbuscular mycorrhizal (AM) symbiosis under these conditions, often have ectomycorrhizal (ECM) fungi as the most important partners for the acquisition of both N and P. ECM fungi are a significant component of ecosystem functioning in forest systems of boreal, temperate and Mediterranean climate zones (Courty et al., 2010). Mediterranean forests, woodlands and scrubs are global biodiversity hotspots, which show the second highest mean plant species richness of all biomes worldwide (Kier et al., 2005) and also host very diverse ECM communities (Azul et al., 2014). However, despite their importance for biodiversity, the response of Mediterranean ecosystems to intense increased N availability remains largely unknown (Dias et al., 2014).

The global trend of plant root colonization by ECM with increasing N addition was found to be negative (Treseder, 2004; Li et al., 2015) and long term responses of ECM fungi to increased N availability can be substantial, including diversity loss, changes in community composition and declines in ECM root tips (Cox et al., 2010). The decline in ECM fungi is most probably connected to a lower plant C allocation belowground (Treseder, 2004) as alleviation of N limitation diminishes the dependence on symbiosis for plant growth. However, in an N and P co-limited system such as the Mediterranean maquis (Dias et al., 2012), this does not necessarily apply, as root C allocation will be driven by the limitation of both nutrients (Ågren et al., 2012). Direct responses of N addition on ECM fungal species frequency and abundance were found to vary by genera and to depend on fungal functional type (Lilleskov et al., 2011). It was thus suggested that the addition of N would favour ECM types that are focusing on the uptake of labile N in contrast to ECM types capable of accessing complex organic N sources (Lilleskov et al., 2011). The mobilization of organic N sources by ECM is dependent on fungal enzyme activities (EA), which are also crucial for the functioning of ECM nutrient supply to the plant (Pritsch and Garbaye, 2011). Nutrient turnover rates in the soil follow a strongly constrained stoichiometry between C, N and P, as the degradation of organic molecules by EA is required to obtain both N and P (Sinsabaugh et al., 2009). The addition of N should thus lead to an increased investment in P cycling through the production of phosphatases in order to delay P limitation for plant productivity (Marklein and Houlton, 2012). While in most systems, this co-limitation in N and P is simultaneous, the Mediterranean system also has a temporal discrepancy between N availability and growth period. While in the mild, more humid Mediterranean spring most of the available N will be taken up and stored in the biotic compartment, N will be released during the late summer due to leaf shedding by summer deciduous species, such as *Cistus ladanifer* L. (Dias et al., 2012). Summer deciduous species, such as *Cistus* spp. are replaced in the post fire succession by evergreen sclerophyllus shrub and tree species, a development accompanied by a shift in soil inorganic N from a NO_3^- to a more NH_4^+ dominated state (Cruz et al., 2003). *C. ladanifer* is a pyrophytic shrub that serves as a bridge for post-fire re-colonization by ECM fungi, which are associated with both shrub and late successional tree species (Martín-Pinto et al., 2006), so induced succession and decline in *Cistus* spp. by anthropogenic N - addition might have indirect negative effects on ECM fungi. Indeed, while there are indications of initial plant biodiversity increase after N addition in Mediterranean scrublands undergoing post-fire succession (Dias et al., 2011), medium term (5 years) changes in plant richness were found to depend on the N form applied: the availability of NO_x is in general increasing aboveground biomass, whereas the availability of NH_y is decreasing the abundance of summer deciduous N-affected plants, for example *Cistus ladanifer* (Dias et al., 2014). This species belongs to the *Cistus* genus, which is comprised of plants that are highly mycorrhizal, as is clear from their potential association with 200 fungal species belonging to 40 genera (Comandini et al., 2006) of which several have economical value (Mediavilla et al., 2016). Thus, we hypothesized that *C. ladanifer* could be a promising candidate to monitor the effects of anthropogenic N addition on ECM fungi and that the negative growth response to ammonium observed in this plant species will be directly related with a decrease in ECM colonization. In contrast, we supposed that NO_x is connected to increased plant growth, which would shift N and P pools from the soil towards the biotic compartment with effects on soil EA and ECM fungal community, postulating that increased N availability in the biotic compartment will lead to an alteration in EA towards P acquisition. These hypotheses were tested by assessing the ECM fungal community structure in mycorrhizal root tips and determining the EA of the bulk soil, the roots and the most common morphotypes of mycorrhizal root tips in order to relate these values with soil nutrient and plant tissue stoichiometry.

2. Materials and methods

2.1. Study site

This study was conducted in a Natura 2000 site located south of Lisbon, Portugal (PTCON0010 Arrábida/Espichel) in Arrábida Natural Park. The plots (38°29′N, 9°01′W) are situated on a southeast-facing slope (5%) at 130 m a.s.l. Soil is skeletal (15–20 cm deep) and consists of 57% silt, 28% clay and 15% sand (silt-sand-loam – Correia, 1988). The site was burned in a fire event in 2003, after which the vegetation regenerated into a dense Mediterranean maquis (Eunis class F5.2 - http://eunis.eea.europa.eu/). In 2007, a reactive nitrogen (N) addition experiment was started, using 3 N - treatments: 40 and 80 kg N ha^{-1} yr^{-1} of ammonium nitrate NH_4NO_3 (termed “40AN” and “80AN” respectively) and 40 kg N ha^{-1} yr^{-1} of ammonium in 1:1 mix of NH_4Cl and $(NH_4)_2SO_4$ (termed “40A”). Background nitrogen deposition is 5.2 kg N ha^{-1} yr^{-1} (2.9 kg NO_x + 2.3 kg NH_y - http://webdab.emep.int/Unified_Model_Results/AN/). Each treatment and the control have 3 replicate plots in squares of 400 m^2, but in order to avoid boundary effects sampling was performed in a central 100 m^2 square. The summer deciduous *Cistus ladanifer* was used in this study as it is the dominating shrub species and known to form ectomycorriza.

