

Contrasting effects of nitrogen addition on soil respiration in two Mediterranean ecosystems

Mauro Lo Cascio^{1,2}  · Lourdes Morillas¹ · Raúl Ochoa-Hueso³ · Silvana Munzi⁴ · Javier Roales¹ · Niles J. Hasselquist⁵ · Esteban Manrique⁶ · Donatella Spano^{1,2} · Renée Abou Jaoudé¹ · Simone Mereu^{1,2}

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Abstract Increased atmospheric nitrogen (N) deposition is known to alter ecosystem carbon source-sink dynamics through changes in soil CO₂ fluxes. However, a limited number of experiments have been conducted to assess the effects of realistic N deposition in the Mediterranean Basin, and none of them have explored the effects of N addition on soil respiration (R_s). To fill this gap, we assessed the effects of N supply on R_s dynamics in the following two Mediterranean sites: Capo Caccia (Italy), where 30 kg ha⁻¹ year⁻¹ was supplied for 3 years, and El Regajal (Spain), where plots were treated with 10, 20, or 50 kg N ha⁻¹ year⁻¹ for 8 years. Results show a complex, non-linear response of

soil respiration (R_s) to N additions with R_s overall increasing at Capo Caccia and decreasing at El Regajal. This suggests that the response of R_s to N addition depends on dose and duration of N supply, and the existence of a threshold above which the N introduced in the ecosystem can affect the ecosystem's functioning. Soil cover and seasonality of precipitations also play a key role in determining the effects of N on R_s as shown by the different responses observed across seasons and in bare soil vs. the soil under canopy of the dominant species. These results show how increasing rates of N addition may influence soil C dynamics in semiarid ecosystems in the Mediterranean Basin and represent a valuable contribution for the understanding and the protection of Mediterranean ecosystems.

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✉ Mauro Lo Cascio
mlocascio@uniss.it

- ¹ Department of Science for Nature and Environmental Resources (DipNET), University of Sassari, Via Enrico de Nicola, No. 9, 07100 Sassari, Italy
- ² Euro-Mediterranean Centre on Climate Change (CMCC), IAFES Division, Via Enrico de Nicola, No. 9, 07100 Sassari, Italy
- ³ Department of Ecology, Autonomous University of Madrid, Darwin St., 2, 28049 Madrid, Spain
- ⁴ Centre for Ecology, Evolution and Environmental Changes (cE3c), Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisbon, Portugal
- ⁵ Department of Forest Ecology and Management, Swedish University of Agricultural Sciences, SE-901 83 Umeå, Sweden
- ⁶ Department Biogeography and Global Change, Spanish National Research Council (MNCN-CSIC), National Museum of Natural Sciences, C/Serrano 115 Dpdo, 28006 Madrid, Spain

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Introduction

Soil carbon dioxide (CO₂) emissions, mostly derived from the microbial processing of organic matter, represent the second largest carbon (C) flux in terrestrial ecosystems (Raich and Schlesinger 1992) accounting for 70–90% of total ecosystem respiration (Schlesinger and Andrews 2000). Therefore, any alterations in R_s can have large cascading effects on atmospheric CO₂ concentration and thus on the global C balance. Nitrogen (N) additions are known to strongly influence the abundance, composition, and activity of soil microbial communities (Reich et al. 2006b; Ferretti et al. 2014), which are the main drivers of soil-atmosphere CO₂ fluxes. Nitrogen

deposition can also affect organic matter decomposition through changes in soil faunal and microbial community structure (Hungate et al. 2003).

In order to predict the responses of the global C cycle to climate change and atmospheric N pollution, several studies investigated the role and metabolic efficiency of microorganisms in converting available organic substrates by calculating the ratio between respiration and C uptake (Sinsabaugh et al. 2013; Eberwein et al. 2015). This parameter is known as carbon use efficiency (CUE), defined as the ratio of microbial community growth over C assimilated by the ecosystem (Bradford and Crowther 2013). High CUE values indicate a prevalent growth of microbial biomass with respect to soil C stabilization, while low CUE values indicate an increase in R_s rate (Manzoni et al. 2012).

Carbon use efficiency is sensitive to environmental changes. In particular, the higher temperature and frequency of drought events predicted for the next future might reduce the CUE (Tucker et al. 2013), whereas increasing availability of N and nutrients are expected to increase it (Ågren et al. 2001). Indeed, a reduction of R_s is the most widely reported response to N addition, highlighting a functional dependence of the soil processes on nutrient additions, phenomenon which could increase the net ecosystem exchange, thus potentially mitigate rising atmospheric CO₂ concentration with a consequent positive feedback on climate change (Reich et al. 2006a; Janssens et al. 2010). In line with this, a meta-analysis of experimental N manipulation studies showed a general reduction in R_s of ca. 15% in response to N additions (Janssens et al. 2010). However, this response can range from no effect at all to positive or negative depending on ecosystem type, age, dominant plant species, soil chemical characteristics (Pregitzer et al. 2008; Rodriguez et al. 2014; Zhu et al. 2016), N loads in the soil in combination with climatic conditions (Alster et al. 2013), and experimental duration (Zhou et al. 2014). In another study, Zhang et al. (2014) reported a positive correlation between N addition and R_s in a semiarid ecosystem in China, whereas Bowden et al. (2004) showed that N addition suppressed R_s in a Californian grassland. Thus, despite the general assumption that N additions lead to a reduction in R_s , numerous studies have shown a complex and non-linear relationship between N additions and R_s . Moreover, most of these studies have been conducted in temperate (Thomas Quinn et al. 2010), boreal (Allison et al. 2008; Allison et al. 2009; de Vries et al. 2009), tropical, and subtropical (Fan et al. 2014) forested ecosystems, whereas semiarid ecosystems are scarcely investigated. In addition, the peculiarities of arid and semiarid ecosystems prevent to extrapolate findings and patterns from other ecosystem types (e.g., boreal, temperate). Recently, some efforts have been made to fill this gap and researchers have focused on Mediterranean ecosystems and semiarid regions. Alster et al. (2013) reported that drought events and N have an impact on soil functionality, measured

in terms of soil extracellular enzyme activity, while Eberwein et al. (2015) found that under both N enrichment and temperature change, the C availability remains the major driver of R_s .

