UNIVERSITÀ DEGLI STUDI DI SASSARI

CORSO DI DOTTORATO DI RICERCA
ScienzeAgrarie

Curriculum: Agrometeorologia ed ecofisiologia dei sistemi agrari e forestali

Ciclo XXIX

A BIOGEOCHEMICAL ANALYSIS OF MEDITERRANEAN ECOSYSTEMS AND THE RESPONSE TO NITROGEN DEPOSITION

An insight on the carbon and nitrogen cycle in Mediterranean Ecosystems

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Anno accademico 2015- 2016

Molto più importante di quello
che sappiamo o non sappiamo è
quello che non vogliamo sapere.

Eric Hoffer

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Ringraziamenti

I documenti per l'iscrizione al concorso arrivati mezz'ora prima della scadenza del bando, il messaggio di mio fratello che mi annunciava la dipartita di nonna Cristina poco prima della partenza Lisbona – Alghero, il primo risultato del concorso non del tutto entusiasmante, insomma una seria di segnali né confortanti né rassicuranti. Invece un giorno si è "ribaltato tutto" una totale inversione degli eventi che mi hanno portato qui, oggi a scrivere questi ringraziamenti. Questi tre anni di dottorato sono volati, è stata in generale una bella fase della mia vita, impegnativa, stimolate ma soprattutto allegra, grazie al mio carattere ma soprattutto alle persone che ho incontrato in questo percorso. Probabilmente qualcuno non sarà citato, in tal caso siete invitati a inoltrare le lamentale alla mia segretaria ☺. Ancora custodisco gelosamente quella carta per l'imballaggio sul quale con Silvana Munzi abbiamo scritto la prima bozza del progetto di dottorato, grazie di cuore per aver creduto nelle mie capacità. Doveroso è il ringraziamento al gruppo del DipNet, ok non proprio a tutto. Chiaramente grazie a Simone Mereu che si dal primo giorno, con fare amichevole mi ha schiavizzato insegnandomi molto dal punto di visto scientifico e (forse non accorgendosene) qualche cosa dal punto di vista umano. Grazie a Lourdes, con la quale tra una incomprensione caratteriale e una culturale in modo amichevole abbiamo sempre collaborato per obbiettivi comuni e grazie per avermi dato molti spunti per la mia crescita professionale. Sento di ringraziare la prof. Donatella Spano, che seppur prettamente impegnata in politica, ha sempre creduto nel lavoro che ho svolto sostenendolo. Questi anni chiaramente sono stati contornati da molta burocrazia, però devo dire che in un modo o nell'altro tutto ha funzionato. Sono rimasto positivamente colpito da tutta la "macchina" dell'ufficio relazioni internazionali dell'UNISS. Tramite diversi programmi d'internazionalizzazione ho potuto collaborare con diverse università e istituti di ricerca esteri. Grazie a Esteban Manrique Reol del CSIC- MSUN (Madrid – Spagna) che tra una uscita in campo e una birretta mi ha amichevolmente guidato durante il periodo di raccolta dati e grazie a Jorge Curiel Yuste per avermi fatto scoprire la parte non turistica della capitale. Rimanendo sulla penisola Iberica ringrazio la prof Cristina Cruz per avermi ospitato nei suoi laboratori e il gruppo de trabalho da Universidade de Lisboa, “tenho sempre muita saudade”. Volando dall’altra parte del mondo sento di ringraziare Raúl Ochoa-Hueso, ho apprezzato molto la sua professionalità e disponibilità a condividere il sapere, cosa non da tutti. Chiaramente ringrazio anche Lilia, Juan e Manuel che tra un sushi night e una gita al mare mi hanno fatto sentire più vicino alla mia cultura anche se lontano migliaia di km da casa. Porterò sempre nel cuore il viaggio in Australia soprattutto perché l’ho condiviso con una persona molto cara, che anche in questa occasione, mi ha dimostrato di essere una persona forte, confermandosi ancora una volta un sostegno importante nella mia vita personale, grazie Giuseppina. Parlando di affetti personali devo ringraziare i miei genitori in generale per aver creduto in me sin dal giorno che mi hanno accompagnato alla stazione di Paola per il mio trasferimento a Bologna. Sono passati quasi 15 anni però tutto è iniziato da quella stazione. Grazie a papà che con i sui consigli e il suo drastico sdrammatizzare, riduce e minimizza le mie incertezze e a mamma che se pur vive in un mondo “fantascientifico parallelo” dispensa saggezza e tenacia 100% calabrese. Però senza dubbio quello che più mi capisce in famiglia e per assurdo più mi contrasta è mio fratello…Ermanno ci siamo già capiti, non credo sia necessario aggiungere altro. Detto ciò voglio ringraziare la Sardegna per avermi accolto, devo dire che mi sento un privilegiato per aver lavorato in posti magnifici come Capo Caccia e aver mangiato tanti piatti buoni (in particolare quelli cucinati dalla signora Maria Grazia). La città di Sassari forse non sarà il mio punto di arrivo, chi lo sa, ma comunque in questi anni mi ha dato molto, però un cinema e soprattutto una piscina a regola d’arte per il mitico #beerpoloteam ancora manco. Ragazzi senza i nostri allenamenti, grigliate e chiaramente birrette, sarebbe stato tutto molto insipido. In conclusione, ma sicuramente non per ordine d’importanza, ma casomai per ordine d’ arrivo, c’è la giovane Sakè, che mi fa capire quando è ora di mollare il lavoro perché lanciare un ramo per giocare è la cosa più importante della vita.

25/04/2017

Capo Caccia experiental site - Alghero, Italy - Lunch break. (Ph M. Lo Cascio)

Chapter 1: Introductive chapter

General introduction

The relatively stable environment of the Holocene has allowed agriculture and consequently society to develop and flourish (Nordhaus et al. 2012). However, the social and economic development model remains largely unaware of the risk of environmental disasters which we are going to meet. The human induced changes to the Earth's climate, land, oceans and biosphere are now so great and so rapid that the concept of a new geological era in which humans are the main driver of change in the Earth systems has been defined the Anthropocene (Zalasiewicz et al. 2011). The exponential growth of human activities result in an increasing pressure on the Earth systems, destabilizing the biophysical balance and triggering abrupt or irreversible environmental changes that would be catastrophic for the welfare of the planet and of man himself. Recently, a new approach to global sustainability has been proposed setting planetary boundaries that should not be transgressed if we are to avoid unacceptable global environmental change. A preliminary analysis indicates that humanity has already transgressed three of these boundaries, namely climate change, the rate of biodiversity loss, and the rate of interference with the nitrogen (N) cycle (Rockström et al. 2009).

About 80% of the Earth's atmosphere is triple-bonded N gas (N_2), but this huge reservoir of N is not biologically available, to most organisms. The natural N_2 fixation, estimated around ~ 100 Tg N yr^{-1} for the terrestrial ecosystems (Galloway and Cowling 2002), is accomplished mainly by some microorganisms that, thanks to their specific metabolism, can produce biologically active reduced forms of N such as ammonia (NH3), amines, and amino acids, the structural constituents of proteins (e.g. enzymes) and nucleic acids of every living organism. (Vitousek et al. 1997; Vitousek et al. 2002). The human modification of the N cycle is profound (Galloway et al. 2013), since the beginning of the industrial revolution the natural N cycle has been rapidly accelerating because of human activities.

During the late 19th century, farmers used many natural sources of N fertilizers, including, livestock manure, guano and mineral nitrate deposits. However, in 1909, the German scientist Fritz Haber developed a process to synthetize ammonia at a small scale. Few years later, in 1913 the German chemist and engineer Carl Bosch, extended the Haber's processes to an industrial scale (Galloway et al. 2013). These processes currently convert more atmospheric N_2, into reactive forms (~ 140 Tg N yr^{-1}) than all the natural Earth's terrestrial processes combined (Galloway and Cowling 2002).

The main forms of nitrogenous air pollutants include nitric oxide (NO), nitrogen dioxide (NO_2) and ammonia (NH_3) as dry deposition, and nitrate (NO_3^-) and ammonium (NH_4^+) as wet deposition (Boring et al. 1988; Camargo and Alonso 2006). Once the reactive N (Nr) is introduced in the ecosystems, it is subject to a sequence of effects defined as the N cascade (Galloway et al. 2003). The same atom of Nr can move along its biogeochemical pathway causing multiple effects in the atmosphere, in terrestrial ecosystems, in freshwater and marine systems, and on human health. The negative impacts are numerous:

- eutrophication of oligotrophic ecosystems (i.e. nutrient poor), altering their biodiversity and functioning, often in the form of higher net primary productivity and lower species diversity (Jones et al. 2014), especially losses of plants adapted to efficient N use.
- Acidification of soils produces toxicity effects on organisms due to exceedance of biological and chemical thresholds of soil pH and increased mobilization of toxic ions such as $Al^{3+.}$ (Barak et al. 1997).
- Through leaching process, groundwater contamination by nitrate, with negative impacts on human health (Jones et al. 2014).
- Overall reduction in the respiration rate of soil microorganisms, although this response is not universal and may depend on the ecosystem observed (Treseder 2008).

- Nr depositions modulate the stoichiometry of soil extracellular enzymatic activity. The response is not linear and it depends on the cycling of different nutrients such as carbon (C), N or phosphorus (P) (Sinsabaugh et al. 2008; Wang et al. 2015).
- Slower microbial decomposition, due to changes in bacterial community composition, and a shift from bacterial to fungal dominance of the decomposer community (Rousk et al. 2010).
- Formation of greenhouse gas, contributing to the climate change (CC) process. There is a high correlation between greenhouse gas effect emissions and Nr production.

Moreover, Nr's unwanted consequences can be further aggravated by CC, and vice versa. It should be evident that humans must drastically reduce anthropogenic N inputs to the environment to avoid ecological and toxicological effects, especially given the great uncertainties of how these effects may interact with changes in global climatic conditions (Suddick et al. 2013). In a scenario, according with the last IPCC report, where the global mean surface air temperature has increased by 0.3°C to 0.6°C over the last 20th century and a reduction of the precipitation is predicted (Cubasch et al. 2013), the adaptation of ecosystems to the cumulative effects of multiple stressors is a major challenge of current ecological research (Serengil et al. 2011).

From one hand, according to Davidson and Janssens (2006) higher temperatures will provide sufficient activation energy for rapid substrate degradation via enzymatic reactions by soil microbes, resulting in a higher CO_2 soil emission which amplify the effects of global warming. On the other hand contrasting effects are reported in semiarid areas, where water availability is a key driver for ecosystem functioning (Sherman et al. 2012). Due the increased levels of infrared radiation on Earth's surface from global warming have elevated the rate of evaporation. The dry soil conditions limit microbial activity and prevent nutrient diffusion, both of which are unfavorable for soil respiration (Falloon et al. 2011).

Overall it is widely recognized that CC and air pollution are both affecting ecosystem health and vitality (De Marco et al. 2014). In addition the high seasonal variability specific of the Mediterranean climate (García-Ruiz et al. 2011) promotes an increase of the N dry deposition which becomes bioavailable with the first winter rain event (Fenn et al. 2003; Ochoa-Hueso and Manrique 2011). This increased load of nutrients could have an impact on plant communities reducing the biodiversity (Báez et al. 2007), particularly of the nitrogen-sensitive species and negatively interfering with soil processes due a nutrients overload (Fenn et al. 203; Allen et al. 2007). The most important links between Nr deposition and climate include:

- N_2O formation (a strong greenhouse gas) during industrial processes (e.g. fertilizer production), combustion, or microbial conversion of substrate containing N.
- Ground level O_3 formation from NOx. O_3 is the third most important greenhouse gas.
- Nr affects net CO_2 uptake from the atmosphere in a positive direction (by increasing productivity or reducing the rate of organic matter breakdown) and negative direction (in situations where it accelerates organic matter breakdown).