2.2. Soil sampling, sample collection and preparation

Sampling took place on the 3rd of July 2013. At each plot, topsoil (10 cm) was sampled at 5 random spots without vegetation cover (termed “soil without plant influence”). Also, 3 *C. ladanifer* plants of ca. 50 cm height were chosen and the bulk soil-root agglomerate around the stem sampled into plastic bags. Samples were stored as bulk in the dark at room temperature (20 °C) until usage within the next 7 days. To calculate dry weight contribution of each mycorrhizal root tip, soil and total roots (Fig. 1), 100 g agglomerate were weighed and then separated into roots and soil (“soil with plant influence”), taking soil subsamples and finishing the separation by carefully rinsing the roots with tap water. Roots were further separated into 5 fractions using a Binocular (Zeiss, Oberkochen, Germany): roots without visible hyphae or mycorrhizal root tip mantle (termed “roots”), highly pigmented root tips that were completely black in appearance (termed “black”), highly branched root tips with a wrinkled surface (termed “coral”), non-pigmented root tips that were translucent (termed “white”) and root tips with

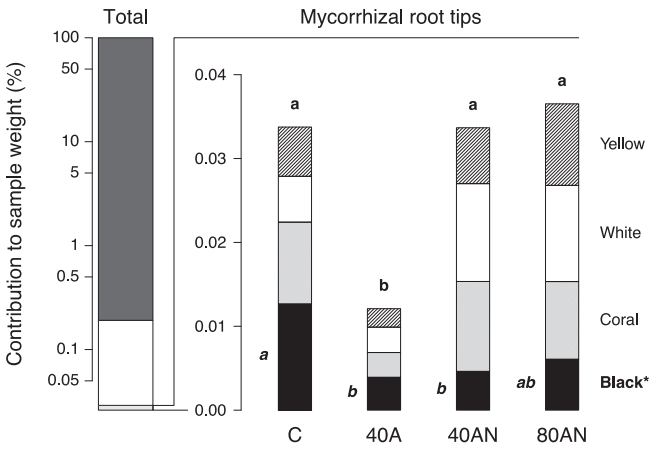

Fig. 1. Total contribution of soil (dark grey), roots (white) and mycorrhizal root tips (light grey) on a log scale to overall sample weight (left) and changes in mycorrhizal root tip composition per treatment (right). Letters above the bars indicate significant differences between treatments for all mycorrhizal root tip combined (Pairwise Welch's *t*-test, n = 4, p < 0.05, Bonferroni - Holm correction). The black morphotype also decreases in the treatments (Pairwise Welch's *t*-test, n = 3, p < 0.05, Bonferroni - Holm correction).

a waxy appearance, which were enlarged in comparison with the main root (termed "yellow"). Using the methodology described by Agerer (1987–2008), morphological characteristics distinguished the "black" morphotype putatively as *Cenococcum geophilum*. It was in most cases monopodial or showed slight dichotomy, had a complete black mantle, no rhizomorphs and only sparse extramatrical hyphal growth. In the following text, the "black" morphotype is subsequently termed *C. geophilum*. After concluding separation, dry weight was determined for each mycorrhizal root tip fraction, total roots and the soil by oven-drying at 60 °C until constant weight and subsequent weighing.

2.3. Organic matter and soil nutrient analysis

Organic matter (OM) of the soil samples was assessed using a modified loss on ignition method (Ball, 1964). Total C and N in soil, roots and mycorrhizal root tips were determined by continuous flow isotope mass spectrometry (CF-IRMS), on an Isoprime (GV, UK) stable isotope ratio mass spectrometer coupled to an EuroEA (EuroVector, Italy) elemental analyser for online sample preparation by Dumas-combustion. Samples were prepared by grinding dry root tips, mycorrhizal root tips and soil to a fine powder in a ball mill (Retsch, Haan, Germany). Five ±0.2 mg of the powder was then packed into tin capsules and subsequently analysed at the Stable Isotopes and Instrumental Analysis Facility (SIIAF) of the Centre for Ecology, Evolution and Environmental Change (cE3c), University of Lisbon - Portugal.

Nitrate, nitrite, ammonium, soluble inorganic phosphorus and pH were determined in 1:10 (wv) soil extracts in ultrapure water (Millipore, Merck, Darmstadt, Germany), using 30 min extraction time on a shaker at room temperature (20 °C). The resulting soil water suspension was centrifuged (5000 g, 4 °C, 15 min) and filtered through 3 layers of medical gauze to obtain a clear extract and pH was then analysed with a glass electrode (pH/mV meter 501, Crison, Barcelona, Spain). Nitrate, nitrite, ammonium and soluble inorganic phosphorus in the soil extract were determined using microscale colorimetric methods. All microscale methods were executed in 96-well plates, which were then read in a microplate reader (Rainbow, Tecan, Männedorf, Switzerland). For each single assay day or microplate, a separate calibration curve was prepared in triplicate. The calibration curves were prepared with the respective salts (KH_2PO_4, $(NH_4)_2SO_4$, KNO_3) as serial dilutions in ultrapure water. Ammonium was determined using a modified Berthelot reaction (Cruz and Martins-Loução, 2000), nitrate and nitrite with the VCl_3/Griess method as described by Hood-Nowotny et al. (2010). As nitrite concentrations were mostly below detection limit, results were omitted here. Inorganic N was calculated as the sum of nitrate and ammonium. Soluble inorganic phosphorus was analysed using a microscale method for soil extracts described by D'Angelo et al. (2001). To determine total phosphorus of soil and roots, the method described by Andersen (1976) was used, after igniting the samples and washing out the ashes with HCl (1 M), phosphorus was analysed as described above.

2.4. Enzyme activity assays

Enzyme activity assays were performed on 3 assay replicates each of root tips, mycorrhizal root tips and soil, taken from the bulk soil-root agglomerate. Roots and mycorrhizal root tips were prepared for analysis by carefully separating root tips from the attached soil, rinsing them in tap water and cutting ca. 2 mm sized root parts from the bulk sample. After separation, the samples were stored in tap water in the fridge until analysis, however, never longer than 24 h. Soil was prepared by weighing 5.2 mg (±0.03 SE) of fresh soil on a fine scale and subsequently transferring the samples to the wells. After the enzyme activity assay procedure, root and mycorrhizal root tip samples were transferred into tin cups, dried to constant weight and weighed on a fine scale to determine dry weight. Soil dry weight was determined from subsamples (5 g, n = 3) taken from the whole soil-root agglomerate at the same time point as the assay samples and water content extrapolated from these subsamples to determine soil dry weight of the samples in the wells.