Understanding the effect of N deposition in Mediterranean ecosystems is particularly relevant as these ecosystems are already experiencing increasing rates of N deposition and projections for 2050 estimate a further increase due to intensification of anthropogenic activities. This is particularly true for terrestrial ecosystems in the Mediterranean Basin, where N inputs are expected to increase from the 6.5 kg N ha⁻¹ year⁻¹ of mid-1990s to 12 kg N ha⁻¹ year⁻¹ in 2050 (Phoenix et al. 2006). Semiarid ecosystems are also regarded as particularly important controllers of between-year variations in the global C cycle due to extremely pronounced intra- and inter-annual climatic variations (Poulter et al. 2014). The strong climatic seasonality of Mediterranean ecosystems causes a strong limitation of biological processes during the rainless summer, and therefore, the effect of N additions may be limited during this period. Additionally, N does not infiltrate into the soil at homogeneous rates during the year, as is commonly observed in temperate ecosystems. Instead, in Mediterranean ecosystems, N tends to accumulate on surfaces during the dry periods and only with the first annual rain events does it infiltrate to deeper soil layers (Welter et al. 2005). Although this seasonal pattern has been recognized as an important driver of R_s in Mediterranean environments (Curiel Yuste et al. 2003; Almagro et al. 2009), manipulative experiments assessing the effects of N addition on R_s , and consequently on the C balance in the Mediterranean Basin, are still missing (Rey et al. 2002).

In this study, we present results from two N addition experiments in Mediterranean ecosystems, one in a coastal macchia (Italy) and one in a semiarid shrubland (central Spain), in which the amount of added N mimicked both N deposition rates projected for the Mediterranean Basin in 2050 (Ochoa-Hueso et al. 2013) and N deposition rates measured in other Mediterranean-type ecosystems (Fenn et al. 2003b).

The main objective was to investigate how dose and duration of chronic N additions affect soil chemistry and in situ R_s fluxes in two Mediterranean ecosystems. Moreover, measurements of R_s were taken beneath shrub canopies as well as in open bare soils (BSs) to determine if R_s responses depend on the spatial heterogeneity of aboveground vegetation.

We hypothesized that (i) R_s would be negatively influenced by N addition in a manner strongly dependent on dose and duration of N additions and C availability and that (ii) the effect of N addition on R_s would strongly depend on the seasonality typical of Mediterranean ecosystems. We specifically predicted that R_s responses to N would be stronger in the spring growing season, when soil moisture and temperature are optimal for microbial activity and plant growth.

Material and methods

Site description

Capo Caccia, Italy

Capo Caccia is a coastal ecosystem (74 m above sea level (asl)) located within the National Reserve “Portoconte-Capo Caccia” in northwestern Sardinia (Italy). The climate is sub-humid Mediterranean, with cold, wet winters and warm, rainless summers with most of the precipitation occurring in spring and autumn. The average annual rainfall during the study period was 47.8 mm, and the maximum mean precipitation was registered in January 2013 with a monthly average of 178.9 mm (Fig. 6a). Annual mean air temperature was 16.8 °C. The lowest temperature recorded was in February 2012 with a minimum monthly average of 7.2 °C, while the highest temperature was in August 2012 with a maximum monthly average of 24.9 °C (Fig. 6a). The soil is Lithic Xerorthent and Typic Rhodoxeralfs (Soil Survey Staff 1999) with a clayish structure. On average, vascular vegetation cover is ca. 80% and is dominated by *Juniperus phoenicea* L., while the remaining 20% are BS colonized by a well-developed biological soil crust (BSC) community composed by common species in Mediterranean soils (Table 3; see Morillas et al. (2017) for an accurate description of the BSC community). According to the Meteorological Synthesizing Centre–West (MSC-W) of the European Monitoring and Evaluation Programme (EMEP 2015) (Simpson et al. 2012), natural oxidized nitrogen (NO_x) deposition from 2000 to 2013 was approximately 0.96 kg $\text{NO}_x \text{ ha}^{-1} \text{ year}^{-1}$, while the last EMEP report (Gauss et al. 2016) stated that in 2014, natural N deposition was between 1.7 and 3.7 kg N $\text{ha}^{-1} \text{ year}^{-1}$.

A meteorological station, located 20 m away from the closest plot, has been acquiring data since 2004 and includes measurements of air temperature (T_{air}) and humidity (HMP45C, Campbell Scientific Inc., USA) at 2-m height, as well as wind speed and direction, global and net radiation, precipitation (ARG100, Environmental Measurements Limited, UK), and soil moisture at 20-cm depth. All sensors are connected to a data logger (CR1000, Campbell Scientific Inc., USA) that acquires data every minute and stores it as half-hour averages.

In March 2012, eight plots (7.5 × 7.5 m) were randomly established. Each plot contained at least one fully developed individual of *J. phoenicea* L. and a portion of bare soil. Six permanent polyvinyl chloride (PVC) collars (inner diameter of 20 cm) were installed within each plot for soil CO_2 efflux measurements, three under the canopy of *J. phoenicea* (hereafter referred to as UC microsite) and three in the bare soil (hereafter referred to as BS microsite) ($n = 48$ total PVC collars). Starting in April 2012, four plots were treated with 30 kg N $\text{ha}^{-1} \text{ year}^{-1}$ over background N deposition, whereas the other four plots served as unfertilized controls. The first

application (spring 2012) was of 22.5 kg N $\text{ha}^{-1} \text{ year}^{-1}$, while the next applications were of 7.5 kg N $\text{ha}^{-1} \text{ year}^{-1}$ at the beginning of each season. Nitrogen was applied by dissolving ammonium nitrate (NH_4NO_3) in 20 L of water and applying it to the soil surface using a backpack sprayer. Control plots received 20 L of water as well.

Soil CO_2 efflux measurements were performed in situ using a portable Li-8100 (LI-COR Inc., Lincoln, NE, USA) containing an infrared gas analyzer system equipped with a flow-through LI-COR 8100–103 closed chamber connected to the central unit. Measurements took 105 s per collar. The headspace of each collar was measured and taken into account to quantify CO_2 efflux rates. Soil respiration measurements were taken at least 3 days after a rainfall event to avoid measuring at times when soil water content (SWC) was abnormally above its maximum field capacity. From August 2012 to October 2015, measurements of soil CO_2 efflux were always taken between 12:00 p.m. and 3:00 p.m. (local time, GMT + 1). Although measurements did not follow any specific frequency during this period, they were collected over the widest range of combinations of soil temperature (T_s) and SWC.

In order to assess local environmental differences, six T_s and soil moisture probes (EC-5, Decagon Devices Inc., Pullman, USA) were inserted at 5-cm depth, three in the UC and three in the BS microsite of each plot.

