Title: Soil nutrients and microbial biomass in three contrasting Mediterranean forests

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ABSTRACT

Aims: The extent to which the spatial and temporal patterns of soil microbial and available nutrient pools hold across different Mediterranean forest types is unclear impeding the generalization needed to consolidate our understanding on Mediterranean ecosystems functioning.

Methods: We explored the response of soil microbial, total, organic and inorganic extractable nutrient pools (C, N and P) to common sources of variability, namely habitat (tree cover), soil depth and season (summer drought), in three contrasting Mediterranean forest types: a *Quercus ilex* open woodland, a mixed *Q. suber* and *Q. canariensis* woodland and a *Pinus sylvestris* forest.

Results: Soil microbial and available nutrient pools were larger beneath tree cover than in open areas in both oak woodlands whereas the opposite trend was found in the pine forest. The greatest differences in soil properties between habitat types were found in the open woodland. Season (drought effect) was the main driver of variability in the pine forest and was related to a loss of microbial nutrients (up to 75% loss of N_{mic} and P_{mic}) and an increase in microbial ratios (C_{mic}/N_{mic}, C_{mic}/P_{mic}) from Spring to Summer in all sites. Nutrient pools consistently decreased with soil depth, with microbial C, N and P in the top soil being up to 208%, 215% and 274% larger than in the deeper soil respectively.

Conclusions: Similar patterns of variation emerged in relation to season and soil depth across the three forest types whereas the direction and magnitude of the habitat (tree cover) effect was site-dependent, possibly related to the differences in tree species composition and forest structure, and thus in the quality and distribution of the litter input.

Keywords: soil fertility, plant-soil interactions, soil carbon, nitrogen, phosphorus

INTRODUCTION

Soil microorganisms, in their role in organic matter decomposition, have the capacity to both mineralize and immobilize nutrients (Singh et al. 1989) thereby influencing soil nutrient availability and plant growth (Lambers et al. 1998). Spatial and temporal changes in soil microbial biomass may determine the patterns of availability of limiting nutrients such nitrogen (N) and phosphorus (P), thus having profound influence on plant communities and ecosystem functioning (Ettema and Wardle 2002; Gallardo and Schlesinger 1994; Sardans et al. 2005; van der Putten et al. 2009).

Spatial and temporal variations of soil microbial biomass and activity are related to different biotic and abiotic factors that modulate the temperature, moisture conditions and substrate quality and availability. For instance, vegetation composition and structure control the spatial distribution, quality and quantity of nutrients inputs *via* litter and root exudates (Aponte et al. 2011; Huang et al. 2013; Prescott and Grayston 2013; Ushio et al. 2010). Soil nutrients and microbial activity usually decrease as soil depth increase due to a decline in the quality and quantity of organic matter (Gaudinski et al. 2000; Xiang et al. 2008). Seasonal changes in temperature, water and substrate availability also have a large impact on soil microbial activity and nutrient cycling (Corre et al. 2002; Quilchano and Marañón 2002; Schmidt et al. 1999). In highly seasonal ecosystems, such as Mediterranean forest, the effects imposed by seasonal variations, in particular associated to the summer drought, are especially important for ecosystem functioning (Aponte et al. 2010b; Marañón-Jiménez et al. 2011; Matías et al. 2011).

Many studies have described soil nutrient heterogeneity in Mediterranean forests; however, most of them have been conducted at local spatial scales, focused on a single forest type (Barcenas-Moreno et al. 2011; Carreira et al. 1994; Gallardo 2003; García et al. 2006; Maltez-Mouro et al. 2005; Monokrousos et al. 2004). The detection of general patterns across different forest types is necessary to fully understand microbial biomass and nutrients dynamics and their consequences for plant community. At the same time, the emergence of site-dependent effects will be of interest from a modelling and management perspective, to properly determine nutrient pools at wide geographical scales including a mosaic of forest types. Patterns of microbial biomass and nutrient heterogeneity across different forest types have been largely investigated in temperate, boreal and tropical forest (e.g. Hackl et al. 2005; Lindo and Visser 2003; Liu et al. 2012; Zhong and Makeschin 2006), while remain far less studied in Mediterranean forest (but see García et al. 2002; Goberna et al. 2006). This coordinated study addressed this knowledge gap and aimed to evaluate whether the effects of main sources of variability, namely habitat (i.e. tree cover), soil depth and season, in the soil nutrients and microbial C, N and P pools could be generalised across three contrasting Mediterranean forests: a *Quercus ilex* open woodland, a mixed *Q. suber* and *Q. canariensis* woodland and a *Pinus sylvestris* forest. While this study builds upon previous knowledge on soil nutrient heterogeneity at local scales (Aponte et al. 2010b; Matías et al. 2011), it focuses on the comparison among forests with different structure and species composition, thus taking a step forward towards understanding general patterns of soil microbial responses to biotic and abiotic environmental drivers. Explicitly, we aimed to answer the following questions: 1) Is there a common pattern across the three forests in relation to the tree effect, soil depth and seasonal drought? ; 2) Are the interactions between the effects of tree cover, soil depth and season (summer drought) similar across forest types?; 3) What is the quantitative importance of the studied factors (tree effect, soil depth and seasonal drought) on the soil and microbial variables in each forest type? 4) Do the relationships between microbial and soil chemical properties hold when examined across forest types?

METHODS

Study areas

The study was conducted in three different Mediterranean forest types: a mixed woodland of *Quercus suber* L. (evergreen) and *Q. canariensis* Willd. (deciduous) in Los Alcornocales Natural Park in the extreme south, near the Strait of Gibraltar, an open woodland dominated by the sclerophyllous *Quercus ilex* subsp. *ballota* L. and eventually mixed with other *Quercus* species (*Q. suber*, *Q. pyrenaica* Willd., *Q. faginea* Lam.) in Sierra de Cardeña and Montoro Natural Park (Cardeña), in the south mainland, and a forest mainly comprised of *Pinus sylvestris* L. interspersed with *Q. ilex* subsp. *ballota* in Sierra Nevada National Park in the southeast of Spain (Fig. 1). In all three forest types, the main tree species are intermingled with open areas covered by sparse herbaceous vegetation. The study sites vary in altitude, climate and soil conditions (Table 1). The general climate of the three sites is Mediterranean-type, characterized by hot and dry summers, and cold and wet winters with most rainfall occurring from October to May. The sites in Cardeña and Sierra Nevada experience more extreme temperatures due to their continental and altitudinal locations (respectively), while temperatures in Alcornocales site are milder due to the lower elevation and proximity to the Mediterranean Sea and Atlantic Ocean. Mean annual rainfall follows a rising gradient from Cardeña to Alcornocales (Table 1). The sites in Alcornocales and Cardeña stand on a bedrock of sandstone and granite, both producing acidic sandy soils. On the contrary the site in Sierra Nevada stands on limestone, which gives rise to basic loamy soils. Cambisols dominated in Alcornocales and regosols in Cardeña (nomenclature follows WRB 2006), indicating a greater soil development i.e. soil depth, structure, water holding capacity and chemical fertility in the former than the later.

Experimental design

At each forest site 10-20 replicates (depending on the site, Table 1) of two main habitat types were identified within a stand: beneath the canopy of the dominant tree species (*Q. suber* and *Q. canariensis* in Alcornocales, *Q. ilex* in Cardeña and *P. sylvestris* in Sierra Nevada), and in open areas with bare soil or sparse herbaceous cover and no tree cover. These habitat types will be referred as 'Tree' and 'Open' respectively hereafter. At each replicate point, four soil cores (0-16 cm) were extracted using an auger after removing the litter layer, divided between 'Top soil' (0-8 cm) and 'Deeper soil'(8-16 cm) and homogenized within the same depth to obtain a composite soil sample per habitat type replicate and depth. Soil samples were taken in Spring (May-June) and Summer (August-September) 2007, coinciding with the moment of maximum soil biological activity and maximum water stress in soil, respectively. In total 400 soil samples were taken corresponding to 10-20 replicates (Table 1) of 2 habitat types x 2 soil depths x 2 seasons x 3 forest sites. Litter, i.e. dead plant material relatively undecomposed standing on the ground, was collected once in all sampling points using a 10 x 10 cm quadrat (in Sierra Nevada) or a 30 x 30 cm quadrat (in Alcornocales and Cardeña). Litter samples were oven-dried at 60ºC for 72 h and weighted.