After sample preparation, enzyme activity was determined for the enzymes: β-xylosidase, β-glucuronidase, cellobiohydrolase, N-acetylglucosaminidase, β-glucosidase, acid phosphatase and leucine amino peptidase using fluorogenic substrates with either 4-Methylumbelliferyl (MU) or 7-amido-4-methylcoumarin (AMC) as fluorescent functional group. The substrates, incubation concentrations, times and buffer pH were: MU-xyloside (30 min, 0.5 mM, pH 4.5), MU-glucuronide (30 min, 0.5 mM, pH 4.5), MU-cellobiohydrofuran (30 min, 0.4 mM, pH 4.5), MU-N-acetylglucosamine (15 min, 0.5 mM, pH 4.5), MU-β-glucoside (15 min, 0.5 mM, pH 4.5), MU-phosphate (30 min, 0.8 mM, pH 4.5) and Leucine-AMC (70 min, 0.4 mM, pH 6.5). Enzyme activity of soil, root and mycorrhizal root tip samples was determined in 96-well filter plates (AcroPrepTM 96-filter plate with 30 μm mesh size, Pall, Life Sciences, Crailsheim, Germany) and fluorescence measured at 364 nm excitation and 450 nm emission in a fluorescence microplate reader (BIOTEK FLx800, BioTek Instruments, Winooski, USA). The procedure for EA determination of roots and mycorrhizal root tips was exactly followed as described by Pritsch et al. (2011) and adapted for the soil samples as in Ulm et al. (2017) for rhizospheric soil. In brief, this enzyme assay is carried out repeatedly on the same sample, which is placed in a filter plate, thus allowing for subsequent incubation and washing steps without loosing the sample to be analysed. As Pritsch et al. (2011) only describe this procedure for mycorrhizal root tips, pre-tests were run on the soil samples to assure that no sample was lost during the washing steps and that sequential incubation with substrates does not interfere with the activity measured. The quantity of soil found to be most suitable for this assay was found to be 5.2 mg, as more sample clogs the pores of the sample plate and less sample increases variance due to soil heterogeneity.

The emission values obtained from the microplate reader were

calibrated against a standard curve of 100 µl calibration solutions (0, 1, 2, 3, 4, or 5 µM) and then related to sample dry weight to express EAs per g sample. EAs were then calculated per g C (Table 2; Fig. 3; Fig. 4; Fig. 5) using the values for total C, which were determined as described in section 2.3. EAs were also expressed for the total bulk soil-root agglomerate by multiplying the enzyme activity expressed per gram with the total weight of each compartment (roots, morphotypes and soil) to yield Fig. 2. The total geometric mean of EA g^{-1} C (Fig. 6) was used as general indicator for soil microbial activity, following the reasoning of García-Ruiz et al. (2008), who used the geometric mean of EAs as an indicator for soil quality and related microbial activity. However, instead of expressing the geometric mean per g soil, the geometric mean of EA g^{-1} C was used, as it was shown that relating EA per C yields more comparable results between different kind of assays (Drouillon and Merckx, 2005) as well as between different kinds of substrates (Sinsabaugh et al., 2009).

2.5. Statistical analysis

Statistical analysis was performed using R version 3.3.2 (R Core Team, 2016) using package "stats", if not stated otherwise, and executed on RStudio IDE version 1.0.136. Additional packages used were: "Hmisc" (Harrell et al., 2013), "lawstat" (Gastwirth et al., 2013), "lmtest" (Hothorn and Zeileis, 2013) and "vegan" (Oksanen et al., 2015).

Pairwise comparisons between groups were calculated using Pairwise Welch's *t*-test with Bonferroni - Holm correction (Fig. 1; Fig. 2; Table 1; Table 2). If only two groups were compared, Welch's *t*-test was used (Table 1). Normality assumptions for group wise comparisons were verified using qq-plots and the Shapiro-Wilk Normality Test. If the normality assumption was violated, data was log transformed. Linear regressions (Fig. 3; Fig. 4; Fig. 6) were performed after verifying assumptions using the Breusch-Pagan Test for homoscedasticity and the Shapiro-Wilk Normality Test on the regression model residuals. The redundancy analysis (RDA) was performed on a correlation matrix of the enzyme data using the *rda()* function in the "vegan" package (Fig. 5), together with the *ordiellipse()* function on the site scores to create 95% confidence ellipses for the standard error of the average of the factor scores (control and treatments). Variable selection was performed using the forward selection procedure in the *ordiR2step()* function, which makes use of p-values and adjusted R^2 (Blanchet et al., 2008). The model and the first two axes were then evaluated for significance against an unconstrained model using adjusted R^2 values in permutational significance tests (1000 permutations).

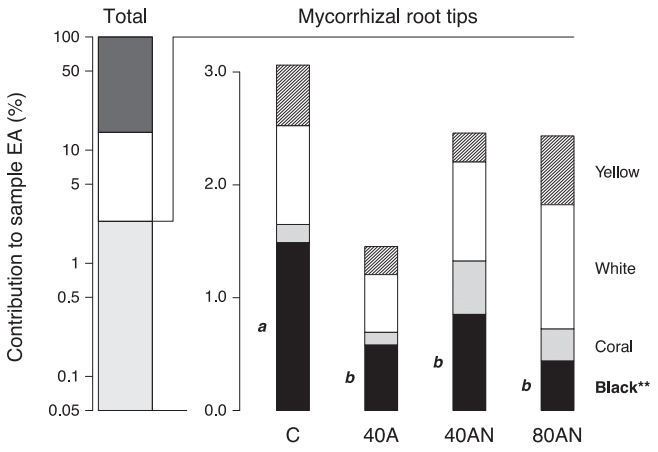

Fig. 2. Contribution of soil (dark grey), roots (white) and mycorrhizal root tips (light grey) on a log scale to absolute EA of the sample (left) and changes in mycorrhizal root tip EA per treatment (right). The black morphotype decreases in the treatments (Pairwise Welch's *t*-test, n = 3, p < 0.05, Bonferroni - Holm correction).

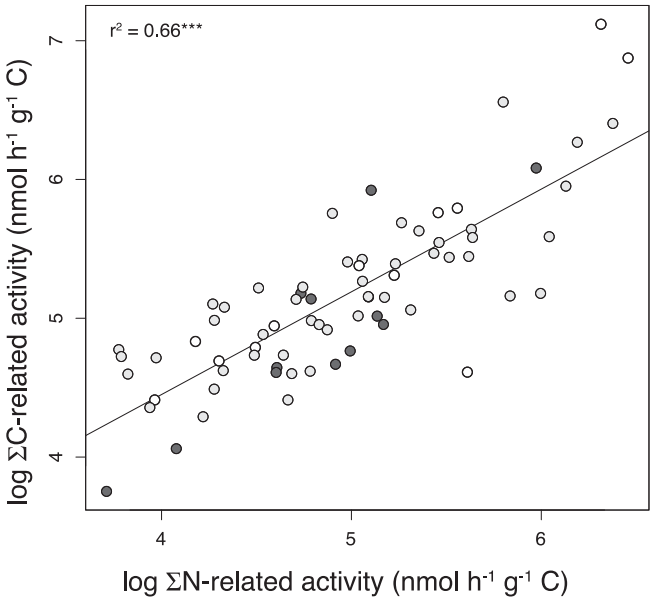

Fig. 3. Correlation between log-transformed ΣN-related (leucine-aminopeptidase and N-acetylglucosaminidase) and ΣC-related (β-glucosidase, cellobiohydrolase, β-glucuronidase and β-xylosidase) specific EA. Significance is denoted with asterisks (*** = p < 0.001). EA values from roots are shown in white, dark grey depicts soil and light grey mycorrhizal root tip values.