El Regajal, Spain

El Regajal is located on a hill (600 m asl) within the Nature Reserve “El Regajal-Mar de Ontígola” in central Spain. The climate is semiarid Mediterranean with cold, wet winters and hot, dry summers. Since the beginning of the experiment (2007), an average annual rainfall of 26.3 mm was recorded with the maximum precipitation of 122.6 mm (monthly average) registered in December 2009 (Fig. 6b). Mean annual T_{air} was 15.5 °C. The coldest month was February 2010 with a minimum monthly average temperature of 2.7 °C, and the hottest month was July 2010 with a maximum monthly average temperature of 36.4 °C (Fig. 6b) (data provided by the AEMT for Getafe, the closest meteorological station at 45 km north from El Regajal n.d). This site is characterized by soil rich in calcium carbonate with a sandy clayish structure. The dominant evergreen species are *Rosmarinus officinalis* L. and *Quercus coccifera* L. (Table 3). The space between shrubs is colonized by a well-developed biocrust (see Ochoa-Hueso et al. 2011 for community description). The MSC-W of the EMEP (Simpson et al. 2012) showed for the period from 2000 to 2013 a natural NO_x deposition of approximately 1.05 kg $\text{NO}_x \text{ ha}^{-1} \text{ year}^{-1}$, while as in Capo Caccia, the last EMEP report (Gauss et al. 2016), for the 2014, reported a natural N deposition in the range of 1.7 and 3.7 kg N $\text{ha}^{-1} \text{ year}^{-1}$.

In September 2007, six replicated blocks were established. Each block consisted of four 2.5 × 2.5-m plots separated by

two perpendicular crossed 1-m buffer bands (24 plots in total). Starting in October 2007, within each block, three randomly selected plots were subjected to N additions of 10, 20, and 50 kg N ha⁻¹ year⁻¹, respectively, as described in Ochoa-Hueso and Manrique (2011) and compared to an unfertilized control plot. Two liters of 0, 19, 37, and 93 mM NH₄NO₃ solution were sprayed on the whole surface of the plots once per month, except during the extreme summer drought periods (July–August). In September, to simulate the peak of N availability occurring with the onset of equinoctial rains (Fenn et al. 2003a), a 3-month total N load was applied to get the final deposition target in each treatment. In 2010, one PVC collar (inner diameter 20 cm) for soil CO₂ efflux measurements was placed at the center of each plot, trying to capture the BSC variability.

Soil respiration was measured six times from April 2015 to October 2015, four times in spring, once in late summer, and once in autumn. For each sampling date, R_s was measured five times in each plot between 8:00 a.m. and 7:00 p.m. (local time, GMT + 1). In order to account for strong diurnal fluctuations, the five R_s measurements in each plot were averaged prior to data analysis. Soil CO₂ efflux measurements were performed with the same protocol and instrument used in Capo Caccia with the exception of the last two measurements (August and October 2015) in El Regajal, when a different Li-8100 (LI-COR Inc., Lincoln, NE, USA) was used. To account for potential differences between instruments, we performed an inter-calibration between the two Licor-8100 instruments under controlled laboratory conditions. Specifically, six PVC collars (inner diameter of 20 cm) were filled with homogenized soil up to 7-cm depth, where some lichen pieces collected from the field and cut into 0.5-cm-side square scraps, were put on top of the soil within the collars, obtaining different lichen coverage, 0–20–30–50–60–90%, respectively, to acquire a wide range of respiration. For both instruments, R_s was measured six times in each collar after watering the “lichen crust.” This data was used ($n = 36$ total measurements) to determine a correction factor for the last two measures at El Regajal to account for any potential differences in instruments.

Starting in April 2015, soil environmental conditions were monitored using a T_s and moisture probe (EC-5, Decagon Devices Inc., Pullman, USA) placed at 5-cm depth between the blocks. Sensor was connected to an external data logger (HOBO 4-channel, U12–006), which acquired data every minute and stored it as hourly averages.

Soil sampling and chemical analyses

In April 2015, one composite soil sample per plot was collected at both sites, for a total of 8 samples in Capo Caccia and 24 samples in El Regajal. Composite samples consisted of five soil cores (2 cm in diameter, 0–4-cm depth) in each plot that

were pooled together to account for spatial variability. Soil samples were dried in an oven at 40 °C for 72 h and later sieved using a <2-mm mesh. Soil pH was determined with a pH meter (1:5 soil:deionized water slurry). Soil organic C content was extracted with potassium dichromate (K₂Cr₂O₇), and after centrifugation, the solution was analyzed by spectrophotometry at 600 nm (Walkley and Black 1934). Total N and total phosphorous (P) were analyzed after a Kjeldahl acid digestion with sulfuric acid (H₂SO₄) and potassium sulfate (K₂SO₄) as catalysts. The results from the digestion were subsequently analyzed using a SAN++ analyzer (Skalar, the Netherlands).

Soil inorganic N and dissolved organic nitrogen (DON) were extracted adding 0.5 M K₂SO₄ at a ratio of 1:5, followed by shaking for 1 h at 200 rpm at 20 °C, and then filtered through a 0.45-mm Millipore filter (Jones and Willett 2006). The NH₄⁺-N concentration was estimated directly via the indophenol blue method using a microplate reader (Sims et al. 1995). The NO₃⁻-N content was first reduced to NH₄⁺-N with Devarda alloy, and its concentration was determined as the difference between the Devarda-incubated and unincubated samples (where the NH₄⁺-N concentration was estimated). Soil inorganic N concentration was calculated as the sum of NH₄⁺-N and NO₃⁻-N. The DON content in the extracts was first oxidized to NO₃⁻-N with potassium persulfate (K₂S₂O₈) in an autoclave at 121 °C for 55 min and then reduced to NH₄⁺-N with Devarda alloy (Sollins et al. 1999). The DON concentrations were estimated as total dissolved N minus inorganic N. Microbial biomass N (MB-N) was determined using the fumigation-extraction method proposed by Brookes et al. (1985). Twenty grams of fresh soil was fumigated with chloroform for 5 days, whereas the non-fumigated replicates were used as controls. Fumigated and non-fumigated samples were extracted with 100 ml of K₂SO₄ 0.5 M and filtered through a 0.45-mm Millipore filter. The extracts were digested as described above. The total N content in the digested extracts was determined by colorimetry using the indophenol blue method through a microplate reader (Sims et al. 1995). The MB-N concentration was calculated as the difference between the total N in fumigated and non-fumigated digested extracts divided by a Kn (fraction of MB-N extracted after the chloroform treatment) of 0.54 (Brookes et al. 1985).