Effort must be continued if we wish to assess the full significance of nitrogenous atmospheric pollutants. These knowledge gaps constitute an important source of uncertainty in several applicative researches for ecosystem management, impact and adaptation strategies to climate change at regional and global scale. To fill this knowledge gap, it is crucial to understand the effect of increased N deposition on ecosystem processes over a representative range of ecosystem. The effect of realistic N loads should be quantified by long-term experimental manipulation of N inputs in unaffected and affected areas. These data would allow the improvement of the biogeochemical cycles modeling processes, necessary to calculate thresholds (critical load) for the long-term effect of atmospheric N deposition and therefore to develop realistic and accurate strategies for reducing N pollutants.

General objectives

Nitrogen atmospheric pollution and CC are synergically and dramatically altering the structure and function of the ecosystems. The impacts on ecosystem stability need to be better understood to develop suitable adaptation and mitigation strategies, both for managed and natural ecosystems. Drylands ecosystems, which cover about 41% of Earth surface (Castillo-Monroy et al. 2011), are particularly under-represented in worldwide networks and scientific literature investigation quality and quantity of the effect of global change. As consequence, environmental policies to protect these important and vulnerable ecosystems are notably inadequate. Moreover, modelling the biogeochemical cycles in Mediterranean ecosystems is rather difficult task as these ecosystems present a complex net of reciprocal relations between the microclimate and vegetation cover. Indeed these ecosystems are often characterized by heterogeneous vegetation cover giving rise to open patches of bare soil of differing sizes and shapes (Rundel and Cowling 2000) that determine strong spatial microclimate variation (e.g. net radiation or soil temperature) and a heterogeneous nutrient distributions. In turn, this spatial variation gives rise to an incredible number of potential niches that are occupied by different species. Additionally these species may be reciprocally neutral, may compete or determine facilitation processes (Chesson et al. 2004).

Besides high biodiversity and spatial complexity, modelling processes in Mediterranean ecosystems is further complicated by the great climatic seasonality, that determines a shift in main ecosystem drivers as temperature and water availability (García-Ruiz et al. 2011). Indeed the results of previous study are contradictory, some studies show that the N excess adversely affects the composition and biodiversity of the biological crust (Ochoa-Hueso and Manrique 2011) and also affects the soil C pool conditioning the organic matter decomposition and soil respiration (Nave et al. 2009; Liu and Greaver 2010). On the other hand, Hagedorn et al., (2012) demonstrated that these effects could be temporary and Pregitzer et al., (2008) proved that they can be correlated to the spatial heterogeneity of the system affected.

On these bases, the main objective of this thesis was to investigate the effects of temperature, water availability, and particularly N deposition on the C cycle in Mediterranean ecosystems to reduce uncertainties in the knowledge and help the establishment of suitable environmental policies.

Specifically, the manuscript is structured around four main chapters, all of them published or in preparation to be published as papers in peer-reviewed international scientific journals. In addition, most of the works, specifically chapters 2, 3 and 4, has been developed with the collaboration of the institutions and experimental sites included within the NitroMed network (see below).

In chapter 2, a literature survey reports on the main atmospheric pollutants of the Mediterranean Basin (N and Ozone), highlighting the main knowledge gaps and consequently underlying the need to reinforce the coordinated research and implement experimental networks (e.g. NitroMed). Successively in chapter 3 have been studied the effect of the soil biological crust (biocrust), an important modulator of the C cycle in the Mediterranean ecosystems, modeling its contribution to soil CO_2 efflux and showing how the biocrust metabolism respond mainly to variation of soil temperature and soil humidity. Then the thesis work continues studying, through the NitroMed experimental network, the effect of the N deposition on soil CO_2 emissions (Chapter 4) and the cause-effect mechanisms that determine changes in litter decomposition under different N loads (chapter 5).

The NitroMed experimental network

The first Caper*med* (Committee on Air Pollution Effects Research on Mediterranean Ecosystems) meeting (Lisbon 3rd – 4th July 2014) highlighted the impelling need to implement coordinated research platforms, along with broader networks of environmental monitoring, to effectively predict and quantify the real consequences of atmospheric pollution and its effects in Mediterranean ecosystems. In general, experimental networks bring many advantages such as support and collaboration for individual scientists, the ability to assess the general applicability of results due the additional statistical power and opportunities to explore interactions with other factors such as climate, ecosystem and soil type and in our specific case, different N loads.

NitroMed is a recent network (Lo Cascio et al. 2016), consisting of three N-manipulation sites located in the Mediterranean Basin (Fig. 1). The main aim of NitroMed is providing "field laboratories" to investigate the effects of N pollution and CC on structure and functioning of Mediterranean ecosystems sharing protocols, experimental design and, whenever possible, equipment.
All three sites present similar characteristics:

- are located in a Mediterranean biome with spontaneous scrub vegetation;
- they host long term experiments (more than 5 years).
- added N loads simulate predicted future loads in the Mediterranean Basin (Phoenix et al. 2006).

The three experimental sites are characterized by different forms and N load:

- Arrábida (Lisbon, Portugal), 40, 80 (kg N $_{ha}^{-1}$ yr^{-1}) in form of NH_4NO_3 and 40 (kg N ha^{-1} yr^{-1}) in N-NH4+ form.
- El Regajal (Madrid, Spain), 10, 20,50 (kg N ha^{-1} yr^{-1}) in form of NH_4NO_3.
- Capo Caccia (Alghero, Italy), 30 (kg N ha^{-1} yr^{-1}) in form of NH_4NO_3.

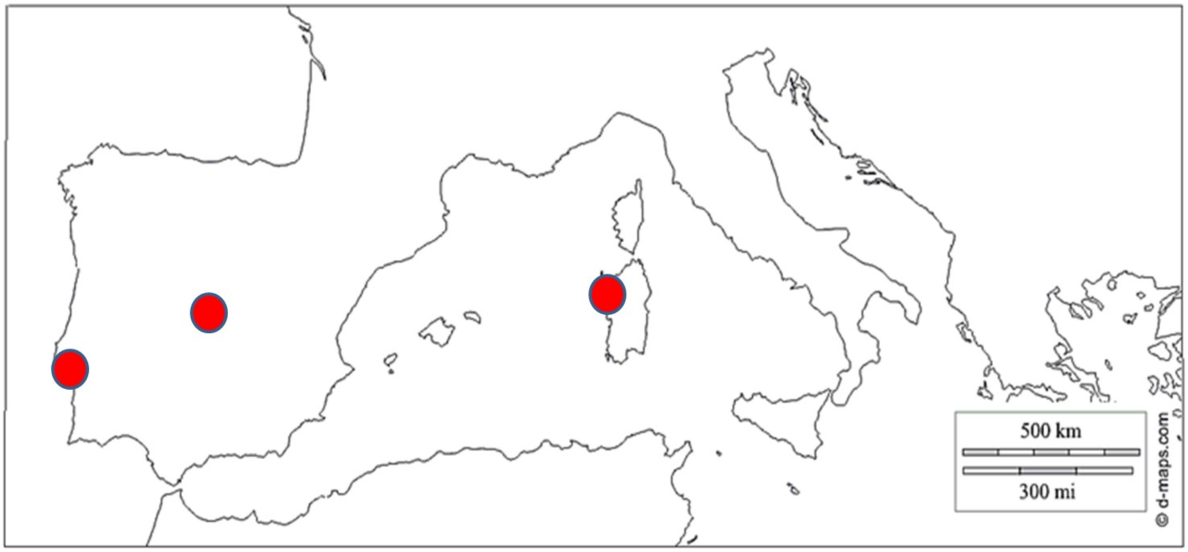

Figure 1: The Mediterranean Basin and the location of the three experimental sites involved in the NitroMed network

Specific objective

Chapter 2 - *Ecological Impacts of Atmospheric Pollution and Interactions with Climate Change in Terrestrial Ecosystems of the Mediterranean Basin: Current Research and Future Directions.*

Manuscript published: *Environmental Pollution (2017)* 227: 194–206.

Most of the studies conducted in the Mediterranean Basin have focused on one global change driver alone but studies on the interaction among global change drivers (e.g. N deposition, O_3 and climate change) are very scarce. During the first Caper*med* 2014, 1st meeting (Lisbon 3rd – 4thJuly), through an extensive bibliographic revision and thanks to an active collaboration among experts on atmospheric pollution and climate changes effects on Mediterranean basin, we had the opportunity to identify the main knowledge gaps and propose a series of possible approach to the issues.

Chapter 3 - *Contribution of biological crust to soil CO2 efflux in a Mediterranean shrubland ecosystem.*

Manuscript published*: Geoderma (2017) 289:11–19.*

The Mediterranean Basin is one of the most vulnerable regions in the world to the impact of CC (Giorgi and Lionello 2008). According to the predicted climate scenarios (IPCC 2007), a pronounced decrease in precipitation and a pronounced warming is expected in this area in the next future. Even if the acknowledgement of the interlink between biosphere, C and water cycles with global and regional climate has been prompted in the last 60 years (Gonzalez-Meler et al. 2014), research on the topic in Mediterranean ecosystems is still scarce. For example, only 8% of articles describing soil CO_2 efflux represent semiarid ecosystems (Castillo-Monroy et al. 2011). In this chapter, through a manipulative experiment, has been developed a soil CO_2 efflux model for the different sub systems that typically compose the Mediterranean site with heterogeneous cover: soil biological crust (BC), bare soil (S), and BC+S. It is also how the biocrust regulates the relationship between CO_2 emission and two soil variables soil temperature and soil water.

Chapter 4 - *Contrasting effects of nitrogen deposition on soil respiration in two Mediterranean ecosystems.*

Manuscript published*: Environmental Science and Pollution Research*
Special issue: "*(E)merging directions on air pollution and climate change research in Mediterranean Basin ecosystems* "

Since the beginning of the industrial revolution, and with the discovery of the Haber-Bosch reaction, the natural N cycle has been accelerating rapidly by human activities. (Galloway et al. 2013). Contrasting are the result how the N pollution modulate the soil CO_2 emission (Zhou et al. 2014), and from our knowledge, no scientific work describes this process in the Mediterranean Basin. In this chapter aiming to reduce this level of uncertainty, soil respiration (Rs) has been measured in two long term N manipulation experimental sites. The datasets have been analyzed with generalized mix model approach, describing how Rs respond to the different N load and to soil temperature and soil water content, two important modulators of the decomposition processes.

Chapter 5 - *Nitrogen do affect soil microbial community abundance and activity with consequence for decomposition and potential carbon sequestration.*

Publish as conference paper: II Caper*med* 2016 - Brescia (IT) and
XIV MEDECOS 2017 - Seville (SP)

Increased N deposition derived from human activities such as fossil fuel combustion and crop and livestock production is a global threat to terrestrial ecosystems worldwide. This excess in atmospheric N deposition can have severe effects on ecosystems, such as nutrient imbalances and soil acidification. In addition, recent works about the effect of N addition on C soil cycling showed that increased soil N can affect C storage, due a strong effect of the pollutant on the soil microbial fauna activity (Manzoni et al. 2012; Sinsabaugh et al. 2013). In this work, we used the structural equation modeling, a powerful statistical tool, to test the effect of different N load, on the C mineralization rate, including in the analysis the complex cause-effect relationship among some different component of the ecosystem, e.g. microbial composition and activity and soil stoichiometry.

People, institution and sites involved for each chapter

Chapter 2 - *Ecological Impacts of Atmospheric Pollution and Interactions with Climate Change in Terrestrial Ecosystems of the Mediterranean Basin: Current Research and Future Directions*

This chapter is a review on the main environmental stressors in the Mediterranean ecosystems. It is the outcome of the Caper*med* 2014, 1st meeting (Lisbon 3rd – 4thJuly). Any field and lab experiment were planned to this chapter.

Chapter 3 - *Contribution of biological crust to soil CO2 efflux in a Mediterranean shrubland ecosystem*

The third chapter has been achieved using data acquired at the experimental site of Capo Caccia, Alghero, (Italy). Data acquired from June 2013 to January 2015.

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Chapter 4 - *Contrasting effects of nitrogen deposition on soil respiration in two Mediterranean ecosystems*

The fourth has been achieved using data acquired from two experimental sites:

- Capo Caccia, Alghero, (Italy). (Data acquired from August 2012 to October 2015).
- El Regajal-Mar de Ontígola, Madrid (Spain). (Data acquired from April to October 2015).