3. Results

3.1. Soil nutrient changes

The soil sampled in this experimental setup was influenced by plant presence, as indicated in Table 1. While OM, NH_4^+, NO_3^- and the ratio between inorganic N (N_{inorg}) and inorganic P (P_{inorg}) were not significantly different between soil with and without plant influence, all other parameters measured here showed significant changes with plant presence. Soil with plant influence was more acidic and showed both higher total P (P_{tot}) and P_{inorg} concentrations. The latter, in combination with slightly higher N_{inorg} levels, lead to a tendency (Welch's *t*-test, p = 0.058) for lower N:P_{inorg} ratios in soil with plant influence, with significantly lower N:P_{inorg} ratios in the 40AN and 80AN treatments.

Soil without plant influence exhibited N:P_{inorg} ratios that were significantly lower in the control compared to all treatments, while N:P_{inorg} ratios of soil with plant influence were the lowest in the control and the highest in the 40A treatments, with intermediate values for the AN treatments. The opposite pattern was observed for P_{inorg} concentrations of soil with plant influence, with the highest values in the control, intermediate values in the AN treatments and the lowest in the 40A treatment. Soil without plant influence exhibited the highest NH_3^+ values in the 40AN treatment, followed by 80AN and slightly lower values for 40A, with the lowest for the control. For NO_3^- control and 40A exhibited lower values than the AN treatments. Also, soil without plant influence in the control plots was more acidic than in the 40A and the 40AN treatments, while the 80AN treatment was significantly different with a neutral pH of 7.

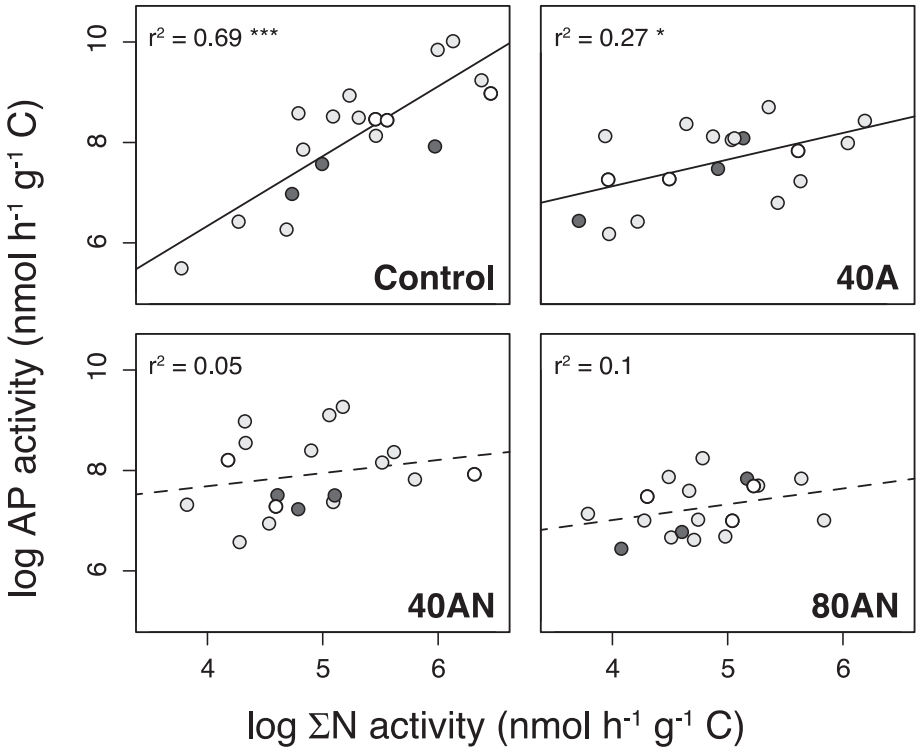

Fig. 4. Correlations between log-transformed ΣN-related (leucine-aminopeptidase and N- acetylglucosaminidase) and AP (acid phosphatase) related EA. Significance is denoted with asterisks (*** = $p < 0.001$). EA values from roots are shown in white, dark grey depicts soil and light grey mycorrhizal root tip values.

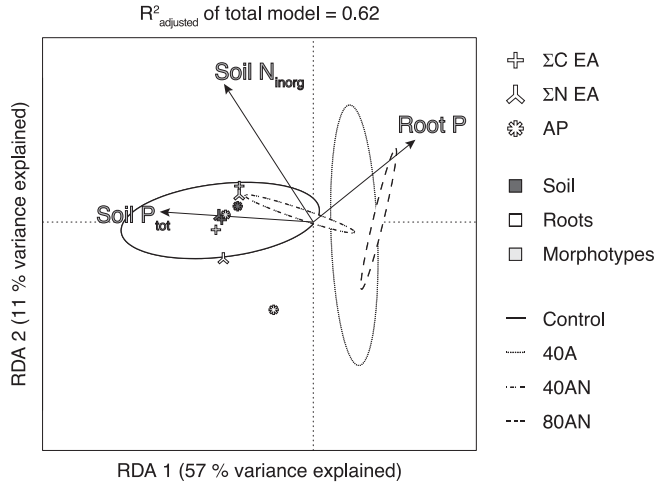

Fig. 5. Redundancy analysis of the specific enzymatic activities of roots, soil and mycorrhizal root tips constrained by stoichiometric values of roots and soil (vector arrows). EA values from roots are shown in white, dark grey depicts soil and light grey mycorrhizal root tip values. ΣC: (β-glucosidase + cellobiohydrolase + β-glucuronidase + β-xylosidase); ΣN = (leucine-aminopeptidase + N- acetylglucosaminidase); AP (acid phosphatase). Ellipses indicate 95% confidence ellipses of the standard error of the site scores.

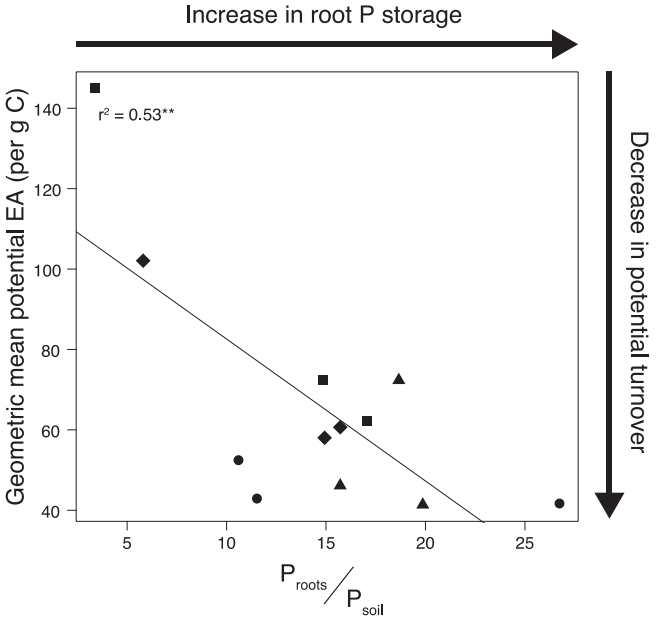

Fig. 6. Correlation between root phosphorus/soil phosphorus ratio and the geometric mean of EA (nmol h^{-1} g^{-1} C). The arrows indicate the shift from lower to higher root P values compared to soil P values and the decrease in potential turnover, respectively. Control plots are marked by squares, 40A plots by triangles, 40AN plots by diamonds and 80AN by circles.