Statistical analyses

All datasets were tested for normality and homogeneity of variance (Shapiro-Wilk and Levene statistics) and were log-transformed when necessary. Statistical significance was defined at the 95% confidence level (p value <0.05). All statistical analyses were performed using R (R Core Team, 2014; package = “lme4” for general linear mixed models (GLMMs) and package = “multcomp” to perform post hoc test).

The statistical analyses to test for differences between fertilized and control plots were performed with the annual N doses for each treatment for both sites (data not shown) and with the cumulative N effect (CML.N) calculated for both sites as sum of each N application, from the first fertilization to the last date of R_s measurements (Table 1). Although results showed similar trends for both the annual N doses and CML.N, we opted for using CML.N due to its clearer effects on R_s .

Since the period covered by R_s measurements and the protocol used to determine it differed between the two sites, they were analyzed separately.

To examine whether R_s differed between fertilized and control within each microsite in Capo Caccia, we used GLMM. In the model, CML.N and microsite (UC and BS) and their interaction were treated as fixed factors and SWC and T_s effects were considered as covariates, whereas the time measurement and the experimental design were treated as random variables

Table 1 Results of the generalized linear mixed model (GLMM) for Capo Caccia, including the two microsities (bare soil and under canopy), and for El Regajal

	<i>F</i> value	<i>p</i> value
Capo Caccia GLMM		
CML.N	2.026*	0.024
ms	1.659	0.219
SWC20	0.001	0.974
T_s	6.701**	0.009
CML.N × ms	2.472**	0.004
Bare soil		
CML.N	1.271	0.261
SWC20	5.457*	0.023
T_s	14.858***	<0.001
Under canopy		
CML.N	1.898*	0.045
SWC20	0.003	0.856
T_s	0.602	0.438
El Regajal GLMM		
CML.N	3.94***	<0.001
SWC5	7.749*	0.050
T_s	4.903*	0.028

In Capo Caccia, the GLMM accounts for the interaction between cumulative N (CML.N) and microsities (ms) as fixed factors and soil water content at 20-cm depth (SWC20) and soil temperature at 3-cm depth (T_s) as covariates on R_s response. In El Regajal, the GLMM accounts for CML.N as fixed factor and soil water content at 5-cm depth (SWC5) and T_s as covariates on R_s response. The measuring date (date) and the respective experimental designs were included in the GLMM as random factors. Statistical differences are indicated as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ and are highlighted in italic. The *F* value test (*F* value) and the level of significance (*p* value) are shown for each model

(Table 1). We also tested how CML.N affected R_s in the two microsities independently using the same GLMM but excluding the microsite effect (Table 1 and Figs. 1 (inset) and 2 (inset)).

We tested how CML.N affected R_s in El Regajal using GLMM. Similar to the previous model, CML.N was the fixed factor and SWC and T_s effect were considered in the analysis as covariates, whereas the time measurement and the experimental design were treated as random factors (Table 1). Model's results were tested by HSD post hoc tests to find the difference among N treatments (Fig. 3, inset).

To identify time of year when N addition affected R_s , at both study, a one-way ANOVA among the treatments was performed for each single date of measurements (Figs. 2 and 3), followed by a LSD post hoc test if necessary.

Soil chemical properties were subjected to a one-way ANOVA, followed by a LSD post hoc test among the study sites only using the data of control plots (Table 2). In Capo Caccia, the N effect was tested on soil chemical properties separately for BS and UC using a one-way ANOVA (Table 2), while in El Regajal, the differences among N treatments were tested by a one-way ANOVA followed by a LSD post hoc test when necessary (Table 2).

Results

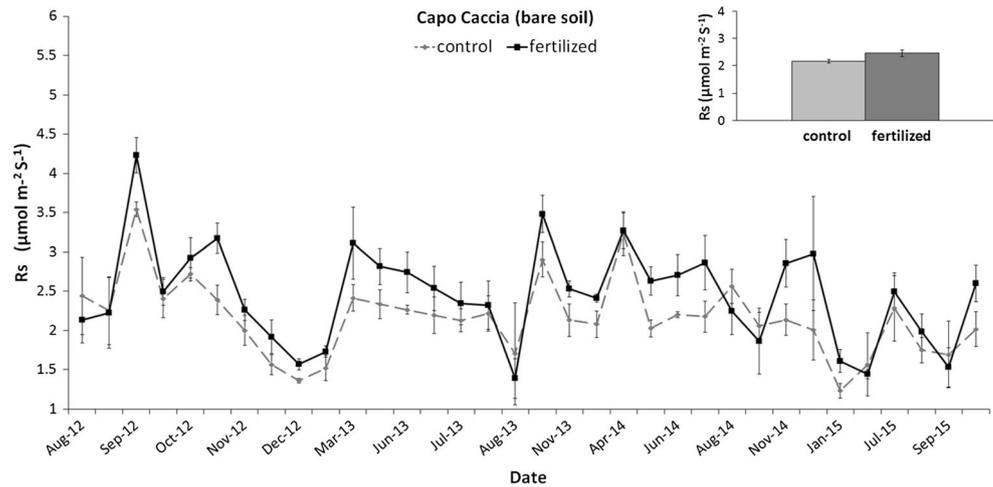
Site and microsite comparison of soil chemistry

Soil pH and NO_3^- in the control plots of BS and UC were significantly lower than in El Regajal (Table 2). Soil NH_4^+ , MBN, and C/N in the control plots in the UC microsite were significantly higher than in El Regajal, whereas DON in the control plots in the BS microsite showed the opposite trend (Table 2).

Capo Caccia

During the experiment, SWC at Capo Caccia followed a seasonal pattern typical of the Mediterranean climate. The BS microsite showed higher SWC in spring, autumn, and winter (26.7 ± 0.3 , 28.1 ± 0.3 , and $35.2 \pm 0.3\%$, respectively) than in summer ($21.0 \pm 0.1\%$; Fig. 4). A similar SWC trend was found for the UC microsite, higher in spring, autumn, and winter (26.1 ± 0.4 , 28.1 ± 0.5 , and $37.7 \pm 0.5\%$, respectively) than in summer ($18.8 \pm 0.1\%$; Fig. 4). Although not significant, SWC was slightly higher in the UC microsite during the rainy season compared to the BS microsite, while this trend was the opposite in the rainless season. Soil temperature in the UC microsite ranged from a maximum of 23.9°C in late July 2015 to a minimum of 6.8°C in mid-February 2012, whereas in the BS microsite, T_s ranged between 32.1°C in mid-July 2015 and 2.5°C in mid-February 2012 (Fig. 4).