Laboratory analyses

Soil samples were brought to the laboratory in an ice-box, fresh-sieved at 2 mm removing stones, roots and other recognizable plant parts and stored at 4ºC for analyses. Water content was determined on a subsample as the difference in weight between fresh and oven dried (105ºC) soil.

Microbial C, N and P were estimated in fresh soils using a chloroform fumigation-extraction procedure (Brookes et al. 1985; Brookes et al. 1982; Vance et al. 1987). Dissolved organic C (DOC) and N (DON) and inorganic P (P_{inorg}) were determined in non-fumigated and chloroform fumigated soil subsamples (24h). Dissolved C and N were extracted with 0.5M K_2S0_4, and their concentration was determined using a Shimadzu TOC-V CSH analyzer. Inorganic P was extracted with either 0.025N HCl+0.03N NH_4F (Bray Kurtz 1 method (Bray and Kurtz 1945) for the acidic soils of Alcornocales and Cardeña) or 0.5M $NaHCO_3$ (Olsen method (Olsen et al. 1954) for the basic soils of Sierra Nevada) and its concentration was determined by colorimetry using the ascorbic acid-molybdenum blue method (Sparks 1996). Microbial C (C_{mic}), N (N_{mic}) and P (P_{mic}) were estimated as the difference in DOC, DON and P between fumigated and non-fumigated samples.

Inorganic nitrogen (N_{inorg}) was extracted from non-fumigated soils using 2M KCl and the extracts were analyzed for NH_4^+ and NO_3^- by the Kjeldhal method (Bremner and Keeney 1965). Soil total C (C_{tot}) and N (N_{tot}) were determined on oven dried soils by combustion at 850ºC (Leco TruSpec autoanalyzer) and total inorganic C (C_{inorg}) was measured by acidification with $HClO_4$ in a TIC analyzer (UIC CM-5014). The difference between C_{tot} and C_{inorg} gave the total organic C (C_{org}).

Data analysis

Differences among habitat, soil depth and season were analyzed using repeated measurement ANOVAs with season as a within-group effect and habitat and depth as between-group effects. Forest site was also included in the analysis to test for potential interactions with the studied factors. Variables were transformed (log, arcsin) when necessary to meet normality assumptions. To control the type I error inflation resulted from repeated tests, the false discovery rate (FDR), i.e. the expected proportion of tests erroneously declared as significant, was controlled at 5% using a step-up procedure (Benjamini and Hochberg 1995; García 2003). The percentage of the total variance explained by the studied factors (habitat, soil depth and season) was calculated for each variable and site using a repeated measurement ANOVA with no interactions. Patterns in pairwise Pearson's correlations between microbial and soil nutrient factions were explored using correlation network analysis (R package igraph, Csardi and Nepusz 2006). Multivariate relationships between variables were analysed using Principal Component Analysis (PCA). The 'Broken stick' method (King and Jackson 1999) was used to select significant components. Habitat, soil depth and season were included in the PCA as supplementary variables, i.e. these factors did not participate in the analysis, but were projected on the multivariate space generated by the PCA for the purpose of interpretation.

RESULTS

Overall, the study forests differed in all the analyzed soil and microbial properties (Table 2 and 3). Cardeña was the least fertile site while Alcornocales had the largest fraction of microbial nutrients (from 3 to 6-fold the values of the other sites) and the largest pool of total and dissolved C and N and organic C (Fig. 2 and 3). Sierra Nevada showed the highest inorganic N and P values (~2-fold to 8-fold the values of the other sites), the highest C_{mic}/N_{mic} ratio (2-fold) and the largest litter pool (~6-fold) (Fig. 3). The ratios of nutrients retained in the microbial biomass *vs.* the pool of available nutrients (N_{mic}/N_{inorg} and P_{mic}/P_{inorg}) as well as the fraction of soil organic carbon and total nitrogen in the microbial biomass (C_{mic}/C_{org} and N_{mic}/N_{tot}) were the highest in Alcornocales and Cardeña and the lowest in Sierra Nevada (Online Resource 1).

Effect of habitat

Soil parameters differed significantly between the two habitat types in all forest sites (Table 3, Fig. 2 and 3). However, the magnitude and direction of those differences varied across sites, as the interaction Site × Habitat was significant for most of the variables (Table 3). In both oak woodlands, Alcornocales and Cardeña, the nutrient pools (microbial, dissolved organic and inorganic) tended to be larger beneath tree canopy than in open areas with the exception of nitrate that showed the opposite trend (Fig. 2 and 3). Greater concentrations of ammonium (156% in both sites), phosphate (120% in Alcornocales and 182% in Cardeña), and microbial nutrients (123 and 166% for C_{mic}; 126 and 227% for N_{mic}; 215 and 175% for P_{mic}) were found beneath tree cover than in open areas. Mean soil organic carbon was also greater beneath tree cover than in open areas in Cardeña (1.7% *vs.* 0.97%; $P<0.0001$) and Alcornocales (4.1% *vs.* 3.6%, not significant difference). A different pattern was observed in the pine forest (Sierra Nevada) where most of the soil nutrient pools were similar between the two habitats or even decreased beneath trees as it occurred with N_{mic} and inorganic N and P (Fig. 2 and 3). Organic and inorganic C also decreased significantly from open areas (3.1%, and 2.85% respectively) to beneath pine tree cover (2.9% and 0.93% respectively). Nevertheless, the amount of litter was larger beneath tree canopy than in open areas in all sites, being the values larger in Sierra Nevada than in the other two forests (Table 1). There were no habitat differences in the fractions of microbial values relative to soil pools (N_{mic}/N_{inorg}, P_{mic}/P_{inorg}, C_{mic}/C_{org} and N_{mic}/N_{tot} ; data not shown).

Effect of soil depth

In general all variables measured showed a consistent pattern with soil depth in the three forest sites, with values decreasing from Top soil to Deeper soil (Fig. 2 and 3). However, there was a significant Site × Depth interaction (Table 3) due to the lack of statistical significance of soil depth for many variables in Cardeña (C_{mic}, P_{mic}, DON, NH_4 and P_{inorg}) (Fig. 2 and 3).

Microbial C, N and P in Top soil were higher than in Deeper soil with the largest variations found in Alcornocales (208, 215 and 274% respectively) and the smallest changes found in Cardeña (128, 155 and 119%) (Fig. 2). On average across sites, the pool of inorganic N and P, DOC, DON and C_{org} was 133% (site mean range 110 – 146%), 155% (129 – 177%), 142% (117-172%), 140% (112-159%) and 118% (114-120%) higher in Top soil than in Deeper soil respectively. As with microbial pools, variation was the least in Cardeña (Fig. 2 and 3). Microbial ratios C_{mic}/C_{org} and N_{mic}/N_{tot} showed the largest decrease with soil depth in Alcornocales but remained constant in Cardeña. The ratio of microbial biomass nutrients (C_{mic}/N_{mic} and C_{mic}/P_{mic}) showed no significant variation from Top soil to Deeper soil in any site. The only exception was found for soils in open areas in Cardeña where C_{mic}/P_{mic} increased with soil depth from 55 to 143, as evidenced by a significant Site × Habitat × Depth interaction (Table 3).