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Chapter 5 - *Nitrogen do affect on soil microbial community abundance and activity with consequence for decomposition and potential carbon sequestration*

Test the overall effects of N on Mediterranean ecosystems by analysing its effect on the correlations among some of its principal components. The fourth chapter will be achieved using data acquired from March 2015 to June 2015 during my short stay abroad.

Three experimental sites are involved:

- Capo Caccia, Alghero, (Italy).
- El Regajal-Mar de Ontígola, Madrid (Spain).
- Arrábida Natural Park, Setúbal (Portugal).

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Chapter 2: Ecological impacts of atmospheric pollution and interactions with climate change in terrestrial ecosystems of the Mediterranean Basin: current research and future directions

Manuscript published: *Environmental Pollution (2017)* 227: 194–206.

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Abstract

Mediterranean Basin ecosystems, their unique biodiversity, and the key services they provide are currently at risk due to air pollution and climate change, yet only a limited number of isolated and geographically-restricted studies have addressed this topic, often with contrasting results. Particularities of air pollution in this region include high O_3 levels due to high air temperatures and solar radiation, the stability of air masses, and dominance of dry over wet nitrogen deposition. Moreover, the unique abiotic and biotic factors (e.g., climate, vegetation type, relevance of Saharan dust inputs) modulating the response of Mediterranean ecosystems at various spatiotemporal scales make it difficult to understand, and thus predict, the consequences of human activities that cause air pollution in the Mediterranean Basin. Therefore, there is an urgent need to implement coordinated research and experimental platforms along with wider environmental monitoring networks in the region. In particular, a robust deposition monitoring network in conjunction with modelling estimates is crucial, possibly including a set of common biomonitors (ideally cryptogams, an important component of the Mediterranean vegetation), to help refine pollutant deposition maps. Additionally, increased attention must be paid to functional diversity measures in future air pollution and climate change studies to stablish the necessary link between biodiversity and the provision of ecosystem services in Mediterranean ecosystems. Through a coordinated effort, the Mediterranean scientific community can fill the above-mentioned gaps and reach a greater understanding of the mechanisms underlying the combined effects of air pollution and climate change in the Mediterranean Basin.

Introduction

Human activities and natural processes have shaped each other over ca. eight millennia within Mediterranean Basin ecosystems (Blondel, 2006). This coevolution, together with the heterogeneous orography and geology, the large seasonal and inter-annual climatic variability, the refuge effect during the last glaciations, and the crossroad location between European temperate ecosystems and North African and Asian drylands, has resulted in the high diversification of the flora and fauna that we observe today, making Mediterranean ecosystems a hotspot of biodiversity, but also of vulnerability (Schröter *et al.* 2005; Blondel 2006; Phoenix *et al.* 2006). Moreover, the Mediterranean Basin is one of the world's largest biodiversity hotspots and the only one within Europe, otherwise dominated by temperate natural and semi-natural grasslands, temperate deciduous forests and boreal conifer forests (Myers *et al.*, 2000). Species-rich ecosystems exclusive to the Mediterranean Basin include Spanish *matorrales* and *garrigas*, Portuguese *matos*, Italian *macchias*, Greek *phryganas*, and agrosilvopastoral ecosystems of high natural and economic value such as Spanish *dehesas* and Portuguese *montados* (Cowling *et al.*, 1996; Blondel, 2006). However, the biodiversity and other ecosystem services of this region are currently at risk due to human pressures such as climate change, land degradation and air pollution (Schröter *et al.*, 2005; Scarascia-Mugnozza & Matteucci, 2012). Air pollution in the Mediterranean Basin is primarily in the form of particulate matter, nitrogen (N) deposition and tropospheric ozone (O_3) (Paoletti, 2006; Ferretti *et al.*, 2014; García-Gómez *et al.*, 2014). Production of pollutants is mainly associated with industrial activities, construction, vehicle emissions and agricultural practices and, within the European context, is characteristically exacerbated by more frequent droughts and the typical stability of air masses in the region, with important consequences for ecosystem and human health (Millán *et al.*, 2002; Vestreng *et al.*, 2008; Izquieta-Rojano *et al.*, 2016a). This also has important social consequences for the Mediterranean region, where approximately 480 million people live, and where more frequent droughts, extreme climatic events and wildfires will only reinforce the current migrant and humanitarian crisis (Werz & Hoffman, 2016).

Environmental pollution interacts synergistically with climate change (Alonso *et al.*, 2001, 2014; Bytnerowicz *et al.*, 2007; Sardans & Peñuelas, 2013). This is particularly true for seasonally dry regions like the Mediterranean Basin (Baron *et al.*, 2014), but the effects of this interaction on the structure and function of Mediterranean ecosystems are not adequately quantified and, therefore, the consequences are poorly understood (Bobbink *et al.*, 2010; Ochoa-Hueso *et al.*, 2011). Projections for 2100 suggest that mean air temperatures in the Mediterranean Basin region will rise from 2.2°C to 5.1°C above 1990 levels and that precipitation will decrease between –4 and –27% (Christensen *et al.*, 2007 and Figure 1). The sea level is also projected to rise, and a greater frequency and intensity of extreme weather events (e.g., drought, heat waves and floods) are expected (EEA, 2005). These changes will exacerbate the already acute water shortage problem in the region, particularly in drylands (Terray & Boé, 2013; Sicard & Dalstein-Richier, 2015), impairing their functionality and ability to deliver the ecosystem services on which society and economy depend (Bakkenes *et al.*, 2002; Lloret *et al.*, 2004). Functions that will be synergistically impaired by air pollution and climate change include reductions in crop yield and carbon sequestration (Maracchi *et al.*, 2005; Mills & Harmens, 2011; Shindell *et al.*, 2012; Ferretti *et al.*, 2014). In addition, a higher fire risk is attributed to higher temperatures and more frequent droughts coupled with an N-driven increase of grass-derived highly-flammable fine fuel (Pausas & Fernández-Muñoz 2012).

In the last decades, atmospheric concentrations of major anthropogenic air pollutants such as particulate matter and sulphur dioxide (SO_2) have decreased in Southern Europe due to emission control policies and greener technologies (Querol *et al.*, 2014; Barros *et al.*, 2015; Aguillaume *et al.*, 2016; Àvila & Aguillaume, 2017). However, mitigation strategies have not been equally effective with other compounds such as reactive N and tropospheric O_3 (Figure. 2; Paoletti, 2006; García-Gómez *et al.*, 2014; Sicard *et al.*, 2016). For example, recent increases in N deposition, particularly dry deposition of NO_3, have been detected in North-eastern Spain, where N deposition is estimated in the range of 15-30 kg N ha^{-1} yr^{-1} (Avila & Rodà, 2012; Camarero & Catalan, 2012; Aguillaume *et al.*, 2016). This has been attributed to increased nitrogen oxide (NO_x) and ammonia (NH_3)

emissions and changes in precipitation patterns (Aguillaume *et al.*, 2016). Background O_3 pollution is typically high in Mediterranean climates due to the meteorological conditions of the area (Paoletti, 2006) and recent reviews have demonstrated that while O_3 in cities has generally increased, no clear trend, or only a slight decrease, has been detected in rural areas (Sicard *et al.*, 2013; Querol *et al.*, 2014); the annual average at rural western Mediterranean sites over the period 2000-2010 was 33 ppb, with a modest trend of -0.22% year^{-1} (Sicard *et al.*, 2013). The Mediterranean Basin is also exposed to frequent African dust intrusions, which can naturally increase the level of suspended particulate matter and nutrient deposition, changing the chemical composition of the atmosphere (Escudero *et al.*, 2005; Marticorena & Formenti, 2013; Àvila & Aguillaume, 2017). This has profound impacts on the biogeochemical cycles of both aquatic and terrestrial ecosystems (Mona *et al.*, 2006), further exacerbating the negative consequences of air pollution and climate change on ecosystem and human health.

In this review, originated as a result of the 1st CAPER*med* (Committee on Air Pollution Effects Research on Mediterranean Ecosystems; http://capermed.weebly.com/) Conference in Lisbon, Portugal, we (i) summarize the current knowledge about atmospheric pollution trends and effects, and their interactions with climate change, in terrestrial ecosystems of the Mediterranean Basin, (ii) identify research gaps that need to be urgently filled, and (iii) recommend future steps. Due to lack of information for other regions within the Mediterranean Basin, we mainly focused our review on studies carried out in south-western European countries (France, Italy, Portugal and Spain). In contrast, we discuss information generated through a variety of experimental approaches (field manipulation experiments, greenhouse studies, open top chambers [OTCs], observational studies, modelling, etc.) from studies carried out in a wide range of representative natural (e.g., shrublands, grasslands, woodlands and forests) and semi-natural (e.g., *montados* or *dehesas*) ecosystems.

Measurement and modelling of atmospheric pollution and deposition

Estimating pollutant deposition loadings, particularly dry deposition, still presents important uncertainties and challenges, both in terms of modelling and measurements (Simpson *et al.*, 2014). This is particularly true in studies at small regional scales and in regions with complex topography or under the influence of local emission sources (García-Gómez *et al.*, 2014), which is very often the case in the Mediterranean Basin. Dry deposition in Mediterranean ecosystems can represent the main input of atmospheric N, contributing up to 65-95% of the total deposition (Figure 2b; Sanz *et al.*, 2002; Avila & Rodà, 2012). For example, wet N deposition at the Levantine border of the Iberian Peninsula can be considered low to moderate (2 - 7.7 kg N ha^{-1} yr^{-1}), but total N deposition loads are comparable to more polluted areas in central and northern Europe (10 - 24 kg N ha^{-1} yr^{-1}) when dry deposition is included (Avila & Rodà, 2012). Given that dry deposition is important in the Mediterranean Basin but is also difficult to measure, we should ideally combine modelled dry deposition with wet deposition measures from representative monitoring stations. A recent modelling analysis has also highlighted that mountain ecosystems in Spain, where monitoring stations are even scarcer, are frequently exposed to exceedances of empirical critical N loads (García-Gómez *et al.*, 2014, 2017). Moreover, mountain areas of the Mediterranean Basin also frequently register very high O_3 concentrations that are not recorded in air quality monitoring networks (Díaz-de-Quijano *et al.*, 2009; Cristofanelli *et al.*, 2015; Elvira *et al.*, under review). This observation should encourage the inclusion of monitoring stations in mountain areas in air quality networks in the Mediterranean Basin to protect these highly valuable and vulnerable ecosystems (García-Gómez *et al.*, 2017). Another important aspect to be considered in both deposition monitoring networks and model-based estimates is the quantification and characterization of ammonium (NH_4^+) and the organic N fraction (Jickells *et al.*, 2013; Fowler *et al.*, 2015). Dissolved organic N (DON) can represent a significant component of wet and dry deposition fluxes but it is often overlooked and not routinely assessed (Mace, 2003; Violaki *et al.*, 2010; Im *et al.*, 2013; Izquieta-Riojano & Elustondo, 2017). However, DON fluxes may have significant implications in terms of critical loads, reaching up to 34-56% of the total N deposition (12 kg DON ha^{-1} yr^{-1}) in Mediterranean agricultural areas (Izquieta-Rojano *et al.*, 2016a). The quantification of temporal trends in air pollution is equally important for evaluating the impact of changing precursor emissions and informing local and regional air quality strategies.