Fig. 1 Effect of N addition ($30 \text{ kg N ha}^{-1} \text{ year}^{-1}$) on soil respiration (R_s) in the bare soil microsite at Capo Caccia ($n = 4$). Inset graph shows the grand mean R_s for the fertilized (± 0.11) and control (± 0.07) plots



Although not significant, T_s was slightly higher in the BS microsite compared to the UC microsite, during the rainless period.

Seasonal pattern in R_s at both microsites was dependent on SWC and T_s . In the UC microsite, R_s was negatively and significantly correlated with SWC ($r = -0.15, p = 0.01$), whereas T_s was not significantly correlated with R_s ($r = 0.09, p = 0.11$). In contrast, R_s at the BS microsite was positively correlated with T_s ($r = 0.16, p = 0.01$) but not with SWC ($r = 0.06, p = 0.31$). In the UC microsite, R_s significantly increased under N addition, whereas in the BS microsite, there were no significant differences between the control and 30-kg N treatment (Table 1 and Fig. 2, inset). Consequently, when analyzing both microsites together, N addition resulted in marginally higher R_s (Table 1).

Soil pH in the BS microsite was the only variable within microsite significantly reduced by the addition of N (Table 2).

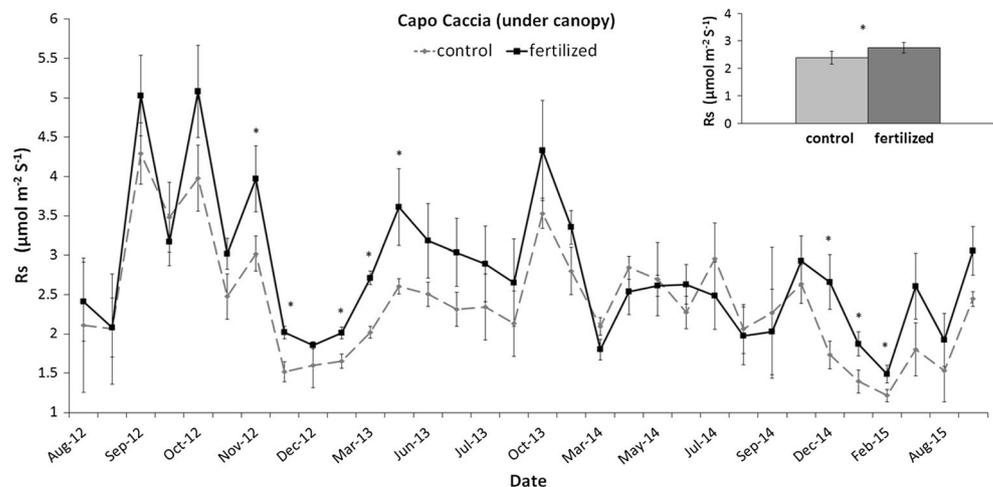
El Regajal

In El Regajal, SWC values were higher in spring and summer (6.7 ± 0.7 and $8.8 \pm 0.4\%$, respectively) compared to early autumn ($6.7 \pm 0.5\%$). Soil temperature at 5-cm depth ranged from $38.5 \text{ }^\circ\text{C}$ in early July 2015 to $3.8 \text{ }^\circ\text{C}$ at the early December 2015 (Fig. 5).

In all treatments, R_s was correlated with changes in both T_s ($r = -0.26, p = 0.01$) and SWC ($r = -0.54, p = 0.01$), which resulted in R_s reaching its maximum during the early growing season before decreasing to lower rates in August and late October (Fig. 3). The treatment effect was not significant in early spring measurements, while all N addition levels (i.e., 10 and $20 \text{ kg N ha}^{-1} \text{ year}^{-1}$) resulted in a significant reduction of R_s ($p < 0.05$ ANOVA, post hoc LSD test) in the dry summer period.

Soil pH showed a not significant decreasing trend responding to increased N doses, while inorganic N content was the only soil chemical property that differed among

Fig. 2 Effect of N addition ($30 \text{ kg N ha}^{-1} \text{ year}^{-1}$) on soil respiration (R_s) under the canopy of *Juniperus phoenicea* L. at Capo Caccia ($n = 4$). Asterisks indicate the significant differences between fertilized and control plots ($p < 0.05$ ANOVA). Inset graph shows the grand mean R_s for the fertilized (± 0.19) and control (± 0.24) plots. Asterisk in the inset indicates the significant differences between fertilized and control plots ($p < 0.05$ GLMM)



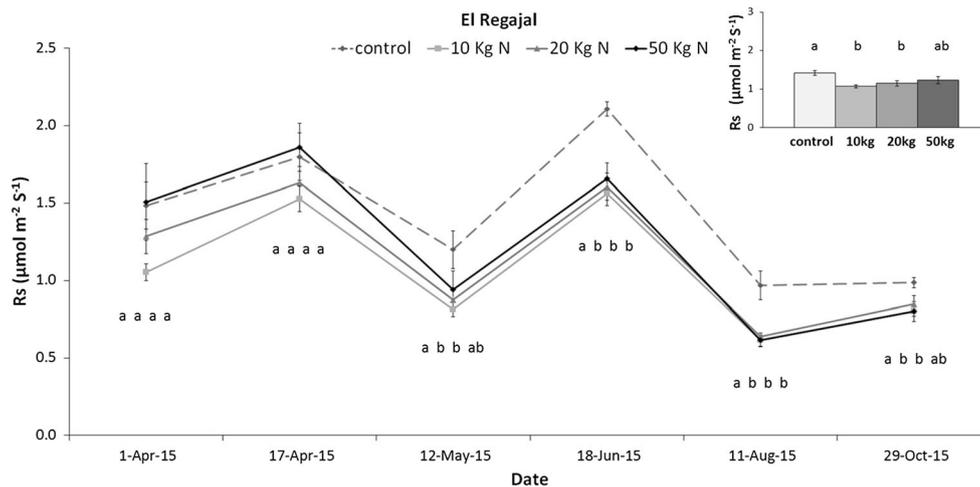


Fig. 3 Mean (\pm SE) soil respiration (R_s) for all N addition treatments (0, 10, 20, and 50 kg N ha⁻¹ year⁻¹) at El Regajal ($n = 6$). Different letters indicate the significant differences among treatments ($p < 0.05$ ANOVA, post hoc LSD test). Inset graph shows the overall mean R_s average for control (± 0.06) and N treatments, 10 (± 0.04), 20 (± 0.07), and 50 kg

N ha⁻¹ year⁻¹ (± 0.10). Different letters in the inset indicate the significant differences among treatments ($p < 0.05$, GLMM, post hoc HSD test). The order of letter for significant differences follow the order of control 10–20–50 kg N ha⁻¹ year⁻¹

treatments and was correlated with the amount of N added, 7.29 ± 0.87 in the control plots to 24.93 ± 3.01 in the plots receiving 50 kg N ha⁻¹ year⁻¹ (Table 2).