3.2. Mycorrhizal root tip abundance and contribution to EA

There were no significant differences of roots or total mycorrhizal root tips in contribution to total weight of the bulk sample in any treatment (Pairwise Welch's *t*-test, > 0.05, n = 3, Bonferroni - Holm correction). As the sampling of the four dominant mycorrhizal root tips was exhaustive, the relative contribution of soil, roots and mycorrhizal root tips to the total sample weight could be calculated and is shown in Fig. 1 (left, note the logarithmic scaling of the Y axis). Soil accounted for more than 99% of the sample weight, while roots for less than 0.5% and mycorrhizal root tips for less than 0.05%. For the mycorrhizal root tips (Fig. 1, **right**), a significant decrease of all the morphotyes measured was observed in

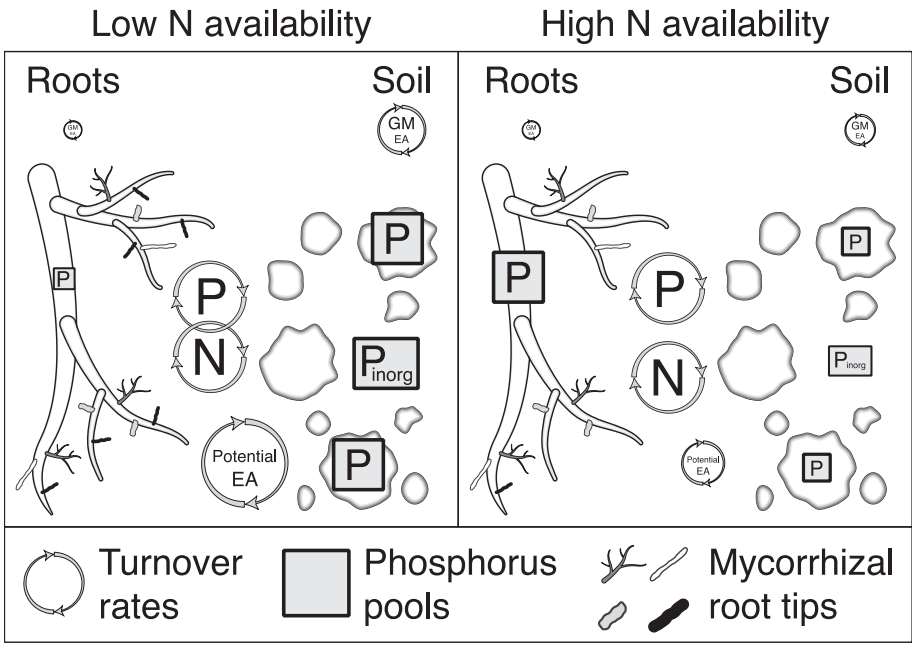

Fig. 7. Simplified model of changes observed at root and soil level before (left) and after (right) N addition. It depicts the decrease in soil phosphorus (P), both for total P and P_{inorg}, at high N availability with a simultaneous increase in root P. Also, while at low N availability N and P turnover are linked, this relationship is not found at high N availability. Systemic specific enzyme activities (EA) are decreased at high N availability, as is the black morphotype (*Cenococcum geophilum*) of mycorrhizal root tips and the geometric mean of soil EA.

Table 1
Parameters measured in soil with and without plant influence, asterisks and points indicate significant differences (Welch's *t*-test, n = 12). Numbers depict mean, standard errors are given in parentheses. Letters indicate significant differences between treatments within each group, highlighted in bold (Pairwise Welch's *t*-test with Bonferroni - Holm correction, n = 3, p < 0.05). Below: Results of a *t*-test between soil with and without plant influence (Welch's *t*-test, n = 3), significant differences are highlighted in bold.

	Treatment	OM (%)	P_{tot} *** ($\mu g\ g^{-1}$)	pH ***	NH_4^+ ($\mu g\ g^{-1}$)	NO_3^- ($\mu g\ g^{-1}$)	P_{inorg} ** ($\mu g\ g^{-1}$)	N:P_{inorg}
Soil with plant influence	Control	7.4 (1.1)	31.6 (16)	5.7 (0.1)	3.2 (0.5)	5.1 (1.3)	**1.8 (0.3)**^{a}	**7.9 (2.7)**^{a}
	40A	6.1 (0.6)	14.9 (2.6)	5.7 (0.1)	5.0 (1.6)	16.3 (4.5)	**0.7 (<0.1)**^{b}	**45.1 (15.2)**^{b}
	40AN	7.0 (0.4)	20.6 (3.5)	6.0 (0.2)	4.9 (0.8)	14.3 (4.8)	**0.9 (0.1)**^{ab}	**26.3 (2.6)**^{ab}
	80AN	7.4 (0.8)	20.2 (4.6)	5.8 (<0.1)	3.8 (0.6)	13.6 (2.5)	**1 (0.2)**^{ab}	**22.5 (7.1)**^{ab}
Soil without plant influence	Control	8.9 (0.5)	8.7 (1.2)	**6.3 (0.1)**^{a}	**2.7 (0.6)**^{a}	**3.1 (0.2)**^{a}	0.7 (0.2)	**14 (1.7)**^{a}
	40A	6.9 (1.9)	8.8 (0.9)	**6.7 (0.1)**^{ab}	**4.0 (0.6)**^{ab}	**6.4 (2.0)**^{a}	0.5 (0.1)	**36.8 (8.8)**^{b}
	40AN	7.9 (2.5)	11.1 (1.0)	**6.5 (<0.1)**^{ab}	**13.3 (2.3)**^{c}	**30.2 (5.6)**^{b}	0.5 (0.1)	**52.3 (8.1)**^{b}
	80AN	9.9 (1.1)	11.3 (1.2)	**7.0 (<0.1)**^{b}	**6.3 (1.0)**^{b}	**44.8 (1.2)**^{b}	0.6 (<0.1)	**74.4 (11.5)**^{b}
Results *t*-test (p-values)								
Results of *t*-test between soil with and without plant influence	Control	0.297	0.148	**0.022**	0.507	0.332	**0.046**	0.228
	40A	0.92	0.085	**0.004**	0.803	0.107	0.287	0.816
	40AN	0.919	**0.042**	0.092	**0.015**	0.124	0.2	**0.039**
	80AN	0.145	0.181	**<0.001**	0.076	**0.018**	0.145	**0.033**

the roots from the 40A treatment. Additionally, *C. geophilum* showed a significant decrease upon N addition in both the 40A and the 40AN treatments compared to the control, with intermediate values in the 80AN treatments.