Effect of season

Soil microbial fractions and nutrient pools varied significantly with the season. However, the seasonal patterns of variation were site-dependent as indicated by a significant Site × Season (Table 3). Seasonal variations were stronger in Sierra Nevada and Alcornocales whereas Cardeña showed the lowest variability between seasons (Fig. 2 and 3). In general, microbial pools were larger in Spring than in Summer, particularly for N_{mic} and P_{mic} which values were on average 237% and 258% higher in Spring (Fig 2). The fraction of microbial C and N relative to soil total pools (C_{mic}/C_{org} and N_{mic}/N_{tot}) decreased from Spring to Summer in Sierra Nevada but not in Cardeña. Microbial ratios (C_{mic}/N_{mic}, C_{mic}/P_{mic}) increased from Spring to Summer in all sites revealing a larger loss of N_{mic} and P_{mic} as compared to C_{mic}.

The seasonal variability of N_{mic}, P_{mic}, C_{org}, DOC and DON was larger in soils beneath tree canopy whereas the variation was subdued in the open habitats (Season × Habitat interaction, Table 3). We also found a strong and significant Site × Season interaction for N_{inorg} and P_{inorg} (Table 3), which was due to opposite seasonal changes across forest types. For example, the pool of available inorganic nutrients (ammonium and phosphate) as well as DON increased from Spring to Summer in Cardeña and Sierra Nevada, whereas the values decreased in Alcornocales (Fig. 3). Despite the discrepancies in the seasonal dynamics of P_{inorg}, the proportion of P_{mic} relative to P_{inorg} was higher in Spring than in Summer in all sites (data not shown). The observed seasonal patterns were similar at the two soil depths.

Variance partitioning among habitat, soil depth and season

As shown in the partition of variance (Fig. 4) and the principal component analysis (Fig. 5) the main drivers of variability differed between sites. Soil depth and season accounted for the largest part of the variability observed in the microbial and soil nutrient pools in Alcornocales and Sierra Nevada. For instance, in

Alcornocales soil depth explained 50, 38 and 30% of the variation of microbial C, N and P respectively and season explained 55 and 39% of the variation of N_{inorg} and $P_{inorg.}$. In Sierra Nevada season was the main driver of microbial variability accounting for 26, 58 and 66% of the variation of microbial C, N and P. In contrast, the variability of soil biotic and abiotic properties in Cardeña was mainly driven by habitat type, which explained 10, 16 and 4% of microbial C, N and P variation respectively and 9 and 12% of N_{inorg} and P_{inorg} variation.

Relations between microbial pools and soil properties

Soil microbial C, N and P were significantly correlated among them in all sites (Fig. 5 and Online Resource 1). Microbial C and N were consistently and strongly coupled (r >0.76 in all sites), whereas P_{mic} was more weakly but still significantly related with C_{mic} (from r = 0.28 in Cardeña to r = 0.69 in Sierra Nevada) and N_{mic} (from r = 0.30 in Cardeña to r = 0.82 in Sierra Nevada). Microbial C, N and P were positively related to most of the measured soil properties in each site (Fig 5). The strongest correlations were found with C_{org}, N_{tot} and soil moisture reflecting microbial biomass dependence on substrate and water availability. Microbial C also showed a significant correlation with P_{inorg} in all sites (r ~0.36). The relationship between litter and C_{org} (r_C) and N_{tot} (r_N) varied across forest sites, being positive in Cardeña ($r_C = 0.43$, $r_N = 0.35$, $P < 0.0001$, seasons and depths pooled), positive in Top soil in Alcornocales ($r_C = 0.28$, $r_N = 0.27$, $P < 0.05$; not significant in Deeper soil) and negative in Sierra Nevada ($r_C = -0.16$, $P < 0.06$; $r_N = -0.36$, $P < 0.0001$). The correlation network was the strongest in Alcornocales, i.e. there was a tight coupling between most variables, and the weakest in Cardeña (Online Resource 1).

The multivariate analyses (PCAs) showed similar patterns of covariation among the nutrient pools for all sites (Fig 5). Two main significant gradients (axes) emerged for each PCA from the analysis based on the 'Broken –stick' method (King and Jackson 1999). For all sites the first axis was strongly correlated to microbial C, N and P, total N and organic C. In Alcornocales and Sierra Nevada the firts axis was also positively related to soil moisture and negatively related to C_{mic}/N_{mic}. The separation of samples along the main axis and the analysis of the supplementary variables indicated that both season and soil depth imposed a similar degree of variability in Alcornocales whereas season was the main driver of variation in Sierra Nevada, which is agreement with our variance partitioning analysis. In Cardeña the first axis was related to litter amount, but not to soil moisture, and separated the samples by habitat type. The second axis in all PCAs was related to the availability of inorganic nutrients (N and/or P), which covariation with other variables was inconsistent across forest types. Higher microbial ratios (C_{mic}/N_{mic}) were consistently associated to lower soil moisture and Summer samples in all sites.

The relationship between litter abundance and microbial and total nutrient pools was positive in Alcornocales and Cardeña, but negative in Sierra Nevada.

Variables covaried similarly when all three sites were combined in a single PCA (Online Resource 2): the first axis accounted for 34% of the variability and was strongly correlated to most nutrient pools (microbial, dissolved organic and total) and soil moisture. The second axis accounted for 27% of the variability and was mostly related to inorganic N and P. The two axes clearly separated between forest sites, with Cardeña at the poorest end of both axes and Alcornocales and Sierra Nevada at the richest end of the first and second axes, respectively.

DISCUSSION

Overall, the three sources of variability considered (habitat, soil depth and season) had significant effects on the soil microbial pools and nutrient concentrations in the studied forests. However the direction and magnitude of these effects varied across forest types and with the soil parameter examined.

The expected positive effect of tree canopy on soil and microbial nutrients was confirmed for the two oak woodlands (Cardeña and Alcornocales) but not for the pine forest (Sierra Nevada), where the soil and microbial nutrients pools were smaller beneath tree canopy than in open areas. The inconsistency of the habitat effect could be attributed to the forests' distinct species composition. Trees generate species-specific effects on soil conditions through multiple pathways, such as changing microclimatic conditions or *via* leaf and root litter input or root exudates (Alameda et al. 2012; Aponte et al. 2013; Aponte et al. 2011; Malchair and Carnol 2009). Tree species changes in soil abiotic properties might in turn affect soil biota (Aponte et al. 2013; Aponte et al. 2010a; Prescott and Grayston 2013). In particular, tree-mediated changes in soil acidity and in the amount and quality of substrate are known to affect microbial communities size and composition (Lucas-Borja et al. 2012; Sagova-Mareckova et al. 2011; Thoms et al. 2010). In Sierra Nevada soil acidity was higher beneath pine cover than in open areas, as evidenced by their distinct pH (7.7 *vs.* 8.1) and C_{inorg} (0.93% *vs*. 2.85% respectively), while clay content was lower (18.5 *vs*. 21.6%). Litter biomass was 15 times greater (8594 *vs.* 559 g m^{-2}) and the amount and quality of the substrate (C_{org}, DOC, N_{tot}, C_{org}/N_{tot}) were significantly lower beneath tree cover (*Pinus*) than in open areas, in agreement with the negative correlation observed between litter and soil C_{org} and N_{tot}. Meanwhile, the opposite was found in the two oak forest sites, i.e substrate quality was higher beneath tree cover (*Quercus*) than in open areas, and it was positively related to litter biomass, thus sustaining the counteracting patterns observed for microbial nutrients. In accordance with our results, previous studies on the effects of tree species on soils have related the lower soil nutrient and microbial values found beneath pine cover, compared to other broadleaves tree species (including *Quercus*), with the poorer quality of the pine litter, and thus to its lower decomposition rate and nutrient release, and its capacity to acidify soils (Augusto et al. 2002; Rutigliano et al. 2004; Smolander and Kitunen 2002; Ste-Marie et al. 2007). Nonetheless, the observed differences in soil and microbial nutrients between habitat types should not be solely attributed to vegetation cover. Other soil physicochemical properties, such as soil depth, structure and texture, which may be the underlying reason for the distinct cover type, can also control microbial development (Hassink 1994).