Discussion

Our results revealed contrasting responses of R_s to N addition (overall increase at Capo Caccia and reduction at El Regajal), suggesting that dose and duration of N additions as well as seasonal variability of SWC and T_s can modulate the R_s rates. Results from El Regajal were in line with previous studies in temperate (Fan et al. 2014) and semiarid ecosystems (Zhou and Zhang 2014), in which even low N addition doses reduced R_s . In contrast, in Capo Caccia, an increase in R_s was observed in the UC microsite but not in the BS microsite, which suggests that the dose of N addition may not be the most important factor influencing R_s . Instead, the cumulative amount of N added may be more important, and it is only after a certain threshold has been reached that one or more ecosystem properties may shift to a new qualitative and quantitative state. Accordingly, in a recent study, He et al. (2015) found a significant reduction of R_s in response to high N doses (112.5 kg N ha⁻¹ year⁻¹) in a semiarid ecosystem, whereas lower N deposition (37.5 kg N ha⁻¹ year⁻¹), leading to a lower global amount of N provided, significantly increased R_s . Since El Regajal has received N additions for 8 years compared to the 3 years at Capo Caccia, it is possible that N additions initially cause an increase in R_s , and after a long period of N addition, a threshold is reached, resulting in reduced R_s .

The different responses of R_s to N additions in the two sites may also be reflecting the different soil N availability (Zhu et al. 2016) and cationic exchange. Pregitzer et al. (2008) reported

how R_s is influenced by soil stoichiometric characteristics and consequently by the native N availability. However, total N in the control plots was only slightly higher at El Regajal than at Capo Caccia, and this minor difference is not likely to fully explain the different responses at the two sites. Instead, the control plots in El Regajal have an NO₃ concentration that is about five times higher than in Capo Caccia and are also richer in DON. These NO₃ and DON indeed represent two forms of N that are actually used by microbes and roots, suggesting that R_s is inhibited in soils rich in these two N forms which is not reflected by a higher concentration of total N. Additional N may inhibit respiration in already N rich systems, while it could be enhanced in nutrient poor sites.

El Regajal, compared to Capo Caccia, represents a N richer system with a potential higher CUE value. With this stoichiometry, soil microbial fauna may be using a larger share of the available C for growth to the detriment of R_s (Liang et al. 2015). The C:N ratios suggest that the decomposer community could be carbon limited in El Regajal and even more in the UC microsite in Capo Caccia. Such differences are even more evident if instead of total N, NO₃ and MBN are considered. If we assume that in El Regajal, the substrate C:N ratio is lower than the critical value for the decomposer community while the opposite occurs in Capo Caccia, added N would promote a higher CUE (more growth and lower respiration) in El Regajal, while it would promote a lower respiration in the least carbon-limited UC microsite (Manzoni et al. 2012). In addition, possible effects of N addition on ecosystem properties include the acidification of the soil (Ste-Marie and Paré 1999), which in turn may induce a shift in the soil microbial community structure (Treseder 2008) and reduce the catalytic activity of enzymes (DeForest et al. 2004; Sinsabaugh et al. 2008). Reduced C allocation to roots has also been reported as a

Table 2 Soil chemical properties (means ± SE) for each N treatment and microsite (bare soil and under canopy) at Capo Caccia (*n* = 4) and for each N treatment at El Regajal (*n* = 6)

	Bare soil		Under canopy		EL Regajal			
	Control	Treated	Control	Treated	Control	10°N	20°N	50°N
pH	7.5 ± 0.14 a, A	7 ± 0.07 b	7.35 ± 0.14 A	7.5 ± 0.14	8.08 ± 0.08 B	7.91 ± 0.11	7.87 ± 0.12	7.76 ± 0.13
Organic C (%)	3.76 ± 0.24	3.82 ± 0.27	4.07 ± 0.24	4.94 ± 0.20	3.07 ± 0.18	2.70 ± 0.18	2.62 ± 0.16	2.97 ± 0.17
Total P (mg/g)	0.22 ± 0.08	0.27 ± 0.06	0.25 ± 0.05	0.28 ± 0.06	0.25 ± 0.04	0.23 ± 0.03	0.25 ± 0.04	0.26 ± 0.04
C:N	2.13 ± 0.32 AB	2.11 ± 0.36	2.31 ± 0.54 A	2.48 ± 0.63	1.55 ± 0.54 B	1.62 ± 0.61	1.38 ± 0.12	1.46 ± 0.31
Total N (mg/g)	1.48 ± 0.20	1.84 ± 0.20	1.77 ± 0.09	2.07 ± 0.16	2.20 ± 0.16	1.68 ± 0.13	1.90 ± 0.13	2.29 ± 0.15
NH4 (µg/g)	5.07 ± 1.43 AB	5.18 ± 1.65	6.97 ± 1.94 A	6.6 ± 0.73	3.13 ± 1.99 B	1.91 ± 1.13	3.17 ± 2.81	2.94 ± 1.17
NO3 (µg/g)	1 ± 0.43 A	0.64 ± 0.9	0.7 ± 0.74 A	2.18 ± 2.04	5.52 ± 2.17 B	7.07 ± 2.46	13.01 ± 3.72	22.51 ± 6.71
Inorganic N (µg/g)	5.82 ± 0.41	5.65 ± 0.45	7.48 ± 1.30	6.04 ± 2.26	7.29 ± 0.87 a	8.97 ± 1.41 a	16.17 ± 2.09 b	24.95 ± 3.01 c
DON (µg/g)	8.6 ± 0.89 A	10.97 ± 0.51	13.54 ± 8.09 AB	9.55 ± 0.62	16.69 ± 3.55 B	17.8 ± 6.85	16 ± 9.57	17.57 ± 8.24
MBN (µg/g)	167.29 ± 54.03 AB	187.84 ± 66.96	160.92 ± 28.24 A	144.88 ± 45.05	120.78 ± 74.51 B	109.73 ± 47.62	120.31 ± 63.73	98.07 ± 69.76