By combining results of the enzyme activity (EA) analysis per g C and the respective weights from Fig. 1, EA could be expressed per compartment (Fig. 2), which is termed "absolute EA" thereafter. Soil EA accounted for more 85.6% of the absolute EA, while roots accounted for 12% and mycorrhizal root tips for 2.4% (Fig. 2**, left**, note the logarithmic scaling of the Y axis). Absolute EA in the soil and roots of the samples did not change in the treatments and among the mycorrhizal root tips, only *C. geophilum* showed a significant decrease in the treatment plots. While there were no changes observed in absolute EA of the soil, ΣN-related EA (Leucine-aminopeptidase and N-acetylglucosaminidase) was significantly higher in the control than in the 40 AN treatment, with intermediate values in 40A and 80AN treatments (Pairwise Welch's *t*-test, > 0.05, n = 3, Bonferroni - Holm correction). The reduction in absolute EA of *C. geophilum* was mainly related with acid phosphatase (AP) activity (r = 0.88, <0.001, Pearson correlation), which also strongly decreased in all treatments (Pairwise Welch's *t*-test, > 0.01, n = 3, Bonferroni - Holm correction). In contrast to absolute EA values, specific EA values per g C did not show significant differences in the treatments, however, root AP showed a significant decrease in the 40A and 80AN treatments in contrast to the control, with intermediate values for 40AN plots (Table 2). The root AP

Table 2
Differences for mycorrhizal root tips, soil and roots in enzymatic activity of ΣN-related (leucine-aminopeptidase and N-acetylglucosaminidase), ΣC-related (β-glucosidase, cellobiohydrolase, β-glucuronidase and β-xylosidase) and AP (acid phosphatase). Letters indicate significant differences between treatments within each group, highlighted in bold (Pairwise Welch's *t*-test with Bonferroni - Holm correction, n = 3, p < 0.05). Geometric mean is given for all compartments combined, letters indicate significant differences between treatments (Pairwise Welch's *t*-test with Bonferroni - Holm correction, n = 9, p < 0.05).

EA (nmol h^{-1} g^{-1} C)		Control	40A	40AN	80AN
Mycorrhizal	ΣC	220.5 (49.4)	204.3 (52.3)	224.8 (44.7)	166.6 (9.9)
Root	ΣN	225.5 (82.3)	195.5 (57.5)	153.9 (28.3)	142.5 (7.5)
Tips	AP	6817.5 (3112.4)	2867.3 (30.2)	4358.5 (1097)	1675.9 (329.8)
Roots	ΣC	538.2 (215.1)	101.2 (11)	500.6 (367.5)	176.2 (33.8)
	ΣN	376.9 (130.3)	138.5 (68.3)	238.9 (157.1)	138.1 (33.4)
	AP	**5771.1 (1075.8)**[a]	**1796.7 (364.5)**[b]	**2633 (643.3)**[ab]	**1688.7 (319.2)**[b]
Soil	ΣC	244.6 (98.5)	100 (31.4)	216.1 (81)	100.2 (24.2)
	ΣN	218 (87.9)	115.8 (38.7)	128.3 (19.1)	111.5 (34.2)
	AP	1924.1 (487.2)	1879.4 (758.4)	1675.3 (146.9)	1347.1 (598.7)
Geometric mean		**121.6 (26.9)**[a]	**52.4 (8)**[b]	**87.2 (21.5)**[ab]	**47.7 (4.8)**[b]

activities are also highly related with the geometric mean of the whole soil-root system (r = 0.8, <0.01, Pearson correlation).

3.3. EA correlations and redundancy analysis

The enzyme activity (EA) analysis revealed a strong correlation (Fig. 3) between ΣC-related (β-glucosidase, cellobiohydrolase, β-glucuronidase and β-xylosidase) and ΣN-related EA (leucine-aminopeptidase and N-acetylglucosaminidase). Using an ANCOVA, no significant interaction was found between treatment and the relation between ΣC-related and ΣN-related EA (F = 0.9, >0.1, df = 3). In contrast, there was a strong interaction between acid phosphatase (AP), ΣN-related EA and treatments (F = 4.91, <0.01, df = 3) (Fig. 4). While in the control a strong correlation between AP and ΣN-related EA was found, this correlation decreased in the 40A treatment and was lost in the 40AN and 80AN treatments.

In order to relate soil nutrient contents (Table 1, soil with plant influence), plant and soil stoichiometry as well as isotopic ratios (Supplementary Table 1) with EA patterns, a redundancy analysis was performed on the correlation matrix of the specific EA values that were used in Figs. 2, 3 and 4. After selecting a parsimonious set of variables (see 2.7) only 3 variables (soil N_{tot}, soil P_{tot}, roots P_{tot}) were left from the original 10 (OM, soil N_{tot}, soil P_{tot}, soil N:P_{tot}, soil N_{inorg}, soil P_{inorg}, soil ^{13}C, roots N:P_{tot}, roots N_{tot}, roots P_{tot}). This constrained ordination had an adjusted R^2 = 0.62 (Fig. 5) and its first two axes RDA1 and RDA2 explained 68% of the total variance and 93% of the fitted variance. Using permutation tests, the constrained model was significantly different from an unconstrained model by < 0.01 and both RDA1 (explaining 57% of the total variance) and RDA2 (explaining 11% of the total variance) were found to be significant (<0.05). RDA1 was mainly negatively associated with soil P_{tot} values and to a lesser extend positively with root P_{tot}, while RDA2 was mainly associated with soil N_{tot}. The 95% confidence intervals of control and treatments indicate a gradient of EA along RDA1, with the control showing the highest EA, being associated with higher soil P_{tot} and lower root P_{tot} concentrations. The treatment closest to the control was 40AN, followed by the treatments receiving more ammonium, which are the 40A and 80AN, which show lower EA. Both 40A and 80AN are aligned along the RDA2 axis, which indicates a larger variation in soil N_{tot} levels in these treatments, especially in the 40A treatment, which in both cases is also associated with a larger variation in soil AP activity. The RDA1 axis also showed a very strong correlation with the total geometric mean of EA per g^{-1} C (r^2 = 0.98, <0.001, F = 794.5, df = 10). As RDA1 was related with both soil and root P_{tot}, the ratio of P_{tot} concentration in the roots vs. P_{tot} concentration in the soil was plotted against the total geometric mean of EA per g^{-1} C as a general indicator for microbial activity (Fig. 6), which showed a strongly negative relationship.