Interestingly our results also revealed a difference in the magnitude of the positive tree-effect on soil nutrients between the two oak woodlands, Cardeña and Alcornocales. These two sites significantly differed in their soil type and nutrient content: Cardeña sited over Regosols, i.e. weakly developed soils with a low organic matter content and water holding capacity (WRB 2006) (Table 1). In contrast, soils in Alcornocales were cambisols (also known as Brown forest soils, WRB 2006), they were well structured and presented a thick humic horizon (15-20cm beneath tree canopy; Garcia et al, unpublished data), and a relatively high soil organic matter content (11% in 0-25cm upper soil, Polo 2006). Mean site C_{org} was greater in Alcornocales (3.9%) than in in Cardeña (1.3 %), clay content was 7 times higher in the former (36%) than in the later (5%), and CEC (cation exchange capacity) was two-fold in Alcornocales than in Cardeña (Table 1), all of which supported the distinct soil fertility and microbial nutrient levels observed in both forest sites (Table 2). These two sites also differed in their stand structure, with a lower tree density in Cardeña than in Alcornocales (131*vs*. 219 stems ha^{-1}). It is possible that the interaction between their distinct soil types and stand structure could be determining why habitat type was the main driver of variability of soil microbial properties in Cardeña but it was of lesser importance in Alcornocales. The intensity of tree effects on soil properties is modulated by the spatial distribution of tree canopies (Bennett et al. 2009; Ushio et al. 2010). It is well-known that oak trees in Mediterranean savannah-like systems (dehesas) generate islands of fertility beneath their canopies where the leaf litter and root exudates accumulate and build up the soil organic matter that sustain microbial biomass and nutrient cycling (Alameda et al. 2012; Gallardo 2003). In sparse forests, such as Cardeña, trees are scattered in a matrix of open areas and their footprints on soil fertility are expected to be more intense beneath the canopy. In contrast, a more diffuse footprint occurs in dense forests where open areas are intermingled in a matrix of trees. This is consistent with C_{org} and C_{mic} being greater beneath tree cover than in open areas by a factor of 1.7 and 1.7 in Cardeña and a factor of 1.1 and 1.2 in Alcornocales respectively. In addition, the small concentrations of substrate (C_{org}, DOC, N_{tot}, DON) in Cardeña could be a limiting factor for microbial biomass and a tree-mediated increase in its availability would render a larger boost of microbial growth than in more fertile sites.

Microbial C, N and P showed a common and seasonal pattern, with values decreasing from Spring to Summer in response to summer drought. This response was the weakest in Cardeña, where changes were not significant. Seasonal variation was larger for N_{mic} and P_{mic} than for C_{mic}, rendering a shift in the microbial ratios, as evidenced by the multivariate analyses. The change in C_{mic}/N_{mic} was the largest in Sierra Nevada (from 9 in Spring to 34 in Summer), where a decrease in C_{mic}/C_{org}, a proxy for microbial C assimilation efficiency (Sparling 1992), was also observed. Soil microorganisms in Mediterranean ecosystems have adapted to withstand the seasonal variation in water availability and temperature that define the Mediterranean-type climate (Goberna et al. 2007). Seasonality, in particular the summer drought, may influence microbial biomass directly by inducing microbial metabolic responses to changes in soil moisture and temperature (Chen et al. 2003 ; Jensen et al. 2003), or indirectly by influencing plant productivity, organic matter release and C diffusion in soil, and hence substrate availability (Rey et al. 2002; Xiang et al. 2008). The high microbial values found in Spring may reflect favourable environmental conditions and more labile substrates derived from roots or from materials incorporated into the soil whereas the decrease in Summer might indicate a loss in the total number of organisms. This is consistent with previous work conducted in the same forest stand in Alcornocales, which showed higher soil enzyme activity during the rainy season than in summer (Quilchano and Marañón 2002). On the other hand, summer increases in microbial ratios can be related to an increasing proportion of fungi vs. bacteria (Jensen et al. 2003), since fungi have a higher carbon to nitrogen ratio (C_{mic}/N_{mic}) (related to the their lower efficiency, Cleveland and Liptzin 2007); and are more drought-tolerant than bacteria(Wilkinson et al. 2002). In addition at low water potentials, fungi are able to increase their cytoplasmic C (thus further increasing C_{mic}/N_{mic} and C_{mic}/P_{mic}) to reduce osmotic pressure and maintain hydration (Schimel et al. 2007). We propose that the loss of N_{mic} and P_{mic} as compared to C_{mic} in all sites could be explained by a net decrease in the size of the microbial biomass, driven by lower substrate (C_{org}) and water availability, together with an increase in the proportional abundance of fungi. However, neither microbial activity nor community composition indicators were measured in our study, thus the underlying mechanisms for the observed seasonal changes remain unclear.

Although the microbial pool showed a common trend affected by the summer drought, we observed significant discrepancies on the seasonal dynamics of the available pools. In Alcornocales, nutrient availability was higher in Spring, whereas the opposite was found for the other two sites. Net nutrient pools size is the result of the nutrient release through mineralization, nutrient immobilization and uptake by microorganisms and plants. The rates of N mineralization and nitrification can be more influenced by soil type and soil organic matter quality than by changes in temperature, and the effect of temperature on the rate of P mineralization can vary among soil types (Nadelhoffer et al. 1991). The more severe summer drought in Cardeña and Sierra Nevada might reduce plant uptake capacity (Kozlowski and Pallardi 2002), and increase the proportion of nutrients in the soil when compared to Alcornocales. In a climate change study conducted in the same forest site in Sierra Nevada, Matías et al. (2011) observed that under a dry scenario (30% summer rainfall reduction) soil available nutrients increased and plant and microbial nutrient pools decreased. Thus the contrasting seasonal patterns observed could be the result of different interacting factors such as the activity rates of soil microorganisms, the substrate availability and accessibility, the soil acidity and texture, and the plant nutrient uptake.

The effect of soil depth, i.e. decreasing soil and microbial nutrient content from Top soil to Deeper soil, was similar in all forest types. However the magnitude of the change varied among forests, with Cardeña showing the smallest changes. This effect of soil depth has been previously reported for Mediterranean and other forest types (Aponte et al. 2010b; Raubuch and Joergensen 2002; Ross et al. 1996; Wang et al. 2004), the main causes being a decrease in the labile C pools and an increase in the concentration of recalcitrant compounds (Fierer et al. 2003; Goberna et al. 2006). In our study C_{org} decreased with soil depth in all sites, the largest change observed in Alcornocales (from 4.6% to 3.2%) and the smallest one in Cardeña (from 1.5% to 1.2%).The stronger vertical development of cambisol soils in Alcornocales, as evidenced by the deeper soil layer having a significantly lower amount (C_{org}) and quality (C_{org}/N_{tot}) of carbon compounds than the top soil, explains the larger variability of soil properties associated to soil depth observed in this site. This is consistent with the changes observed in microbial C and N values related to soil total pools (C_{mic}/C_{org} and N_{mic}/N_{tot}) in Alcornocales, which are an indicator of a lower efficiency of the microbial biomass to assimilate C and N possibly due to a higher proportion of the soil organic matter being highly recalcitrant (Sparling 1992). Meanwhile, the dominance of shallower and more weakly developed soils in Cardeña (i.e. regosols) underpin the low importance of soil depth as a driver of soil and microbial nutrient content.

The size of the microbial pool fell within the ranges observed in other Mediterranean forests (Gallardo et al. 2000; Goberna et al. 2006) although it differed significantly between sites. It was not within the scope of this study to investigate the overall differences between forest types, but in general differences among the studied forest types were probably underpinned by the variation in soil types, the amount and quality of soil organic matter, the soil texture and water content, all of them factors constraining the size of the soil microbial biomass

(Bohlen et al. 2001; Nielsen et al. 2009). For example, clay content was the highest in Alcornocales (site mean of 35% *vs*. 8% in Cardeña). Clay content is positively related to microbial biomass and soil organic carbon because it protects microbial biomass from predation by creating refuge microsites. Furthermore, it increases soil organic matter stabilization and soil water retention thus enhancing soil conditions for microbial development (Insam et al. 1989; Sparling 1992).