Impacts of atmospheric pollution and climate change on natural and semi-natural terrestrial ecosystems

The ecological impacts of air pollution (particularly for N deposition and O_3) on natural and semi-natural ecosystems have been primarily studied in the temperate and boreal regions of Europe and North America and, more recently, in steppe and subtropical areas of China (Paoletti, 2006; Xia & Wan, 2008; Bobbink *et al.*, 2010; Ochoa-Hueso, 2017). In contrast, much less is known for Mediterranean Basin ecosystems, which differ from these better-studied ecosystems in critical aspects that justify their separate consideration, such as their much-higher levels of biodiversity (particularly for plants) and their higher-than-average levels of biologically-relevant spatial and temporal environmental heterogeneity, including the characteristic summer drought period (Cowling *et al.*, 1996; Myers *et al.*, 2000). Most studies on the impacts of atmospheric pollution in terrestrial ecosystems from the Mediterranean Basin have been carried out in just a small part of the geographic area (i.e. certain localities in Italy, Portugal and Spain) and have used different experimental design and methodologies (Fig. 1 and Supplementary Table 1). Similarly, instead of taking advantage of the development of statistical methods to integrate responses at the ecosystem level (e.g., structural equation modelling; Eisenhauer *et al.*, 2015), studies have typically focused solely and independently on plants (community or, more frequently, individual species), lichens (community or, again more frequently, individual species) and soil properties (soil biogeochemistry, structure and functioning; Supplementary Table 1). One notable exception to this is NitroMed, a unique network of three comparable N addition experimental sites (Capo Caccia [0 and 30 kg N ha^{-1} yr^{-1}], Alambre [0, 40 and 80 kg N ha^{-1} yr^{-1}], and El Regajal [0, 10, 20 and 50 kg N ha^{-1} yr^{-1}]; see Figure 3b, f and h) that is currently using common experimental methodology and structural equation modelling to understand the cause-effect mechanisms that determine changes in gas (CO_2) exchange and litter decomposition and stabilization rates in response to N deposition in semiarid Mediterranean ecosystems (see Ochoa-Hueso and Manrique 2011 and Dias et al. 2014 for further details on experimental methodologies). Preliminary results suggest that N deposition increases soil N availability and reduces soil pH which, in turn, has an effect on microbial community structure (lower fungi to bacteria ratio) and overall enzymatic activity, direct responsible for reduced litter decomposition and higher stabilization rates (Lo Cascio *et al.*, 2016). Similarly, a new coordinated project is looking at the effects of N addition at realistic doses (20 and 50 kg N ha-1 yr-1), in conjunction with P, on alpine ecosystems from five National Parks in Spain. Moreover, most of these studies addressed the impact of one global change driver alone (often increased N availability, mostly the N load, or O_3) and so comprehensive studies on the interaction between global change drivers (e.g., air pollution and climate change) are few. However, recent studies have described a heterogeneous response of annual pasture species to O_3 and N enrichment, with legumes being highly sensitive to ozone but not N, while grasses and herbs were more tolerant to O_3 and more responsive to N (Calvete-Sogo *et al.*, 2016). Thus the interactive effects of O_3 and N can alter the structure and species composition of Mediterranean annual pastures via changes in the competitive relationships among species (González-Fernández *et al.*, 2013 and references therein; Calvete-Sogo *et al.*, 2014, 2016). Similarly, only a few studies have addressed the impacts on edaphic fauna and above- and below-ground biotic interactions such as mycorrhiza, biological N fixation, herbivory or pollination in ecosystems from the Mediterranean Basin (Supplementary Table 1 and references therein), despite the relevance of ecological interactions to healthy, functional ecosystems (Tylianakis *et al.*, 2008). For example, Ochoa-Hueso *et al.* (2014a) found that edaphic faunal abundance, particularly collembolans, increased in response to up to 20 kg N ha^{-1} yr^{-1} and then decreased with 50 kg N ha^{-1} yr^{-1}, whereas 10 kg N ha^{-1} yr^{-1} were enough to completely supress soil microbial N fixation (Ochoa-Hueso *et al.*, 2013a). Another notable exception is Ochoa-Hueso (2016), who showed how even low-N addition levels (10 kg N ha^{-1} yr^{-1}) can completely disrupt the tight coupling of the network of ecological interactions in a semiarid ecosystem from central Spain, despite the lack of evident response of most of the individual abiotic and biotic ecosystem constituents evaluated (i.e., soils, microbes, plants and edaphic fauna). Ozone and N soil availability can also alter volatile organic compound (VOC) emissions, and thus biosphere-atmosphere interactions, of some Mediterranean tree and annual pasture species. The consequences of these interactions need to be further studied (Peñuelas *et al.*, 1999; Llusià *et al.*, 2002; Llusia *et al.*, 2014). Therefore, a more comprehensive and integrative experimental approach is urgently needed to fully capture the real consequences of air pollution in the Mediterranean region.

Sensitivity of Mediterranean forests to air pollution and climate change

Mediterranean forest ecosystems have naturally evolved cross-tolerance to deal with harsh environmental conditions (Paoletti, 2006; Matesanz & Valladares, 2014). However, climate change, N deposition and O_3 are currently threatening Mediterranean forests in unprecedented and complex manners, with consistent stoichiometric responses to increased N deposition (higher leaf N:P ratios; Sardans *et al.* 2016), but with physiological and growth-related consequences forecasted to vary among the three main tree functional types (i.e., conifers, evergreen broadleaf trees, and deciduous broadleaf trees). As deposition increases, photosynthesis, water use efficiency, and thus growth, often increase in conifers (Leonardi *et al.*, 2012), although under chronic N deposition, other nutrients such as P can become more limiting, counteracting the initial benefits of more N availability (Blanes *et al.*, 2013). Nitrogen deposition could also increase pine mortality rates in response to drought due to a decline of ectomycorrhizal colonization rates, a phenomenon of widespread occurrence in US dryland woodlands (Allen *et al.*, 2010). On the other hand, their low stomatal conductance and their high stomatal sensitivity to vapour pressure deficit and water availability might limit the diffusion of O_3 to the mesophyll (Flexas *et al.*, 2014). Similarly, conservative strategies of water and nutrient-use may also play a key role in allowing conifers to keep a positive balance between assimilation and respiration in response to climate change (Way & Oren, 2010). However, O_3 exposure might be impairing their ability to withstand other environmental stresses such as those triggered by drought, high temperature and solar radiation (Barnes *et al.*, 2000; Alonso *et al.*, 2001).

In contrast, evergreen broadleaf species inhabiting resource-poor ecosystems might be jeopardized by N deposition by shifting biomass partitioning (Cambui *et al.*, 2011) and altering allometric ratios (e.g., leaf area/sap wood or root/leaf biomass), which may have consequences for their ability to deal with water stress, particularly in the context of the characteristic summer drought period and climate change (Martinez-Vilalta *et al.*, 2003; Mereu *et al.*, 2009). Ecophysiological responses to O_3 vary from down-regulation of photosystems (Mereu *et al.*, 2009) to reduced stomatal aperture and increased stomatal density (Fusaro *et al.*, 2016) and sluggishness (Paoletti & Grulke, 2005, 2010). However, Mediterranean vegetation usually has efficient antioxidant defences (Nali *et al.*, 2004), which are key factors in O_3 tolerance (Calatayud *et al.*, 2011; Mereu *et al.*, 2011), and is usually known to be more O_3-tolerant than mesophilic broadleaf trees (Paoletti, 2006). Nevertheless, biomass losses and allocation shifts cannot be excluded, especially as a consequence of synergistic effects of N deposition and drought, although local differentiation may result in significant intraspecific tolerance differences (Alonso *et al.*, 2014; Gerosa *et al.*, 2015).

Responses of deciduous broadleaf species to N deposition may be modulated by water and background nutrient availability (mainly P) but, in general terms, growth is favoured over storage (Ferretti *et al.*, 2014). In contrast, broadleaf tree species are highly sensitive to climate change, particularly to the combination of drought and increased temperature (Lopez-Iglesias *et al.*, 2014), which also suggests relevant interactions between air pollution and climate change. In this direction, De Marco *et al.* (2014) predicted that crown defoliation will increase in Mediterranean environments due to drought events and higher temperatures by 2030, a phenomenon that could be exacerbated by excessive N. Deciduous broadleaf species also have lower capacity to tolerate oxidative stress than evergreen broadleaf species due to traits such as thinner leaves and higher stomatal conductance (Calatayud *et al.*, 2010). Gas exchange and antioxidant capacity in deciduous broadleaves are, therefore, generally more affected by high O_3 concentrations than in evergreen broadleaves (Bussotti *et al.*, 2014). Based on their levels of visible foliar injury and expert judgement, deciduous broadleaf species range from highly to moderately sensitive species such as *Fagus sylvatica* and *Fraxinus excelsior*, respectively (Baumgarten *et al.*, 2000; Tegischer *et al.*, 2002; Gerosa *et al.*, 2003; Deckmyn *et al.*, 2007; Paoletti *et al.*, 2007; Sicard *et al.*, 2016), to O_3-tolerant species like some *Quercus* species (*Q. cerris*, *Q. ilex* and *Q. petraea*; Gerosa *et al.* 2009; Calatayud *et al.* 2011; Sicard *et al.* 2016).

Relatively little is known about the effects of O_3 on annual, perennial and woody understory vegetation of Mediterranean forest ecosystems. Under experimental conditions, some species characteristic of the annual grasslands associated with *Q. ilex dehesas* have high O_3 sensitivity. Interestingly, N fixing legumes, of higher nutritional value, are more O_3 sensitive than grasses (Bermejo *et al.*, 2004; Gimeno *et al.*, 2004), particularly in terms of flower and seed production (Sanz *et al.*, 2007), which could affect their competitive fitness and, ultimately, reduce the economic value of the pasture. Nitrogen availability can partially counterbalance O_3 effects on aboveground biomass when the levels of O_3 are moderate, but O_3 exposure reduces the fertilization effect of higher N availability (Calvete-Sogo *et al.*, 2014). Anyhow, given that O_3 levels are higher in summer, when herbaceous species are dormant, Mediterranean species that are summer-active such as pines and oaks are more likely to be directly affected by O_3 than forbs and grasses. This suggests that the seasonality of O_3 concentrations as well as plant phenology and functional type must be considered if we are to fully understand the consequences of air pollution on the highly diverse Mediterranean plant communities. A unique ozone FACE (free air controlled experiment) is now available in the Mediterranean Basin (Figure 3) to help fill this gap (Paoletti et *al.*, in preparation).

Role of environmental context in the response of biodiversity and C sequestration

The local abiotic (e.g., climate, soil properties) and biotic (e.g., vegetation type, community attributes, etc.) contexts are known to modulate ecosystem responses to environmental drivers at different temporal and spatial scales (Bardgett *et al.*, 2013). Given that plant biodiversity at the regional ($10\text{-}10^6$ km^2) and local (< 0.1 ha) scales in Mediterranean ecosystems ranks among the highest in the world (Cowling *et al.*, 1996), this is a particularly relevant aspect for the region. Various studies in Mediterranean ecosystems have shown that increased N availability may have a positive (Pinho *et al.*, 2012; Dias *et al.*, 2014), negative (Bonanomi *et al.*, 2006; Bobbink *et al.*, 2010) or even no effect (Dias *et al.*, 2014) on plant species richness, which is probably due to cumulative effects and modulating factors such as the ecosystem type, the initial N status of the system, the dominant form of mineral N in the soil (NH_4^+, NO_3^-), and/or the N form added. Positive effects on species richness, however, have only been observed in areas characterized by strong environmental stress and low nutrient availability (e.g., open arid and semiarid Mediterranean ecosystems) and are often associated with an increase in nitrophytic and weedy species (Bobbink *et al.*, 2010; Pinho *et al.*, 2011; Dias *et al.*, 2014). The presence and density of shrubs, as well as the availability of inorganic phosphorus (P) and other macro and micronutrients, can also modulate the response of the herbaceous vegetation to N addition and plant invasion in semiarid Mediterranean areas (Ochoa-Hueso *et al.*, 2013b; Ochoa-Hueso & Stevens, 2015). For example, Ochoa-Hueso & Manrique (2014) found that N addition increased the nitrophytic element, particularly native crucifers, only when these species were present in the seed bank in relevant densities and there was sufficient P, whereas a closed scrub vegetation is known to be less susceptible to invasion by N-loving species than open shrublands, woodlands and grasslands (Dias *et al.*, 2014). The role of soil nutrient availability, typically lower than in other Mediterranean-type ecosystems such as those from Chile (Cowling *et al.*, 1996), in the ecosystem response to extra N can also be linked to induced nutrient imbalances, particularly N in relation to P, and therefore to an alteration of ecosystem stoichiometry (Ochoa-Hueso *et al.*, 2014b; Sardans *et al.*, 2016).