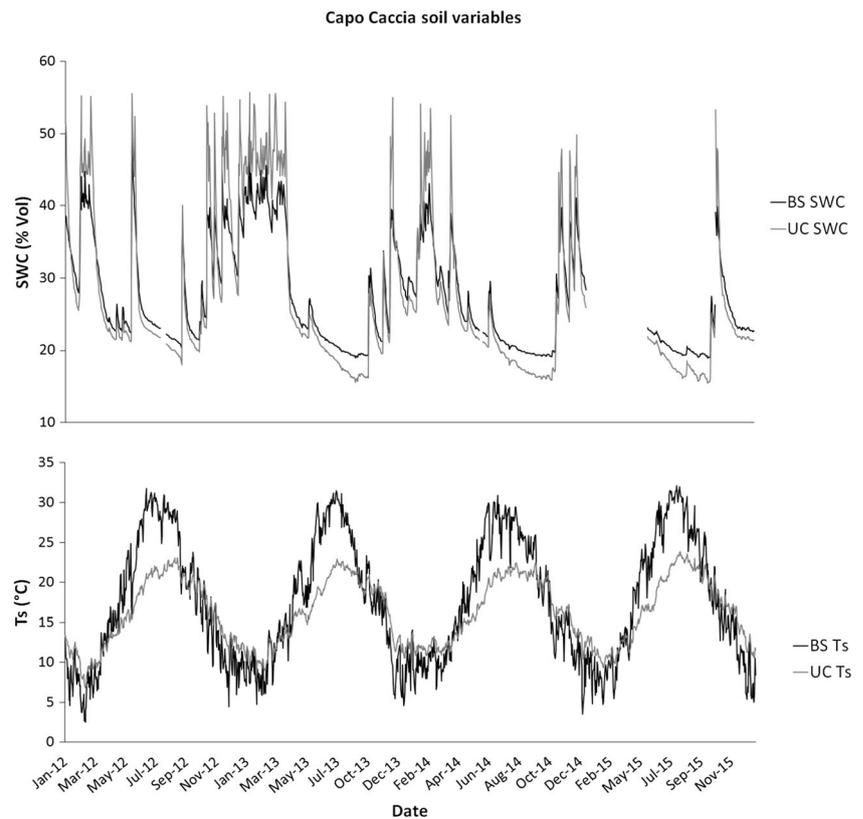
Soil samples were collected in April 2015. Statistical differences are highlighted in italic. Lowercase letters indicate significant differences (*p* < 0.05) between N treatments within the BS and UC microsites at Capo Caccia and among N treatments at El Regajal. Uppercase letters indicate significant differences (*p* < 0.05) among the control of the study microsite-site (one-way ANOVA, post hoc LSD test)

response to added N (Litton et al. 2007), which would reduce the contribution of autotrophic respiration and limit the amount of substrates (root exudates and dead fine roots biomass) for soil microbes.

Higher rates of *R_s* in the UC microsite could also be due to a greater availability of C substrates for microbial communities due enhanced litter fall under shrubs compared to BS microsite. With a high C availability plus the N amendments, the soil microbiota tends to reduce the CUE ratio resulting in a reduction of the potential long-term sequestration but in favor of higher respiration rates (Sinsabaugh et al. 2013). Knorr et al. (2005) performed a meta-analysis on the effects of N deposition on litter decomposition and concluded that N additions may both inhibit or stimulate litter decomposition depending on litter quality (lignin:N and C:N) and N dose applied. Thus, N addition at Capo Caccia may have stimulated litter decomposition by soil microbes which could help in explaining relatively higher *R_s* increase in the UC compared to the BS. Additionally, organic C is a key factor that determines N retention in soils (Barrett and Burke 2002), and therefore, higher organic C in the UC microsite may have led to greater N retention of the added N in the organic soil layer. Conversely, lower soil organic C in BS microsite may have resulted in a greater leaching of the added N, thereby reducing N accumulation in the soil organic layer and its effects on *R_s*. This would be in line with other studies that have shown that up to 40% of added N may be lost from the ecosystem without ever being involved in any chemical transformation and that this fraction of N lost decreases with increasing soil organic content and canopy cover (Barrett and Burke 2002).

The high seasonal variability in soil temperature and water content also differ dramatically depending on the the presence or absence of cover which in turn affects soil properties and plays a key role in modulating *R_s* rates in Mediterranean ecosystems (Maestre and Cortina 2003). As already discussed, the different soil C contents and the presence/absence of litter may well determine different effects of N on *R_s*; however, soil C content did not add further explanation of variance to the GLMM (data not shown). Instead, the results are in line with other studies in semiarid ecosystems that suggest that SWC and *T_s* are the main environmental drivers of *R_s* (Almagro et al. 2009; Morillas et al. 2017). Despite higher *T_s* in the BS microsite at Capo Caccia, *R_s* was slightly higher in the UC compared to the BS microsite. Such differences could be due to greater autotrophic respiration, assuming higher root biomass under shrubs in the UC microsite compared to the BS microsite. Other studies have reported the importance of the contribution of autotrophic respiration to soil CO₂ efflux (Tang and Baldocchi 2005; Han et al. 2007; Hasselquist et al. 2012) with percentage contribution ranging between 10 and 90% and a mean of 48% (Hanson et al. 2000). Most of these previous studies were conducted in boreal or temperate forests, whereas one of the few studies in Mediterranean

Fig. 4 Daily mean soil temperature (T_s) (upper panel) at 5-cm depth measured for the bare soil (BS) and under canopy (UC) microsites and daily mean soil water content (SWC) (lower panel) at 20-cm depth for BS and UC microsites in Capo Caccia. Data collected from January 2012 to December 2015 ($n = 1460$)



ecosystems found that autotrophic respiration accounts for roughly 25% of total soil R_s in a Mediterranean pine forest (Matteucci et al. 2015).