Conclusions

Our findings revealed that across three contrasting Mediterranean forest types with significant differences in soil abiotic conditions, the microbial nutrient pools showed a consistent response in relation to soil depth and seasonal (drought effect) variability, which is indeed mirrored in many other ecosystems at a global scale. In contrast, the direction and magnitude of the variability associated to habitat (tree effect) varied among forest types suggesting a higher complexity in the biotic interactions between the aboveground and belowground components of these ecosystems. Few consistent interactions between factors (tree effect, soil depth and seasonal drought) were observed across forest types.

Microbial and soil chemical properties showed similar patterns of covariation in all sites, with microbial biomass responding to variations in the amount and quality of soil organic carbon and soil moisture. Thereby, the quantitative importance of the three studied factors on soil microbial nutrients varied across site, being the most important factor in each case that one which alleviated limitations and imposed the largest variability in substrate and water availability. As such, differences in forest structure and species composition between forest types would underpin the observed inconsistent tree effect on soil microbial properties, since they are related to the amount, quality and spatial and temporal distribution of the resources available to soil microorganisms.

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FIGURE CAPTIONS

Fig. 1. Location of the studied forest sites in the Iberian Peninsula.

Fig. 2. Microbial and soil nutrient fractions in Alcornocales (A), Cardeña (C) and Sierra Nevada (SN). Differences between the levels of each factor are as indicated by repeated measurement ANOVAs with season as a within-group effect and site, habitat and depth as between-group effects followed by Tukey's posthoc comparisons (* P<0.05; ** P <0.01; *** P <0.001). Abbreviations are: C_{mic}, microbial C; N_{mic}, microbial N; P_{mic}, microbial P; DOC, dissolved organic C; DON, dissolved organic N.

Fig. 3. Soil inorganic nutrient fractions and organic carbon in Alcornocales (A), Cardeña (C) and Sierra Nevada (SN). Differences between the levels of each factor are as indicated by repeated measurement ANOVAs with season as a within-group effect and site, habitat and depth as between-group effects followed by Tukey's posthoc comparisons (* P <0.05; ** P <0.01; *** P <0.001). Abbreviations are: P_{inorg}, inorganic available P; C_{org}, organic C.

Fig. 4. Percentage of the total variance explained by each of the studied factors, habitat (tree effect), soil depth and season, for each variable in each site. C_{mic}, N_{mic}, P_{mic}: microbial C, N and P, respectively; Corg: organic C; P_{inorg}: inorganic P; N_{inorg}: inorganic N; DOC, DON: dissolved organic C and N, respectively; C_{mic}/N_{mic}, C_{mic}/P_{mic}: ratios between respective variables; Overall: mean across all variables.

Fig. 5. PCA ordination plot showing the distribution of Spring and Summer values of each study sites. C_{mic}, N_{mic}, P_{mic}: microbial C, N and P, respectively; P_{inorg}: inorganic P; DOC, DON: dissolved organic C and N, respectively; N_{tot}: total N; C_{org}: organic C; C_{mic}/P_{mic}, C_{mic}/N_{mic}, N_{mic}/N_{inorg}, P_{mic}/P_{inorg}: ratios between respective variables. Depth, season and habitat (in grey) are supplementary variables included as passive in the analysis.

TABLES

Table 1. Characteristics of the studied forest sites. Values are site means (± standard deviation, when provided) and habitat means in square brackets [Open; Tree].

	Alcornocales	Cardeña	Sierra Nevada
Coordinates	36°31' N, 5°34' W	38° 15´ N, 4° 21´ W	37º05’ N, 3º28’W
Altitude (m a.s.l.)	545	750	1650
Soil			
Bedrock	sandstone	granite	limestone
pH	acidic	acidic	basic
	[6.34; 6.07][a]	5.4 [b]	[8.1; 7.7][c]
Soil type	cambisol	regosol	regosol, cambisol
Texture	sandy	sandy	loamy
Sand (%)	[44; 49] [a]	[80; 79] [d]	[22; 19] [c]
Clay (%)	[39; 33] [a]	[4.8; 4.5] [d]	[29; 37] [c]
CEC (meq $100g^{-1}$)	[23.1; 19.7] [a]	[7.8; 8.6] [d]	[14.7; 18.5] [c]
Litter (g m^{-2})	[45±38; 936±350]	[282± 259; 1200± 875]	[559±362; 8594±5543]
Climate			
Temperature (ºC)			
mean annual	15.5	15.3	12.1
mean min	9.1	7.3	-1.1
mean max	23.6	25.3	29.2
Rainfall (mm)			
annual	1117	752	811
spring	259	151	206
summer	28	39	43
Vegetation			
Tree density (stems ha^{-1})	219	131	787
Basal area (m^2 ha^{-1})	24	13	-
Experimental design (n)	Open (10)	Open (19)	Open (16)
	Q. suber/ Q. canariensis (20)	*Q. ilex* (19)	*P. sylvestris* (16)

[a] Values determined in 0-25 cm deep soil samples (Polo 2006)

[b] Mean value for regosols in the region (0-15cmGil Torres et al. 2003)

[c] Values determined in 0-16 cm deep soil samples (Matías et al, unpublished data)

[d] Values determined in 2-14 cm deep soil samples (Alameda et al. 2012/ Alameda et al., unpublished results).

Table 2. Mean (±SE) values of the measured soil variables across habitat, season and soil depth by site. Letters indicate differences between sites (P < 0.05).

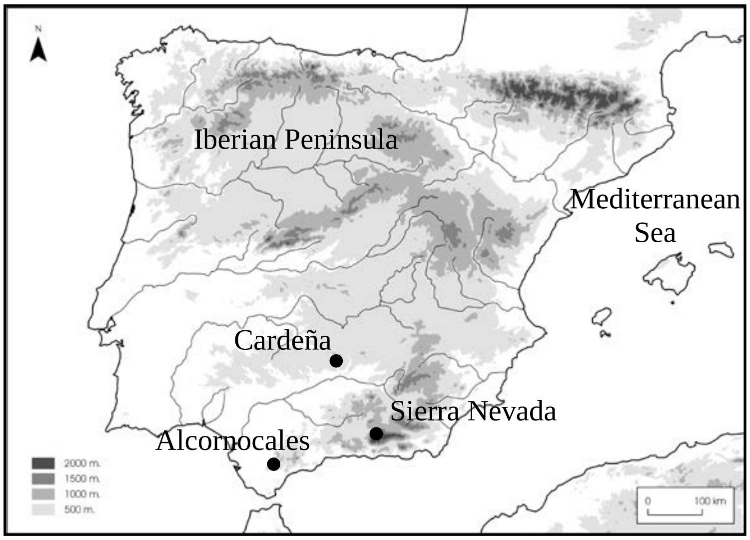
	Alcornocales		Cardeña		Sierra Nevada	
C_{mic} (mg kg^{-1})	378 ± 18	a	58 ± 4	b	63 ± 3	b
N_{mic} (mg kg^{-1})	46 ± 3	a	17 ± 1	b	6.9 ± 0.5	c
P_{mic} (mg kg^{-1})	7.9 ± 0.6	a	1.0 ± 0.1	b	4.0 ± 0.3	c
C_{org} (%)	3.9 ± 0.1	a	1.3 ± 0.1	b	3.0 ± 0.1	c
P_{inorg} (mg kg^{-1})	2.7 ± 0.2	a	1.3 ± 0.1	b	3.4 ± 0.2	c
NH_4 (mg kg^{-1})	8.1 ± 0.6	a	2.6 ± 0.2	b	19 ± 1	c
NO_3 (mg kg^{-1})	2.5 ± 0.2	a	0.7 ± 0.1	b	7.9 ± 0.4	c
DOC (mg kg^{-1})	168 ± 7	a	117 ± 8	b	41 ± 3	c
DON (mg kg^{-1})	27 ± 1	a	6.7 ± 0.3	b	3.9 ± 0.2	c
N_{tot} (%)	0.28 ± 0.01	a	0.09 ± 0.0	b	0.22 ± 0.01	c
C_{mic} /N_{mic}	9.3 ± 0.3	a	7.2 ± 0.7	b	21 ± 4	c
C_{mic}/P_{mic}	108 ± 18	a	145 ± 28	a	23 ± 2	b
Moisture Spring (%)	22 ± 1	a	9.3 ± 0.6	b	13 ± 1	c
Moisture Summer (%)	11.0 ± 0.4	a	2.7 ± 0.2	b	3.4 ± 0.2	c