The behaviour of terrestrial ecosystems as a global C sink or source under increased N deposition or O_3 pollution scenarios is currently a research hot-topic and is of paramount importance for the mitigation of climate change (Felzer *et al.*, 2004; Reich *et al.*, 2006; Pereira *et al.*, 2007). Recent studies have suggested that seasonally water-limited ecosystems, such as those typically found in the Mediterranean Basin, may have a disproportionately big role in the inter-annual C sink-source dynamics at the global scale due to higher C turnover rates (Poulter *et al.*, 2014); this is attributed to their large inter-annual climatic variability, with unusually wet years contributing to strengthen the terrestrial C sink but where multiple processes like fire or rapid decomposition could result in a rapid loss of most of the accumulated C. These aspects are, however, still poorly understood in Mediterranean ecosystems, where different studies have reported contrasting results (Ochoa-Hueso *et al.*, 2013a, 2013c; Ferretti *et al.*, 2014). In Mediterranean ecosystems, ecosystem C storage should, therefore, be evaluated in terms of altered abundance and patterns of rainfall (both within and between years) (Pereira *et al.*, 2007), in relation to the levels of N saturation (NO_3^-) and toxicity (NH_4^+) in soil (Dias *et al.*, 2014), as well as other site-dependent characteristics such as dominant vegetation, soil type (texture and pH), and stand history and age (Ferretti *et al.*, 2014). Experimental and observational field studies suggest that, at least in the short-term, seasonal and inter-annual dynamics may override any potential effect of atmospheric N pollution, despite potential cumulative negative impacts in the long-term due to an overall decline in ecosystem health (Ochoa-Hueso *et al.*, 2013c; Ferretti *et al.*, 2014).

Although within the Mediterranean Basin there is still a large gap in the knowledge of the impacts of atmospheric pollution and climate change on natural and semi-natural ecosystems, taken together, all the scattered information available suggests the particularly key role of spatial and temporal environmental heterogeneity, biotic interactions, and ecosystem stoichiometry in mediating the ecosystem response to air pollution.

Critical loads and levels

The concepts of critical loads and critical levels were developed within the United Nation Economic Commission for Europe (UNECE) Convention on Long-Range Transboundary Air Pollution (CLRTAP) for assessing the risk of air pollution impacts to ecosystems and defining emission reductions. This tool is commonly used to anticipate negative effects of air pollution and, therefore, to protect ecosystems before the changes become irreversible. The derivation of empirical critical loads for nutrient N is based on experimental activities performed on different vegetation types and they are assigned to habitat classes, while the derivation of NH_3 and NO_x critical levels is based on the responses of broad vegetation types such as higher plants or lichens and bryophytes. The pan-European critical level for atmospheric NH_3 is currently set at an annual mean of 1 $\mu g\ m^{-3}$ for lichens and bryophytes and 3 $\mu g\ m^{-3}$ for higher plants, while the NO_x critical level for all vegetation types is an annual mean of 30 $\mu g\ m^{-3}$ (CLRTAP, 2011). Although some modelling approaches exist to define N critical loads, the identification of empirical critical loads is recommended for Mediterranean ecosystems due to its particularities such as co-occurrence with other pressures and high seasonality (de Vries *et al.*, 2007; Fenn *et al.*, 2011). Empirical critical loads of N for European-Mediterranean habitats have only been proposed for four ecosystems: (1) Mediterranean xeric grasslands (EUNIS [European Nature Information System] E 1.3), 15-25 kg N ha^{-1} yr^{-1}; (2) Mediterranean maquis (F5), 20-30 kg N ha^{-1} yr^{-1}; (3) Mediterranean evergreen (*Quercus*) woodlands (G 2.1), 10-20 kg N ha^{-1} yr^{-1}, and (4) Mediterranean *Pinus* woodlands (G 3.7), 3-15 kg N ha^{-1} yr^{-1} (Bobbink & Hettelingh, 2011). However, these critical loads are based on very little information and are thus classified as expert judgement. Similarly, NH_3 critical levels have only been set for Mediterranean evergreen woodlands and dense holm oak forests. Critical levels of atmospheric NH_3 of < 1.9 and 2.6 µg m^{-3} have been estimated for evergreen woodlands surrounded by intensive agricultural landscapes (Pinho *et al.*, 2012; Aguillaume, 2015), while for evergreen woodlands under little agricultural influence but strong oceanic influence, the critical level was estimated to be 0.69 µg m^{-3} (Pinho *et al.*, 2014). Nevertheless, the N critical loads and NH_3 critical levels for many European-Mediterranean ecosystems remain unstudied, despite their relevance for protecting relatively undisturbed and oligotrophic ecosystems. Therefore, long-term manipulation experiments across a range of typical Mediterranean terrestrial ecosystems are desperately needed to obtain a more complete set of reliable empirical critical N loads and levels for the Mediterranean Basin (Bobbink *et al.*, 2010; Bobbink & Hettelingh, 2011). Ozone critical levels have also been proposed for the protection of natural vegetation at European level for two vegetation types, forests and semi-natural vegetation (CLRTAP, 2011). The new flux-based O_3 critical levels allow species-specific physiological conditions and O_3 uptake mechanisms to be included considering the particularities of Mediterranean species. Interestingly, multiple studies performed with Mediterranean tree species recommend higher O_3 critical levels for the protection of Mediterranean forests than the values currently accepted (Calatayud et al., 2011; Alonso et al., 2014; Gerosa et al., 2015). The possible definition of different O_3 critical levels for different biogeographical regions or vegetation types is currently under analysis within the Convention (CLRTAP, 2011).

Cryptogams as indicators of the impact of air pollution and climate change

Lichens and bryophytes (i.e., cryptogams), very often used in the definition of critical loads and levels, are important components of the vegetation in Mediterranean ecosystems. These organisms are key drivers of ecosystem properties (soil aggregation and stability) and processes (C and N fixation and nutrient cycling), particularly in the case of biological soil crusts (hereafter biocrusts), a functionally-integrated association of cyanobacteria, protists, fungi, mosses and lichens inhabiting the first millimetres of soil (Cornelissen *et al.*, 2007; Maestre *et al.*, 2011). Cryptogams are usually extremely sensitive to environmental changes and so they often provide early-warning indicators of impacts before any other constituent of the ecosystem, particularly in the case of N (Pardo *et al.*, 2011; Munzi *et al.*, 2012). For example, mosses have been used in N deposition surveys under the ICP-Vegetation framework (Harmens *et al.*, 2014). The results showed that N concentration in mosses can potentially be used as an indicator of total atmospheric N deposition. Similarly, Root *et al.* (2013) showed that lichens can be a suitable tool for estimating through fall N deposition in forests. However, the relationship between N deposition and tissue N concentration can also be affected by environmental factors such as local climate and the form of N deposition.

Mosses and lichens have been instrumental to the evaluation of the impacts of global change drivers on temperate and boreal ecosystems (e.g., Arróniz-Crespo *et al.* 2008), though the number of studies carried out in Mediterranean ecosystems is very limited. Recent studies have, however, reported significant impacts of increased N deposition on Mediterranean biocrust and epiphytic communities. For example, two studies carried out in the Iberian peninsula found higher tissue N content and a shift from N to P limitation in the terricolous moss *Tortella squarrosa* (=*Pleurochaete squarrosa*; Ochoa-Hueso & Manrique 2013; Ochoa-Hueso *et al.* 2014a). Similarly, an alteration of physiological and chemical responses in lichen transplants (Branquinho *et al.*, 2010; Paoli *et al.*, 2010, 2015) and a shift in epiphytic lichen communities from oligotrophic-dominated to nitrophytic-dominated species have also been reported in Portugal (Pinho *et al.*, 2008, 2009) and Spain (Aguillaume, 2016). Recent studies have also observed a change in the isotopic N composition of mosses due to the impact of N from fuel combustion sources (shift to more positive $\delta^{15}N$ signature) and agricultural activities (shift to more negative $\delta^{15}N$ signature; Delgado *et al.*, 2013; Varela *et al.*, 2013; Izquieta-Rojano *et al.*, 2016b). Cryptogam traits (e.g., morphology, anatomy, life form) are also strongly connected to water availability. For example, mosses from dry habitats are organized in dense cushions, naturally retaining water by capillarity and dehydrating slowly, whereas mosses from moist habitats have a less dense morphology and require the activation of specific mechanisms to survive during dry periods (Arróniz-Crespo *et al.*, 2011; Cruz de Carvalho *et al.*, 2011, 2012, 2014). Similarly, lichen growth form and photobiont type have been shown to be relevant traits in the response to water availability in Mediterranean areas (Concostrina-Zubiri *et al.*, 2014; Matos *et al.*, 2015). Cryptogam traits related to water availability could, therefore, be equally effective biomarkers to detect climate-induced hydrological changes in Mediterranean ecosystems but the application of biomonitoring techniques using cryptogams in the Mediterranean region may be complicated by the fact that cryptogam species are simultaneously exposed to both severe water restriction and pollution, and some biomarkers (e.g., ecophysiological responses) are similarly affected by both stress factors (Pirintsos *et al.*, 2011). Thus, we need to disentangle the multiple environmental drivers (Munzi *et al.*, 2014a), possibly by integrating physiological and ecological data to understand the specific response mechanisms to different ecological parameters and environmental changes (Munzi *et al.*, 2014b).

Anticipating global tipping points using ecological indicators

The fact that ecosystem responses to air pollution and climate change are very often non-linear may complicate the use of bioindicators in the Mediterranean Basin. Non-linear dynamics often manifest in the form of tipping points, defined as ecosystem thresholds above which a larger-than-expected change happens, shifting ecosystems from one stable state to another stable state (Scheffer & Carpenter, 2003). Due to its climatic peculiarities, tipping points may be particularly relevant for the Mediterranean Basin. One example is the ability of soils to store extra mineral N. Above a certain N deposition value, N-saturated soils will start leaching N down into the soil profile. This excessive N can also accumulate as inorganic N in seasonally dry soils and be leached by surface flows that, as in the case before, will eventually reach and, therefore, pollute aquifers and watercourses (Fenn *et al.*, 2008). Another relevant example is related to increased fire risk due the accumulation of highly flammable leaf litter, particularly from exotic grasses, as a consequence of N deposition; above a certain N deposition threshold the probability of a fire to occur increases exponentially, priming the ecosystem for a state change (Rao *et al.*, 2010).

Despite the potential prevalence of tipping point-like dynamics in Mediterranean ecosystems in response to air pollution and climate change, we are not aware of any vegetation-based tools available to predict ecosystem thresholds in the Mediterranean Basin context. A notable exception is the work by Berdugo *et al.* (2017), who suggested that changes in the spatial configuration of drylands may be an early-warning indicator of desertification. However, we suggest that if we are to aim for universal indicators of environmental change (i.e., at wide geographical ranges) and to account for the role of the environmental context as a driver (i.e., across ecosystem types), functional trait-based approaches (e.g., functional diversity and community weighted mean trait values [CWM]) should be preferred over other widely used indicators, including species richness (Jovan & McCune, 2005; Valencia *et al.*, 2015). Functional diversity and CWM are independent of species identity and may be functionally linked to the environmental variable of interest (e.g., oligotrophic species, nitrophytic species, or subordinate species responding to eutrophication, species-specific leaf litter traits, etc.). More research is, however, needed to integrate these concepts (ecological indicators, ecological thresholds and functional diversity) in a meaningful way.