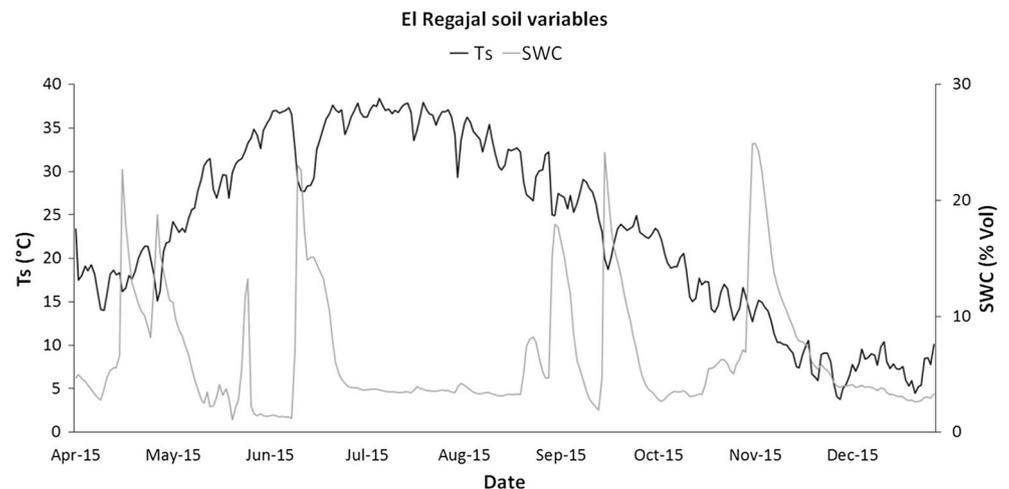
Interestingly, the positive effects of N addition on R_s at Capo Caccia are primarily found when soil moisture is high (above 30%). The lack of an effect of N addition in drier periods suggests that water availability is the main limiting factor for R_s , consistently with other studies (Curiel Yuste et al. 2007) and especially for heterotrophic respiration (Correia et al. 2012). Thus, the effects of N especially on

heterotrophic respiration may be absent during dry periods because microbial activity is strongly inhibited.

Conclusion

Results from this study add some knowledge to the relative scarce information of how the projected increase in N deposition may influence soil C dynamics in semiarid ecosystems in the Mediterranean Basin. This study clearly shows a complex,

Fig. 5 Daily soil temperature (T_s) and soil water content (SWC) at 5-cm depth measured in El Regajal. Data collected from April 2015 to November 2015 ($n = 275$)



non-linear response of R_s to N additions, which is likely the result of the dose and duration of N additions. The addition of N may initially stimulate R_s , whereas over longer time periods, the accumulation of N may reduce R_s . Possibly, the different response observed between the two systems could be due to the contrasting C and N limitations of the decomposer community; while R_s could be inhibited in C-limited sites, it could be enhanced in N-limited sites. However, seasonality of precipitations is the main driver of respiration and largely determines also the effects of N on R_s .

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Appendix

Appendix 1

Table 3 Main characteristics of the study sites Capo Caccia and El Regajal

Site factors	Capo Caccia	El Regajal
Location	Italy, Sardinia 40° 36' 15.2" N, 8° 09' 15.0" E (74 m asl)	Spain, Aranjuez 40° 00' 00.0" N, 3° 36' 00.0" W (600 m asl)
Climate	Subhumid Mediterranean	Semiarid Mediterranean
Ecosystem type	Macchia	Rosemary shrubland
Annual climatic variables	Observation period (1971–2010)	Observation period (1981–2010)
Mean annual temperature (°C)	16.5	20.8
Maximum and minimum temperature (°C)	32.7 and 6.2	40 and 0
Mean annual rainfall (mm)	494	356
Canopy species cover (%)	<i>Juniperus phoenicia</i> L. (<80) <i>Pistacia lentiscus</i> L. (>12) <i>Phyllirea angustifolia</i> L. (>4) <i>Smilax aspera</i> L. (<3) <i>Chamaerops humilis</i> L. (<2)	<i>Quercus coccifera</i> L. (>85) <i>Rosmarinus officinalis</i> L. (<15) Therophytes <i>Limonium echinoides</i> L. <i>Asterolinon linum-stellatum</i> L.
Pedologic substrate	Mesozoic limestone	Cenozoic limestone
Soil type	Lithic Xerorthent Typic Rhodoxeralfs	Gypsum soil

Appendix 2

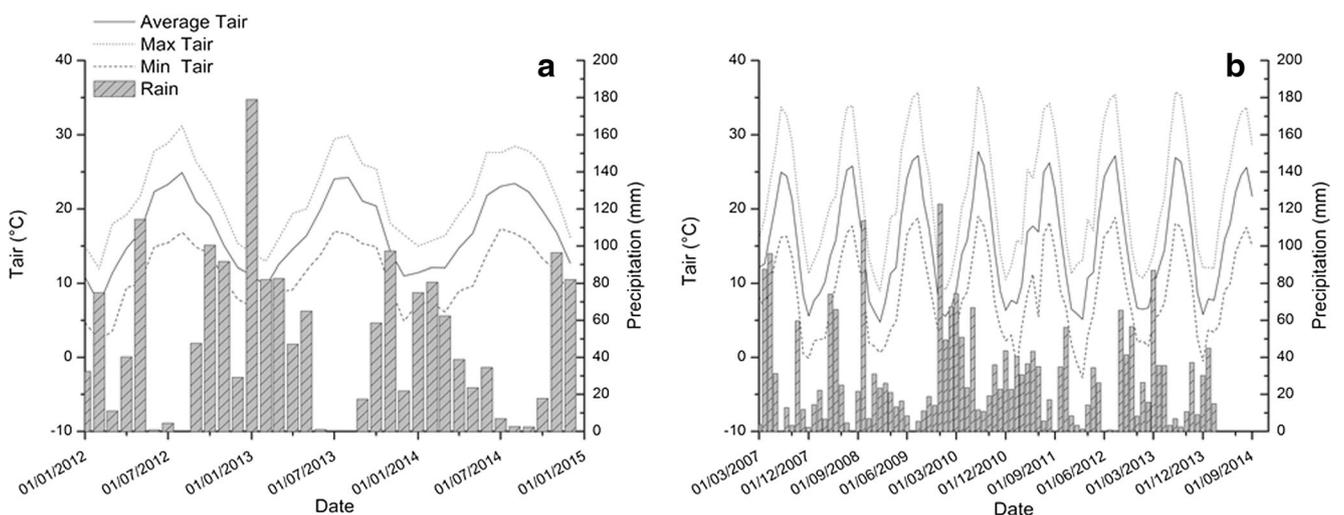


Fig. 6 Air temperature (T_{air}) and precipitation for the two sites since their establishment. **a** Monthly climate data from the meteorological station located within the experimental site of Capo Caccia (January 2012–

October 2015). **b** Monthly climate data from the closest meteorological station to El Regajal (March 2007–September 2015)

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