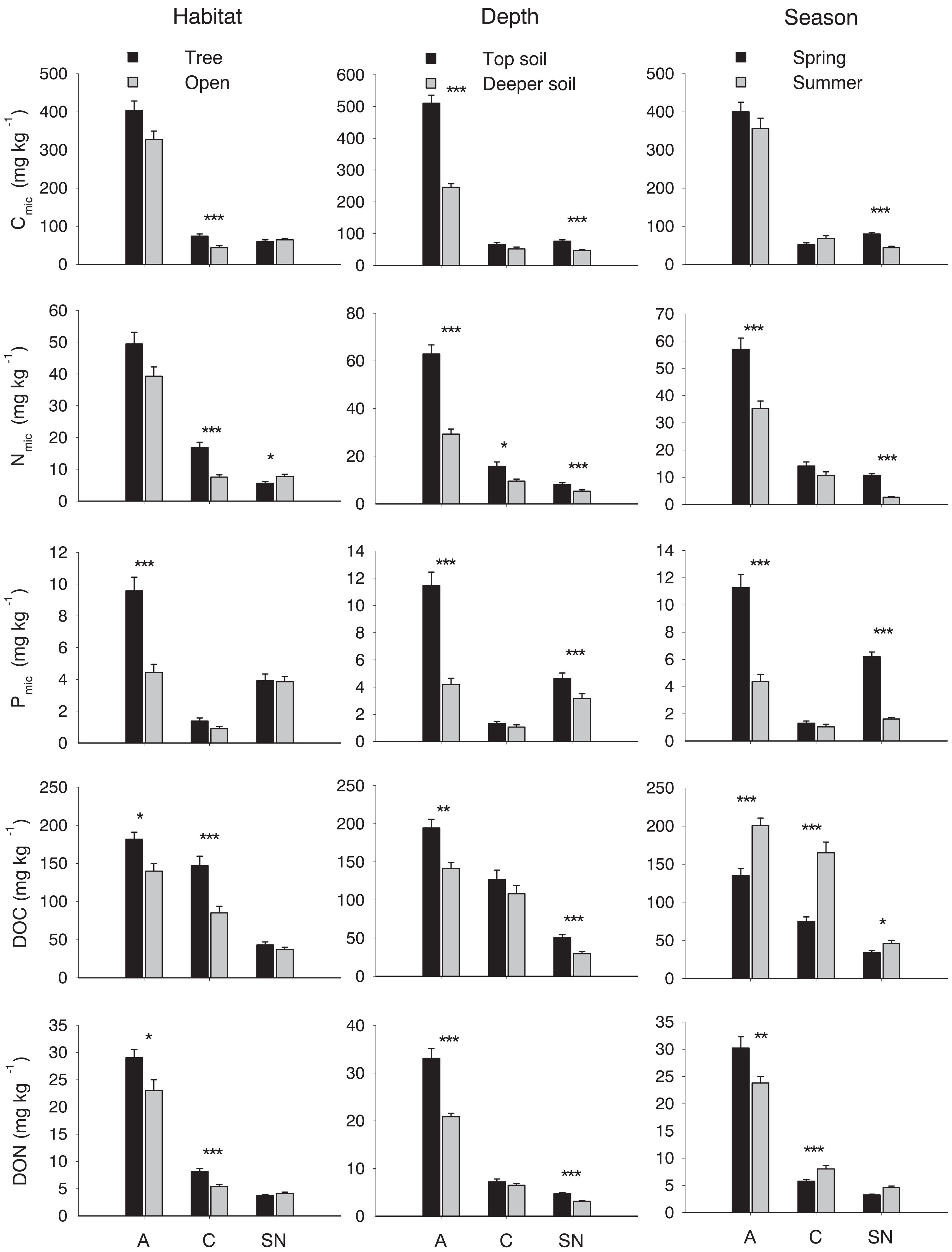
Table 3. Repeated measurements ANOVA for the studied soil properties. F and *P*-values for between effects (forest site, habitat and soil depth), within effect (season) and three way interactions are presented. Significant effects are marked with asterisks (* P <0.05; ** P < 0.01; *** P < 0.001). C_{mic}, N_{mic}, P_{mic}: microbial C, N and P, respectively; C_{org}: organic C; N_{inorg}: inorganic N (NH_4 + NO_3); P_{inorg}: inorganic P; DOC, DON: dissolved organic C and N, respectively.

	C_{mic}		N_{mic}		P_{mic}		C_{org}		P_{inorg}		N_{inorg}		DOC		DON		C_{mic}/N_{mic}		C_{mic}/P_{mic}		Moisture	
Effect	F	*P*	F	*P*	F	*P*	F	*P*	F	F	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
Site	342***		368***		170.4***		11.2***		75.6***		619***		303.3***		590.2***		65.6***		126.9***		265.4***	
Habitat	12.1***		7.79**		14.9***		15.4***		3.83		12.5***		39.2***		16.6***		1.13		5.82*		7.96**	
Depth	41.5***		69.2***		63.5***		6.93**		31.1***		18.3***		45.3***		37.6***		1.73		10.9**		0.93	
Site×Habitat	9.7***		22.3***		13***		7.55**		6.61**		7.36***		3.15		9.12***		6.79***		18.2***		1.71	
Site×Depth	8.1***		3.67*		22.2***		3.96*		3.25		1.87		6.2**		4.76*		0.1		4.86**		8.93***	
Habitat×Depth	0.97		2.14		0.01		0.01		0.02		0.07		2.54		3.52		0.19		8.68**		0.91	
Site×Habitat×Depth	2.54		1.37		0.47		4.05*		2.67		2.58		3.17		1.35		0.22		7.93***		0.9	
Season	8.83**		116.8***		154***		0.14		5.65		0.97		144.9***		11.2***		92.1***		36.4***		1283***	
Season×Site	23.3***		27.2***		31.7***		45.8***		105***		193***		21.2***		22.9***		12.3***		0.40		13.6***	
Season×Habitat	0.05		4.41*		11.4***		6.42*		0.04		5.11*		10.9***		8.13**		6.27*		7.67**		0.06	
Season×Depth	0.04		0.09		0.5		1.54		5.32*		4.27		0.52		0.75		0.89		0.00		13.4***	
Season×Site×Habitat	0.18		0.02		2.45		3.36*		7.48***		3.17		8.67***		5.95**		0.7		0.65		1.45	
Season×Site×Depth	0.28		1.7		0.24		1.46		2.32		3.79		8.61***		5.98**		0.03		0.03		6.61**	
Season×Habitat×Depth	0.08		0.27		3.21		0.84		0.63		0.51		2.97		0.45		0		1.26		0.38	