4. Discussion

Soil microorganisms, roots and mycorrhizal root tips are involved in the degradation of organic matter (OM), which is a process needed for both N and P acquisition. As many ecosystems are co-limited by these nutrients, increased N input without simultaneous P input might lead to an exacerbation of P limitation (Peñuelas et al., 2012). Also, increased N input has been shown to have detrimental impacts on many ECM fungi (Lilleskov et al., 2011), which could have secondary effects on soil functioning. Thus, it becomes more and more clear that nutrient limitations are difficult to assess only on plant level and that soil N:P stoichiometry is an important variable to consider for ecosystem nutrient imbalance (Cleveland and Liptzin, 2007). The work presented here aims to add further evidence to human induced N:P imbalance by observing plant-soil interactions in a medium term N-fertilization experiment in a Mediterranean ecosystem.

4.1. Effect of N additions on soil with and without plant influence

Strong spatial heterogeneity of soil surface characteristics is a known feature of Mediterranean soils (Lepart and Debussche, 1992). As these systems are highly resource limited through biological activity (Thompson, 2005), especially the spatial heterogeneity additionally imposed by plant cover is significant (Cruz et al., 2008). In the system observed here, this heterogeneity manifested in higher P_{tot} and P_{inorg} levels in soil with plant influence compared to soil without plant influence (Table 1). While these changes corroborate findings of P_{inorg} increase under the canopy of *C. ladanifer* growing under natural conditions (Rolo et al., 2012), the same authors suggest that the accumulated P is not available to neighbouring plants. They argue that this occurs either due to soil water depletion by *C. ladanifer*, or due to low OM decomposition rates, which could both be the case in the study presented here, as sampling took place in summer (July). Also, while it is known that the plants accumulate N derived from fertilization over the summer months due to low physiological activity (Dias et al., 2012), root P_{tot} values found here (Supplementary Table 1) were very low compared with *C. ladanifer* plants grown in soil with higher soil P concentrations (Kidd et al., 2007). Indeed, soil from treatment plots exhibit similar P_{inorg} values with and without plant influence, indicating decreased P availability in plant proximity by the N addition. Similarly to plant N, it is known that N_{inorg} in the bare soil exhibits a strong increase in the summer months, especially in the 40AN and 80AN treatments (Dias et al., 2012). As a result, N:P_{inorg} was significantly higher in the treatments, indicating a strong P_{inorg}

depletion both in the soil with and without plant influence.

4.2. Changes in mycorrhizal root tip abundance and EA

As hypothesized, we found strong changes in the percentage of mycorrhizal root tips from *Cistus ladanifer* plants growing in the 40A plots (Fig. 1**, right**), which was most likely related to the low ammonium tolerance observed in *Cistus* (Dias et al., 2015). However, *C. ladanifer* has also been shown to exhibit decreased growth in the 80AN plots (Dias et al., 2011), which was not translated into a loss of mycorrhizal root tips. A possible explanation for this inconsistency is the ability of ECM to form a symbiosis with several hosts simultaneously, e.g. *Cistus* spp. and evergreen late successional species (Martín-Pinto et al., 2006). Plant cover in general decreases in 40A plots (Dias et al., 2014), which might be related with decreasing host C input belowground and thus increasing pressure on the ECM community. On the contrary, late successional evergreen sclerophyllous species benefit from the ammonium-nitrate addition (Dias et al., 2013), which might give the ECM fungi a range of hosts to associate with, thus maintaining high colonization levels even in plants being more affected by the N treatments, such as *C. ladanifer*.

Contrary to the other morphotypes, the abundance of the black morphotype, which we identified putatively as *C. geophilum* using morphological characteristics, diminished strongly upon addition of N (Fig. 1), which makes it an interesting candidate for further evaluation. This fungus is known to be extremely abundant worldwide with a wide range of hosts and has been found in nearly all studies conducted about ECM fungi (Horton and Bruns, 2001). It has been implicated in drought stress resistance due to its high degree of melanin production (Fernandez and Koide, 2013), which makes it an important species in all successional stages of Mediterranean landscapes (Buscardo et al., 2010). While it has not been found in a previous study concerning *Cistus* ECM symbionts, the authors (Comandini et al., 2006) did not rule out its possible association with *Cistus* spp. and it was also found to be abundant in sites dominated by *C. ladanifer* (Buscardo et al., 2010).

The strong decrease of *C. geophilum*, both in abundance and EA per compartment (Fig. 2), was somewhat unexpected, as generalist species such as *C. geophilum* tend to show low changes in their enzyme profiles upon receiving stress (Herzog et al., 2013). However, it was reported that the interaction of drought and N addition might have a detrimental effect on *C. geophilum* (Nilsen et al., 1998), and Kjøller et al. (2012) suggested that *C. geophilum* prefers low N sites. Also, it is known that *C. geophilum* stores both N and P in the mantel hyphae, which raised the hypothesis that phosphate is needed as a compensating anion (Kottke et al., 1995). As mentioned in section 3.2, the decrease in absolute EA of *C. geophilum* was mainly related with AP activity, and as P_{inorg} levels are lower around plants growing in plots with N addition (Table 1), this compensation could be diminished, eventually leading to decreased growth. Additionally, *C. geophilum* is a short distance exploration type (Agerer, 2001), which are ECM types that in general lack strong hydrolytic capacities and require to scavenge for soluble and labile nutrients (Alvarez et al., 2012). As ECM accumulate polyphosphates in the mantel hyphae of hartig nets (Cairney, 2011), it could be argued that AP in the mycorrhizal root tips zone might thus be more related with the transfer of P to the plant host by hydrolysis of polyphosphates (Werner et al., 2007; Alvarez et al., 2012) than with the hydrolysis of P_{org} from soil OM. Interestingly, a putative consequence of the decline in *C. geophilum* abundance might be increased fungal litter with high melanin content, which could decrease nutrient turnover due to its strong recalcitrance and its ability for EA inhibition (Fernandez et al., 2016). Also, *C. geophilum* tends to produce large amounts of extramatrical mycelia and sclerotia (Fernandez et al., 2013), which may make up long lasting fungal necromass in the soil.

4.3. Absolute EA and changes in soil and root EAs

Using the dry weights of each compartment (soil, roots and mycorrhizal root tips) and their respective EA per g C, the contribution of each compartment to absolute EA per sample could be estimated (Fig. 2). While no differences between treatments were found, except for a strong decrease in absolute EA of *C. geophilum* (see section 4.2), the values obtained give an interesting insight into the importance of each compartment *in situ*, as they estimate the potential flux in the soil-root system. Using a volume-based procedure, other authors (Meier et al., 2015), also found no response of whole compartments to 6 years of N addition and similarly to the results obtained here, they determined soil EA as the main contributor to EAs, followed by rhizosphere and hyphosphere.