Linking functional diversity to the provision of ecosystem services

The universal applicability and ecological relevance of the functional trait diversity concept makes it equally valuable to establish possible connections between global environmental change and the loss of ecosystem services. Ecosystem services that may be impaired by air pollution and climate change and that may be particularly associated with changes in functional diversity include C sequestration, soil fertility and nutrient cycling and pollination, among many others. However, research on the link between functional diversity and ecosystem services is lagging behind in the Mediterranean region where only a few controlled experiments exist (Hector *et al.*, 1999; Pérez-Camacho *et al.*, 2012; Tobner *et al.*, 2014; Verheyen *et al.*, 2016), species trait databases are still incomplete (Gachet *et al.*, 2005; Paula *et al.*, 2009), and field surveys along climatic and air pollution gradients are only recently starting to emerge (De Marco *et al.*, 2015; Sicard *et al.*, 2016).

The few studies available within the Mediterranean Basin context have shown that N deposition has already induced changes in functional diversity of epiphytic lichens along a NH_3 deposition gradient in Mediterranean woodlands, with a drastic increase and decrease of nitrophytic and oligotrophic species, respectively, (Pinho *et al.*, 2011). Similarly, a continuous increase of nitrophytic species (plants, lichens, mosses) has been detected in the Iberian Peninsula for the period 1900-2008 using the Global Biodiversity Information Facility (GBIF) database (Ariño *et al.*, 2011). Increased N availability in nutrient-poor ecosystems like Mediterranean maquis can also alter plant functional composition (e.g., higher proportion of short-lived species in relation to summer semi-deciduous and evergreen sclerophylls), leading to changes in litter amount and quality (e.g. higher proportion of evergreen sclerophyll litter from affected shrubs and a general increase in lignin and N content in litter and a decrease in lignin/N ratio) and microbial community (e.g., reduction in biomass and activity), thus affecting nutrient cycling (an ecosystem function) and, therefore, soil fertility (including soil C accumulation, an ecosystem service) (Dias *et al.*, 2010, 2013, 2014). In another study, Concostrina-Zubiri *et al.* (2016) showed that livestock grazing greatly affected the abundance and functional composition of moss–lichen biocrusts in a Mediterranean agro-silvo-pastoral system, with direct negative consequences on microclimate regulation and other ecosystem processes (CO_2 fixation, habitat provision and soil protection). This also affected the cork-oak regeneration processes, one of the traditional and most economically valuable services in these systems. Given the negative impacts of air pollution on cryptogamic biocrusts, a similar effect of air pollution on the cork-oak regeneration processes mediated by biocrusts might be expected.

Common experimental design, data sharing and global networks

The understanding of the ecological impacts of pollution and climate change across the Mediterranean region would improve through co-ordinated efforts and networks, which could take several forms. One possible approach is the use of large-scale regional surveys on existing pollution gradients representative of the current range of pollution loads (e.g., from big cities and/or extensive agricultural areas to their periphery). This approach was successfully used to survey 153 acid grasslands in ten countries across the Atlantic biogeographic zone of Europe (significantly less biodiverse than their Mediterranean counterparts) (Stevens *et al.*, 2010), where each partner surveyed sites in their local area according to an agreed protocol. Other networks have been successful using experimental approaches. For example, the Nutrient Network (NutNet) is a global network of over 90 sites following a common experimental protocol for nutrient addition and grazing (Borer *et al.*, 2014). Similarly, the previously presented NitroMed network, originated within the CAPER*med* platform, aims at using the same experimental protocols to integrate results from three comparable experiments in semiarid Mediterranean ecosystems. Other experimental networks have not used common experimental protocols, but through coordinated analyses have added value to individual experiments (Phoenix *et al.*, 2012). Co-ordinated experimental networks (e.g., low-cost N addition experiments) bring many advantages such as the ability to assess the general applicability of results, additional statistical power resulting from well-established and robust statistical methods (e.g., linear mixed effects models, hierarchical Bayesian models, structural equation modelling), and opportunities to explore interactions with other natural and human-caused gradients such as climate, ecosystem and soil type, land use, atmospheric pollution (including O_3 gradients), etc. They can also provide support and collaboration for individual scientists. An inventory of the existing sites with manipulation experiments in the Mediterranean Basin would provide added value to the individual sites through the implementation of common protocols and experiments.

In the Mediterranean region, another path to follow may be to build upon existing research and to participate more in already existing large-scale initiatives, in which the Mediterranean research community is not particularly well-represented. For example, interacting with the International Long Term Ecological Research (ILTER) network or with the International Cooperative Programme (ICP), established under the United Nation Economic Commission for Europe (UNECE) "Convention on Long-Range Transboundary Air Pollution" (CLRTAP) that includes several initiatives such as ICP Forest, ICP-Vegetation, and ICP-IM, would facilitate the collection of large-scale spatial and temporal data series. Cooperation with other more specific networks like NitroMed (N deposition), ICOS (C cycle), and GLORIA (Alpine environments) would also help to establish a wider and more collaborative research community focused on air pollution impacts in Mediterranean terrestrial ecosystems.

The need of more coordination and investment to better understand the Mediterranean responses to climate change and air pollution has already been acknowledged by several groups of scientists both at the European (e.g. CAPER*med*) and global scales (e.g. MEDECOS). These groups not only represent suitable arenas to discuss scientific results, but can also provide leading members able to manage the above-mentioned research and networking activities. However, all the above mentioned presented approaches require considerable funding and determined political support to foster the exchange of information and best practices across the entire Mediterranean region and, thus, to promote the development of concrete projects and initiatives. In this context, the European Commission, through funding programs like Horizon 2020, could and should have, in our opinion, a pivotal role in supporting research projects (as it happened with the CIRCE project) and to provide the logistic means for transferring the scientific knowledge to the society.

Increasing awareness about the effects of climate change and pollution among stakeholders and society is encouraging the development of several European and Pan-European Programs (e.g. UNECE/ICP, Climate-ADAPT). One important step towards the coordinated action of the Mediterranean-basin countries in relation to Adaptation to climate change was the creation of "The Union for the Mediterranean Climate Change Expert

Group" (UfMCCEG), a partnership promoting multilateral cooperation between 43 countries (28 EU Member States and 15 Mediterranean countries). These initiatives show that opportunities do exist for countries to make progress. Due to campaigning, and partially because of the considerable losses from extreme weather events in recent years, public awareness in Mediterranean countries about risks associated with climate and air pollution increased. Governments and organisations at the EU level, national and sub-national level, have developed or are in the process of developing adaptation strategies. Therefore, there is an opportunity to make progress by actively engaging actors from all sections of the Mediterranean society.

Conclusions and future directions

The comparatively fewer number of studies on the effects of air pollution and its interactions with climate change on terrestrial ecosystems from the Mediterranean Basin is particularly noteworthy considering the high biodiversity, cultural value, and unique characteristics of this region such as high O_3 levels, dominance of dry deposition over wet deposition, and long dry periods. Therefore, we emphasize the need to urgently implement common and coordinated research and experimental platforms in the Mediterranean region along with wider and more representative environmental monitoring networks. In particular, a robust connection between N deposition monitoring networks and modelling estimates is crucial. Ideally, monitoring and assessment programs should regularly include a set of common biomonitors such as local and/or transplanted cryptogams to identify local pollutant sources and, thus, help refine pollutant deposition maps (physiological indicators) and to provide early warning indication of potential critical thresholds (community shifts). Only by filling these gaps can the scientific community reach a full understanding of the mechanisms underlying the combined effects of air pollution and climate change in the Mediterranean Basin and, consequently, provide the science-based knowledge necessary for the development of sustainable environmental policies and management techniques and the implementation of effective mitigation and adaptation strategies. Finally, CAPERmed, a bottom-up initiative (from the researchers to the institutions), can be the longed-for catalyst that brings the Mediterranean community together and, therefore, represents an excellent opportunity to make all this happen.

Acknowledgements

This manuscript resulted from the 1st "Committee on Air Pollution Effects Research on Mediterranean Ecosystems" (CAPERmed) Conference celebrated in Lisbon, 2014 (http://capermed.weebly.com/). All attendants to this conference are, therefore, greatly acknowledged. Fundação para a Ciência e Tecnologia (FCT) through the Post Doc grant SFRH/BPD/85419/2012 to Teresa Dias and the Investigador grant to Silvana Munzi. ADM, EP and PS thank the LIFE10 ENV/FR/208 project MOTTLES. EP thanks also the Fondazione Cassa di Risparmio di Firenze (2013/7956). The authors declare no conflicts of interest.

Figures

Figure 1. Modeled nitrogen deposition for the Mediterranean region based on the European Monitoring and Evaluation Programme (EMEP) model at 0.1º-0.1º longitude-latitude resolution (EMEP MSC-W chemical transport model [version rv4.7; www.emep.int]). Modelled N deposition is based on 2013 emissions data. (a) Total N deposition (oxidized + reduced; dry + wet), (b) percentage of dry deposition, (c) percentage of wet deposition, (d) percentage of oxidized deposition and (e) percentage of reduced deposition.

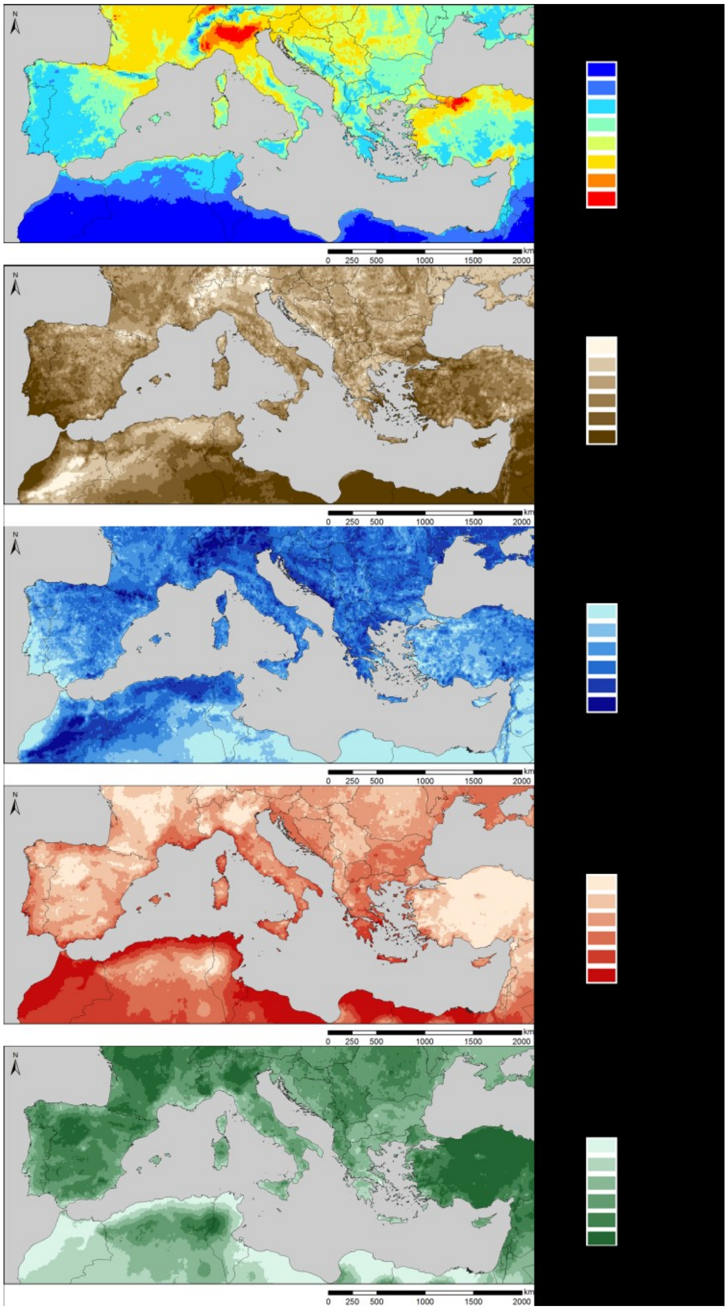


Figure 2. (a) Mean annual precipitation (MAP) and (b) temperature (MAT) for the year range between 1960-1990. Projected (c) MAP and (d) MAT for the year 2070 based on predictions from the CCSM4 model considering the RCP 8.5 (no mitigation of emissions) IPCC5 scenario. Data obtained from http://www.worldclim.org/version1 (Hijmans *et al.*, 2005).