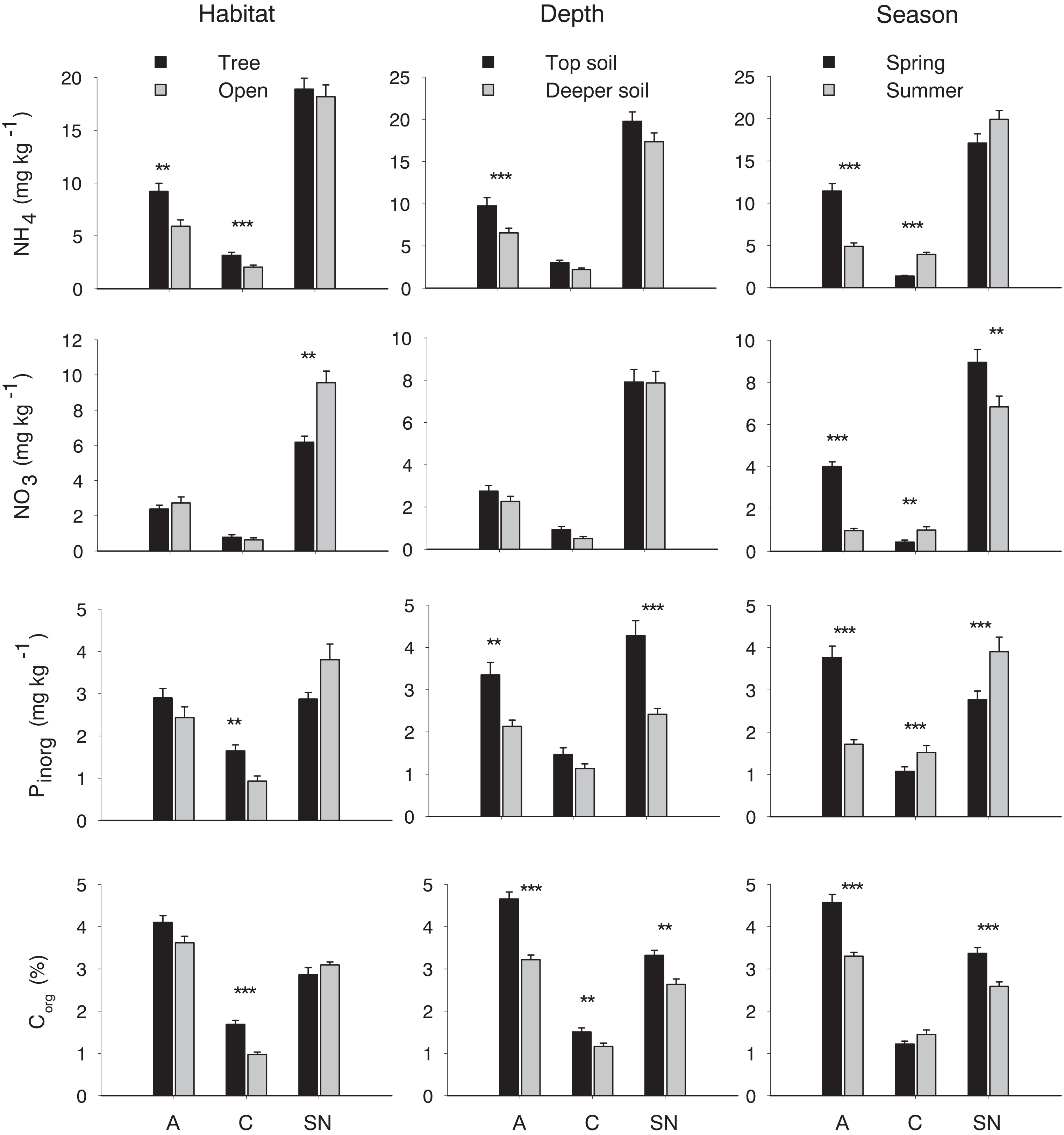
Site: Alcornocales, Cardeña and Sierra Nevada
Habitat: Tree and Open;
Depth: Top soil (0-8cm) and Deeper soil (8-16cm);
Season: Spring and Summer .

Fig. 1.

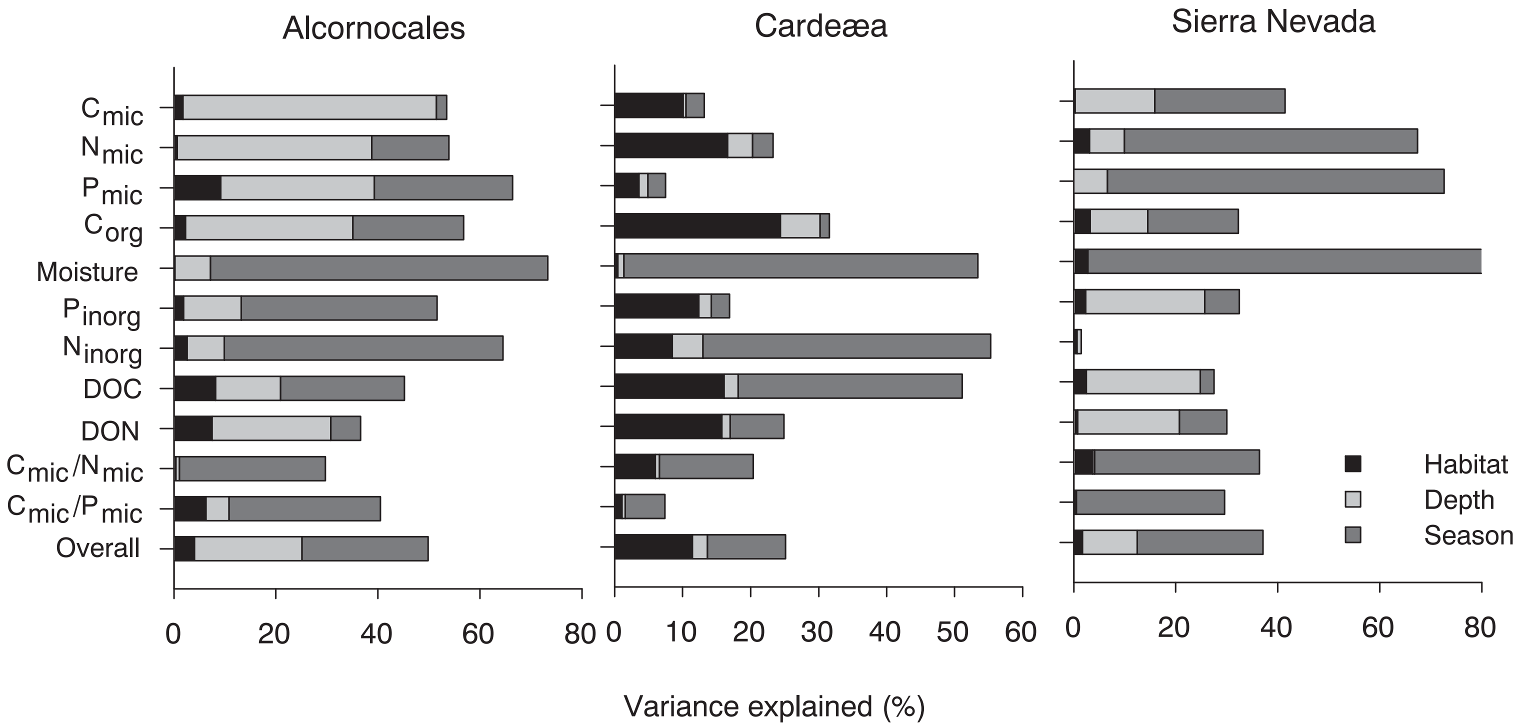

Iberian Peninsula
Mediterranean Sea
Cardeña
Sierra Nevada
Alcornocales
2000 m.
1500 m.
1000 m.
500 m.
0
100 km



Habitat
Depth
Season
Tree
Open
Top soil
Deeper soil
Spring
Summer
C_{mic} (mg kg^{-1})
N_{mic} (mg kg^{-1})
P_{mic} (mg kg^{-1})
DOC (mg kg^{-1})
DON (mg kg^{-1})
A
C
SN