While the whole root and soil compartments show no significant changes in the treatments, AP decreased substantially on the root surface (Table 2) and was positively correlated with P_{inorg} and P_{tot} in the soil with plant influence ($r = 0.69$ and $r = 0.66$, respectively, $p < 0.05$, Pearson correlation). This is a situation normally associated with non fertilized soil (Olander and Vitousek, 2000) and in contrast to a comparable Mediterranean based N addition experiment with a shorter span (3 years), where these changes were not found for root phosphatase and the correlation between available P and AP activities was negative (Ochoa-Hueso and Stevens, 2015). Indeed, the general consensus is that N addition leads to an increase in phosphatase activities in roots and soil among various types of ecosystems (Marklein and Houlton, 2012), however, in some cases either no or a negative response was found for root surface AP activity (e.g. Johnson et al., 2005). These exceptions are not contradictory to the general model, as they might be explained by a decreased plant P demand (Johnson et al., 2005), which, in the case of *C. ladanifer*, could be due to lower plant growth as a consequence of ammonium toxicity (Dias et al., 2015). Also, low root AP activities might be a function of localized P_{tot} depletion in the rhizosphere, as it is known that rhizosphere distance is an important variable to consider for EAs (Tarafdar and Jungk, 1987). Indeed, while there was no significant difference of soil P_{tot} between control and treatment plots, there was a tendency for lower P_{tot} in soil with plant influence in the treatments (see section 4.1), which might indicate the onset of P depletion around the plant. Also, as P_{inorg} levels were generally very low, product inhibition by increased P_{inorg} is implausible (Chen, 2003) and higher P_{inorg} levels as seen in the control plots are likely to be the result of high AP activities, thus highlighting the importance of substrate availability vs. feedback inhibition by P_{inorg}.

Apart from the loss of AP at the root surface, also N related EA in the soil compartment decreases (section 3.2). Decreases in N related EA upon N treatment were reported before for litter (Sinsabaugh et al., 2002) and soil (Saiya-Cork et al., 2002). N related EAs, such as N-acetylglucosaminidase, are known to be indicators of fungal presence in the soil (Miller et al., 1998) and as 82% of the N related EA determined in this study was derived from N-acetylglucosaminidase, a decrease in N related EA might point to a suppression of fungal activity by N addition, resulting in reduced OM decomposition capabilities (Ramirez et al., 2012). In the system observed here, plant community composition changed with N addition, resulting in the input of low quality plant tissue with delayed OM decomposition (Dias et al., 2013). This might be a common occurrence in N fertilized systems, due to the suppression of microbial N mining, which is tightly coupled with OM decomposition (Craine et al., 2007).

4.4. The decoupling of N and P cycles and P concentrations as putative indicators of N:P imbalance

Coupling of EAs is a phenomenon observed over a wide variety of ecosystems and is due to the strong stoichiometric constraints of microorganisms in nutrient acquisition (Sinsabaugh et al., 2009). Indeed, the decoupling of N and P occurs only if one of the nutrients is limiting and processed faster than the other, for example because of time lags in nutrient uptake or due to nutrient storage limits (Appling and Heffernan, 2014). Thus, plant P uptake can be decoupled by N addition, but also from C and P mineralization itself (Kritzler and Johnson, 2010). However, N and C related EAs were highly correlated across the different compartments and treatments (Fig. 3), while on the contrary, we found that N and P cycles decoupled in the N treatments (Fig. 4), and as high soil N:P_{inorg} ratios in the treatments indicate (Table 1), there was a significantly higher N availability compared to P availability. While we did not measure the abundance of fungal types in the soil, the strong relationship between C and N cycles might be attributed to increasing numbers of saprotrophs, as N addition favours saprotrophs and supresses ECM fungi (Högberg et al., 2003), which degrade OM mainly for N acquisition (Lindahl and Tunlid, 2015). On the contrary, a direct link between *C. album* ECM symbionts and P status of the plant was evidenced in the redundancy analysis (RDA), which revealed a shift from higher soil P_{tot} levels in the control plots to higher root P levels upon N addition (Fig. 5). High soil P_{tot} and low root P values, as found in the control, were correlated with higher EAs of mycorrhizal root tips and soil, indicating that higher soil P is connected with higher microbial turnover. This was also further exhibited by changes in soil quality, expressed as the geometric mean of EA per g C (García-Ruiz et al., 2008), which was negatively correlated to the ratio between root and soil P (Fig. 6), emphasizing P status as an important factor for the plants' investment in symbiotic OM degradation. The decoupling of AP and N related EAs could thus be due to changes in the microbial community from an oligotrophic to a copiotrophic status (Ramirez et al., 2012), which does not necessarily translate in lower turnover *per se*, but implies changes in OM chemistry and degradability (Grandy et al., 2008), selecting for microorganisms more related to labile C and N pools (Lilleskov et al., 2011). The decoupling of N and P cycles found here might thus be the first signs of negative effects on parts of the ECM community and a decrease of ECM fungi would have long-term detrimental consequences not only on *C. ladanifer* itself, but also on the plant community structure as a whole, as *C. ladanifer* serves as a bridge between early and late successional ECM symbionts (Martín-Pinto et al., 2006).

5. Conclusions

This work explored the effects of medium term (6 years) N additions on the ECM fungal community, the root tips and the soil surrounding an abundant Mediterranean ECM plant (*C. ladanifer*). As hypothesized, there was a negative effect of NH_4^+ on mycorrhizal colonization, as well as a decrease of root AP activity in the treatments. This was linked to high N:P_{inorg} ratios and thus probably caused by a limitation in rhizospheric P availability. Also, *C. geophilum* exhibited a strong reduction in root colonization and EA, similarly to the effects seen on roots AP, thus highlighting this species as an interesting candidate for further research. Additionally, we found a general decrease in N related activity in the soil as well as a decoupling of N and P cycles, but not N and C cycle. The strong coupling between EA and plant P vs. soil P status was posed as a further indicator for the regulation of the microbial turnover in the soil by the plant host. We hypothesize that the decoupling of N and P turnover might be related to the onset of change in microbial community from ECM fungi-dominated to a more copiotrophic, saprotrophic fungal composition. While we found first evidence of N:P cycle disruption and changes in the dominant mycorrhizal root tip morphotypes, further work needs to address microbial changes in the soil and also to describe in more detail the nature of the OM found after several years of N addition. Mediterranean ecosystems are expected to experience anthropogenic N addition to a great extend in the near future, and as these systems are often co-limited by N and P, this work is an important contribution to gain insight into changes in both N and P turnover as interacting nutrient cycles.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.05.028.

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