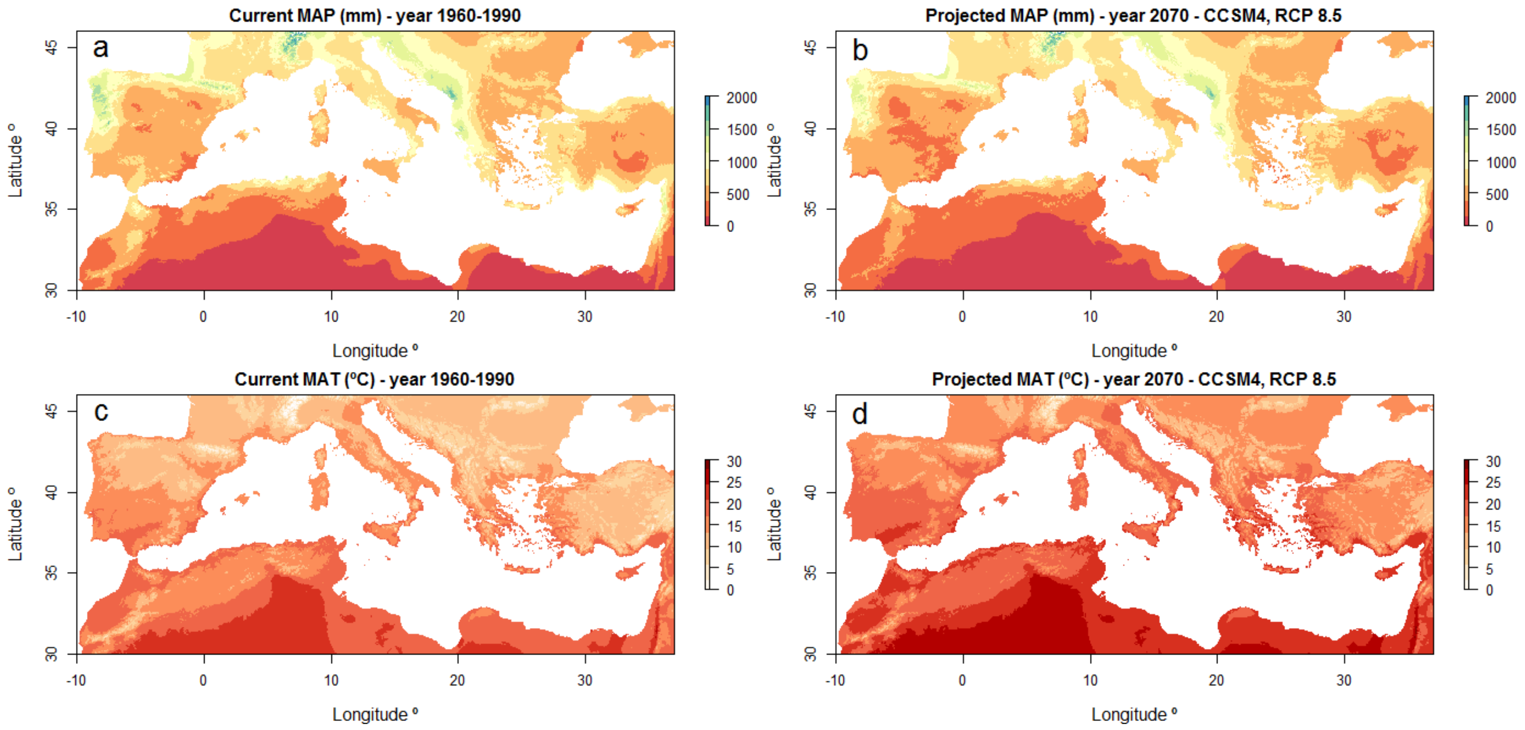
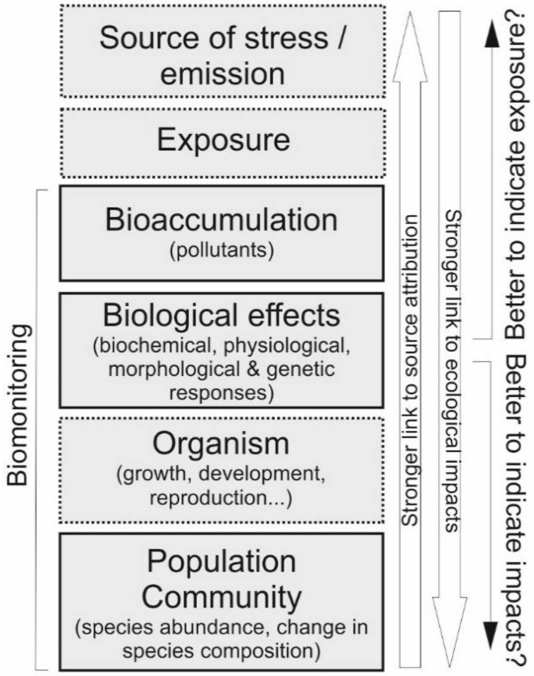


Figure 3. Examples of terrestrial ecosystems and experimental facilities set up to investigate the effects of air pollution and climate change in the Mediterranean Basin: a) Companhia das Lezírias, Samora Correia, Portugal; b) Alambre, Serra da Arrábida, Portugal; c) Herdade da Coitadinha, Barrancos, Portugal; d) Alto de Guarramillas, Madrid, Spain; e) La Higueruela, Toledo, Spain; f) El Regajal, Madrid, Spain; g) Tres Cantos, Madrid, Spain; h) Capo Caccia, Sardinia, Italy; i) La Castanya, Spain; j) Ozone FACE (Free-Air Controlled Exposure) facility, Florence, Italy; k) Fontblanche, Provence, France.

Figure 4. The biomonitoring chain: from the source of stress to ecological impacts. Measurements closer to the source of stress (e.g. bioaccumulation of pollutants) have a stronger link to source attribution, provide an account of exposure, and can be seen as an early warning system for potential impacts. On the other hand, biological effects (biomarkers) and species-based measurements commonly have a close link to impacts on the ecosystem but can have a weaker link to source attribution. Dark frame indicates those levels and measurements most commonly considered in biomonitoring studies.



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Supplementary Table – Examples of the ecological impacts of atmospheric pollution and climate change under natural, semi-natural and greenhouse conditions in the Mediterranean Basin.

Driver	Affected parameter	Effect	Habitat	Observations	Location	Reference
Nitrogen deposition	**Plant richness**	**Increased** (with +80 kg NH_4NO_3-N ha^{-1} yr^{-1} or +40 kg NH_4^+-N ha^{-1} yr^{-1})	Mediterranean maquis (post fire early succession)	Estimated N background deposition < 4 kg ha^{-1} yr^{-1} N manipulation (dose and form) field experiment running since 2007	38º29' N, 9º01' W (Southern Portugal)	Dias et al. 2014
		No effect (with +40 kg NH_4NO_3-N ha^{-1} yr^{-1})				
		Decreased (with + 35 kg CH_4N_2O-N ha^{-1} yr^{-1})	Semi-natural grassland (early successional stage of an old field)	The site was used for agriculture until 15-20 years before the experiment 3-year N manipulation (dose) field experiment running since 2007	43°02' N, 13°00' E (Central Italy)	Bonanomi et al. 2006
	Plant productivity	**Increased** (with + 35 kg CH_4N_2O-N ha^{-1} yr^{-1})				
		Increased (with + 40 kg NH_4NO_3-N ha^{-1} yr^{-1})	Mediterranean maquis (post fire early succession)	Estimated N background deposition < 4 kg ha^{-1} yr^{-1} N manipulation (dose and form) field experiment running since 2007	38º29' N, 9º01' W (Southern Portugal)	Dias et al. 2014
		No effect (with + 40 kg NH_4^+-N ha^{-1} yr^{-1} or 80 kg NH_4NO_3-N ha^{-1} yr^{-1})				
	Epiphytic lichen diversity	**No effect** (shift in the lichen functional groups – nitrophilous and oligotrophic - along the atmospheric NH_3 gradient)	Cork oak woodland	Long-term (> 20 years) atmospheric NH_3 gradient as a result of a cow barn (~200 beef-cattle) The atmospheric NH_3 gradient ranged between 18 and 382 kg N ha^{-1} yr^{-1}	38°44' N, 8°47' W (Southern Portugal)	Pinho et al. 2011
	Arbuscular Mycorrhizal Fungi (mycorrhization rate)	**Increased** (on *Asterolinon linum-stellatum*)	Kermes oak shrubland	Estimated N background deposition ~ 6 kg ha^{-1} yr^{-1} N manipulation (0, 10, 20, 50 kg NH_4NO_3-N ha^{-1} yr^{-1}) field experiment running for 1,5 years	40°00' N, 3°36'W (Central Spain)	Ochoa-Hueso et al. 2013a
		No effect (on *Limonium echiodes*)				
	Biological N fixation	**Decreased** (+ 20 kg NH_4NO_3-N ha^{-1} yr^{-1})	Soils from a Kermes oak shrubland	Greenhouse experiment with soils collected from an N manipulation (0, 10, 20, 50 kg NH_4NO_3-N ha^{-1} yr^{-1}) field experiment Estimated N background deposition ~ 6 kg ha^{-1} yr^{-1}	40°00' N, 3°36'W (Central Spain)	Ochoa-Hueso et al. 2014c

	Soil fauna abundance	**Increased** (~ 20 kg NH_4NO_3-N ha^{-1} yr^{-1})	Semi-arid shrubland	Estimated N background deposition ~ 6 kg ha^{-1} yr^{-1} N manipulation (dose) field experiment running since 2007	40°00’ N, 3°36’W (Central Spain)	Ochoa-Hueso et al. 2014b
	Plant physiology (mesophyll conductance and photosynthesis)	**Decreased** (in *Quercus suber*)	Cork oak woodland	Long-term (> 20 years) atmospheric NH_3 gradient as a result of a cow barn (~200 beef-cattle); the atmospheric NH_3 gradient ranged between 18 and 382 kg N ha^{-1} yr^{-1}	38°44’ N, 8°47’ W (Southern Portugal)	Pintó-Marijuanet al. 2013
	Lichen physiology (*Cladonia foliacea*)	**Increased** (chlorophyll *a* fluorescence increased until + 26kg N ha^{-1} yr^{-1} of total deposition)	Semi-arid shrubland	Estimated N background deposition ~ 6kg ha^{-1} yr^{-1} 2-year N manipulation (dose) field experiment	40º09’ N, 3º29’ W (Central Spain)	Ochoa-Hueso et al. 2011
		Increased (pigments between + 16-56 kg N ha^{-1} yr^{-1} of total deposition)				
	Soil pH	**Decreased** (acidification)	Semi-arid shrubland	Estimated N background deposition ~ 6 kg ha^{-1} yr^{-1} N manipulation (dose) field experiment running since 2007	40°00’ N, 3°36’W (Central Spain)	Ochoa-Hueso et al. 2014b
		No effect	Mediterranean maquis (post fire early succession)	Estimated N background deposition < 4 kg ha^{-1} yr^{-1} N manipulation (dose and form) field experiment running since 2007	38º29’ N, 9º01’ W (Southern Portugal)	Dias et al. 2014
	Soil organic matter	**Increased** (with +80 kg NH_4NO_3-N ha^{-1} yr^{-1})	Mediterranean maquis (post fire early succession)	Estimated N background deposition < 4 kg ha^{-1} yr^{-1} N manipulation (dose and form) field experiment running since 2007 Microcosms experiment under controlled conditions	38º29’ N, 9º01’ W (Southern Portugal)	Dias et al. 2013, 2014
Climate change	**Plant richness**	**No effect** (75% of the mean annual precipitation AND ≠ frequencies)	Sand dune	Annual plant species 1-year field manipulation of precipitation amount (100%, 75% and 50% of the mean annual precipitation) and frequency (1, 2 or 4 weeks interval of drought)	36º49’ N, 2º15’ W 37º00’ N, 2º26’ W 37º05’ N, 2º21’ W (Southeast Spain)	Miranda et al. 2009
		Decreased (50% of the mean annual precipitation)				