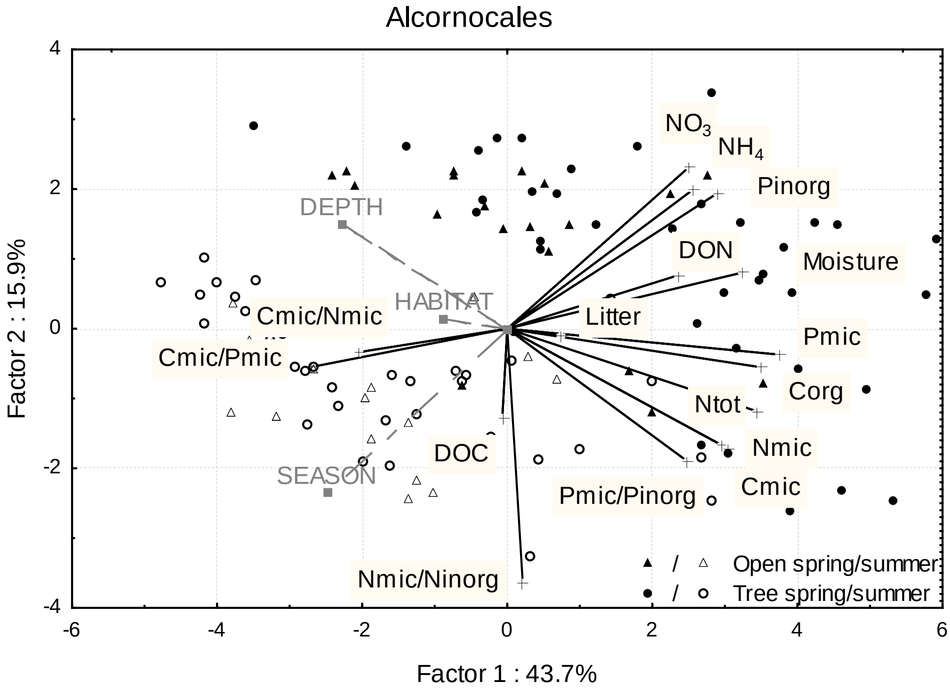


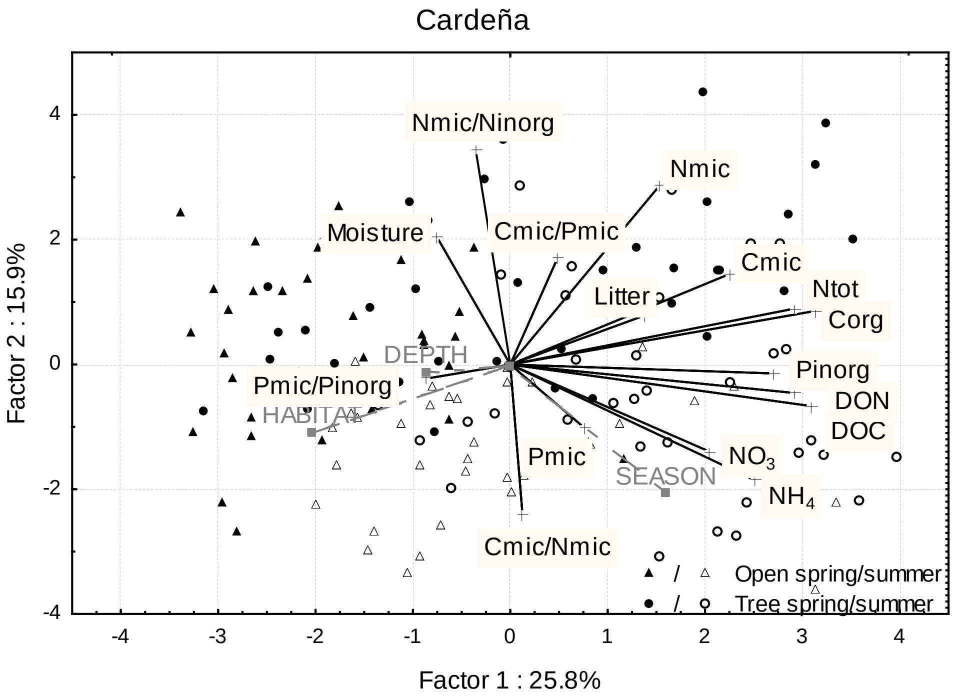
Habitat
Depth
Season
Tree
Open
Top soil
Deeper soil
Spring
Summer
NH4 (mg kg -1)
NO3 (mg kg -1)
Pinorg (mg kg -1)
Corg (%)
A
C
SN

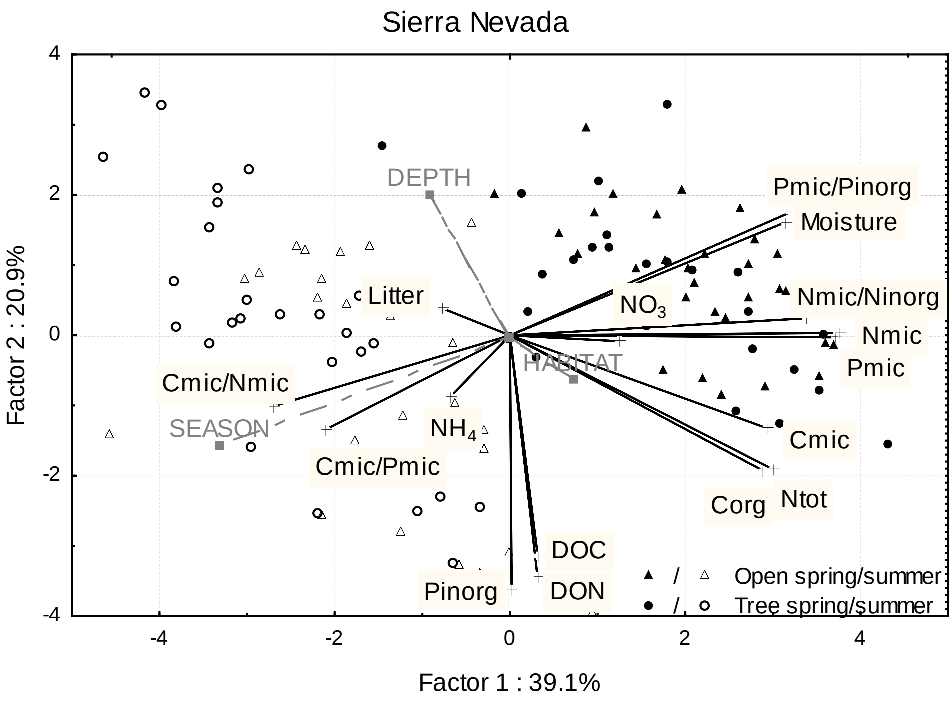


Alcornocales
Cardeæa
Sierra Nevada
C_{mic}
N_{mic}
P_{mic}
C_{org}
Moisture
P_{inorg}
N_{inorg}
DOC
DON
C_{mic}/N_{mic}
C_{mic}/P_{mic}
Overall
Habitat
Depth
Season
0 20 40 60 80
0 10 20 30 40 50 60
0 20 40 60 80
Variance explained (%)

Fig 5.


Alcornocales
Factor 1 : 43.7%
Factor 2 : 15.9%
NO3
NH4
Pinorg
DON
Moisture
Litter
Pmic
Corg
Ntot
Nmic
Cmic
Pmic/Pinorg
DOC
Nmic/Ninorg
Cmic/Nmic
Cmic/Pmic
DEPTH
HABITAT
SEASON
Open spring/summer
Tree spring/summer


Cardeña
Factor 1 : 25.8%
Factor 2 : 15.9%
Nmic/Ninorg
Nmic
Moisture
Cmic/Pmic
Cmic
Litter
Ntot
Corg
DEPTH
Pinorg
Pmic/Pinorg
HABITAT
DON
DOC
Pmic
NO3
SEASON
NH4
Cmic/Nmic
Open spring/summer
Tree spring/summer



Sierra Nevada
Factor 2 : 20.9%
Factor 1 : 39.1%
4
2
0
-2
-4
-4
-2
0
2
4
DEPTH
SEASON
HABITAT
Pmic/Pinorg
Moisture
Nmic/Ninorg
Nmic
Pmic
NO3
Litter
Cmic/Nmic
Cmic/Pmic
NH4
Cmic
Corg
Ntot
DOC
DON
Pinorg
Open spring/summer
Tree spring/summer