		No effect (1-6 weeks drought)	Grassland (cork oak woodland)	Sandy soil and understorey vegetation consists of a mixture of C3 annual species Field manipulation of precipitation patterns (1, 3 or 6 weeks interval of drought)	38º08’ N, 8º20’ W (Southern Portugal)	Jongen et al. 2013a,b
		Decreased (for grasses and legumes under 10 weeks drought)		Sandy soil and understorey vegetation consists of a mixture of C3 annual species 1-year field observation in a year drier than usual		
		Increased (for forbs under 10 weeks drought)				
	Plant productivity	**No effect** (75% of the mean annual precipitation AND ≠ frequencies)	Sand dune	Annual plant species 1-year field manipulation of precipitation amount (100%, 75% and 50% of the mean annual precipitation) and frequency (1, 2 or 4 weeks interval of drought)	36º49’ N, 2º15’ W 37º00’ N, 2º26’ W 37º05’ N, 2º21’ W (Southeast Spain)	Miranda et al. 2009
		Decreased (50% of the mean annual precipitation)				
		No effect (1-6 weeks drought)	Grassland (cork oak woodland)	Sandy soil and understorey vegetation consists of a mixture of C3 annual species 1-year field manipulation of precipitation patterns (1, 3 or 6 weeks interval of drought)	38º08’ N, 8º20’ W (Southern Portugal)	Jongen et al. 2013b,c
		Decreased (for 10 weeks drought)		Sandy soil and understorey vegetation consists of a mixture of C3 annual species 1-year field observation in a year drier than usual (10 mm instead of 280 mm between December and March)		
	Soil respiration	**No effect**	Grassland (cork oak woodland)	Sandy soil, and understorey vegetation consists of a mixture of C3 annual species Field manipulation of precipitation patterns (1 or 3 weeks interval of drought)	38º08’ N, 8º20’ W (Southern Portugal)	Jongen et al. 2013a

	Nutrient availability (nitrogen)	**No effect** (1, 3 week drought)	Grassland (cork oak woodland)	Sandy soil, and understorey vegetation consists of a mixture of C3 annual species Field manipulation of precipitation patterns (1 or 3 weeks interval of drought)	38º08’ N, 8º20’ W (Southern Portugal)	Jongen et al. 2013a,c
		Decreased (10-week drought)		Sandy soil, and understorey vegetation consists of a mixture of C3 annual species 1-year field observation in a year drier than usual (10 mm instead of 280 mm between December and March)		
	Net photosynthesis (biocrust)	**Decreased** (in communities with biocrust cover >50 %)	Semi-arid shrubland (*Stipa tenacissima* and biocrust)	2-years field manipulation of warming (2–3 °C increase), rainfall exclusion (30 % rainfall exclusion) and biocrusts cover (<25 % vs. communities with cover >50 %)	37°05’N, 2°04’W (Southeast Spain)	Miranda et al. 2009
		Increased (in winter in communities with biocrust cover >50 %, no warming and in the rainfall exclusion treatment)		4-years field manipulation of warming (2–3 °C increase), rainfall exclusion (30 % rainfall exclusion) and biocrusts cover (<25 % vs. communities with cover >50 %)	40°02’N, 3°32’W (Central Spain)	
Ozone	**Tree canopy** (crown defoliation and discoloration)	**Increased** (higher in conifers than in broadleaves trees)	Mediterranean forests (deciduous, broadleaf forests and conifers)	Stomatal O_3 fluxes were modelled and correlated to real-world forest-response indicators in 2012 and 2013	South-eastern France (30 plots) and North-western Italy (24 plots)	Sicard et al. 2016
	Tree canopy (visible foliar O_3 injury)	**Increased** (from 0.3% to 15% of foliar surface was affected by visible O_3 injury depending on the species)				
	Tree canopy (crown defoliation)	**Increased** (defoliation increase ranged between 15 and 34.5 % in the period 2000-2002)	Woodland with *Fagus sylvatica* as main species	Different O_3 descriptors were used (AOT40, mean concentration and maximum 2-week concentrations)	Italy, Spain and Switzerland	Ferretti et al. 2007

	Tree canopy (visible foliar O_3 injury)	**Increased** (in *Pinus uncinata*)	Mediterranean montane ecosystem	Region with prolonged summer drought and moderate winter snowpack Ozone concentration collected at the sites was used	Catalonia (North-eastern Spain)	Kefauver et al. 2014
	Leaf traits (leaf mass per area, total leaf thickness, percentage of palisade tissue, nitrogen content)	**Increased**	Adult beech (*Fagus sylvatica*) stands	Beech stands growth at full density with homogeneous structural characteristics Ozone concentration routinely gathered	Italy (North-south gradient)	Bussotti et al., 2005
	Leaf traits (chlorophyll content and photosystem II functionality)	**Decreased** (in *Viburno lantana*)	sub-alpine region of Northern Italy	Sites characterized by different ozone levels (+62.6% at the high-ozone with respect to the low-ozone, in terms of cumulated exposure)	Trentino (North Italy)	Gottardini et al. 2014
	Stomatal uptake of ozone	**Assessment of ozone dose Taken up by forest canopies in several year of measurements**	Mediterranean coastal *Quercus ilex* forest	Stomatal dose is only a minor part of the total ozone absorbed by forest	41°44′N, 12°25′E (Central Italy)	Gerosa et al. 2005,2009b
	Carbon assimilation	**Assessment of carbon uptake (NEE) in relation of stomatal uptake**	Mediterranean forest and orchard sites	Reduction of carbon assimilation due to ozone uptake	38°53′N, 20°37′W (California) 41°44′N, 12 °24′E (Central Italy)	Fares et al. 2013
	Stomatal uptake	**Assessment of ozone dose Taken up by coastal maquis in one summer season**	Coastal Mediterranean maquis	Vegetation not completely developed, ecosystem in a dynamic equilibrium between low maquis and medium maquis Ozone concentrations routinely monitored	41º40’N, 12º23’ E (Central Italy)	Gerosa et al. 2009a
	Visible foliar O_3 injury	**Increased** (but symptoms in the field differed from symptoms under controlled conditions)	*Fraxinus ornus*	Seedlings experimentally exposed to ozone (O_3) in indoor chambers (150 ppb, 8 h d(-1)/7 weeks), and field trees exposed to ambient O_3 (max 93 ppb/one growing season)	43°46′57″N 11°19′14″E (Central Italy)	Paoletti et al. 2009a
	Gas exchange	**Decreased** (but symptoms in the field differed from symptoms under controlled conditions)				

	Photosynthesis	**No effect** (only a 9% reduction was recorded in the O_3-treated leaves)	*Quercus ilex*	Clean air or air with a square wave of 110 ± 10 nmol mol−1 O_3 combined with mean CO_2 concentrations range around 1500 and 400 μmol mol^{-1}	43°26′N, 10°42′E (Central Italy)	Paoletti et al. 2007
	Visible foliar O_3 injury	**Increased**	*Hibiscus syriacus*	Plants were placed in Open-Top Chambers with two O_3 treatments: charcoal-filtered air and non-filtered air plus 30 ppb O_3	39º16'14.8''N, 00º26'59.6''W(Northern Italy)	Paoletti et al. 2009b
	Biomass (leaf and wood)	**Decreased**	*Populus maximoviczii* x *berolinensis*	3-years treatment at ambient conditions O_3 AOT40, M24 and M12 during the growing season were 24.6 ± 0.5 ppm h, 35.7 ± 0.3 ppb and 40.3 ± 0.3 ppb respectively	43°44′N, 11°16′E (Central Italy)	Hoshika et al. 2013
	Mycorrhizas (diversity of ectomycorrhizal types)					Katanic et al. 2014
	Biomass (root and stem)	**Decreased** (butbiomass allocation above ground depend on the tree ontogenetic stage)		Average values of M24 and AOT40 were 35 ppb and 23 ppm h, respectively, with a maximum O_3 concentration of 143 ppb		Carriero et al. 2015
Nitrogen deposition x climate change	**Soil characteristics** (chemistry and enzymatic activity)	**Decreased** Soil C:N ratio in response to high N and low water (greenhouse) Base cation availability (gradient) Nitrogen fixation and mineralization (greenhouse) Non-base cations (greenhouse) pH (greenhouse)	Semiarid shrublands	Greenhouse experiment Background N deposition of sampling site: 6.1 kg N ha^{-1} yr^{-1} Weekly application of 0, 1.3, 2.6 or 6.5 mg of NH_4NO_3 in water, equivalent to 0, 10, 20 and 50 kg N ha-1 yr-1 Two watering levels	40º00' N, 3º36' W (Central Spain)	Ochoa-Hueso et al 2014c
		Increased N availability (both) Phosphatase activity (greenhouse) Base cations (greenhouse)		Five study sites were sampled along an atmospheric N deposition and climatic gradient (3.98-6.05 kg N ha^{-1} yr^{-1})	Central Spain	

	Plant biomass	**Increased** Up to 20 kg N ha-1 yr-1 and then decreased. High water and high N resulted in higher biomass but no interactive effects (response driven by forbs)	Semiarid shrubland	Greenhouse experiment Background N deposition of sampling site: 6.1 kg N ha^{-1} yr^{-1} Weekly application of 0, 1.3, 2.6 or 6.5 mg of NH_4NO_3 in water, equivalent to 0, 10, 20 and 50 kg N ha-1 yr-1 Two watering levels	40º00' N, 3º36' W (Central Spain)	Ochoa-Hueso and Manrique 2010
		Increased High water and high N resulted in higher biomass; interactive effects due to very low vs. high levels of water		Greenhouse experiment Background N deposition of sampling site: 6.1 kg N ha^{-1} yr^{-1} N application in water (Hoagland solution) equivalent to 9.6 and 13.7 kg N ha-1 yr-1 (low N treatments) and 57.4 and 82.1kg N ha-1 yr-1 (high N treatments) under low and high water conditions, respectively. Nitrophytic plant seed bank addition treatment		Ochoa-Hueso and Manrique 2014
		Decreased High N and high water under competition with nitrophytic plants resulted in lower-than-expected forb biomass				
	Moss cover	**Increased** High water, high N and low competition resulted in higher cover		Greenhouse experiment Background N deposition of sampling site: 6.1 kg N ha^{-1} yr^{-1} Weekly application of 0, 1.3, 2.6 or 6.5 mg of NH_4NO_3 in water, equivalent to 0, 10, 20 and 50 kg N ha-1 yr-1 Two watering levels. Vascular plant removal treatment		Ochoa-Hueso and Manrique 2013
	Moss cover	**Decreased** High N coupled with low water resulted in lower moss cover. High N and high water resulted in lower cover at peak plant biomass conditions (20 kg N ha-1 yr-1)				

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Chapter 3: Contribution of biological crust to soil CO2 efflux in a Mediterranean shrubland ecosystem

Manuscript published: Geoderma (2017) 289:11–19.

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Abstract

In Mediterranean ecosystems, the soil biological crust (hereafter biocrust) plays a crucial role in maintaining ecosystem functioning. In these ecosystems, soil water content can often be a stronger driver of soil CO_2 efflux than soil temperature, or at least comparable. However, little is known on the contribution of the biocrust to soil CO_2 efflux or how the respiration of the biocrust responds to soil water content and temperature. A manipulative experiment was performed in a Mediterranean shrubland ecosystem in Sardinia (Italy) to assess the contribution of the biocrust to soil CO_2 efflux and to identify the main environmental drivers of the CO_2 efflux. For 19 months, *in situ* soil CO_2 efflux was measured over two different surfaces: soil deprived of biocrust (hereafter *Soil*) and intact soil (hereafter *Soil+BC*), and estimated by subtraction in a third surface: biocrust (hereafter *BC*). CO_2 efflux emitted by *Soil*, *BC* and *Soil+BC* were uniquely driven by soil moisture and temperature: *BC* respiration was mainly controlled by soil moisture at 5 cm depth, whereas both soil temperature and water content at 20 cm depth determined *Soil* CO_2 efflux. Soil temperature and water content at 5 cm depth drove *Soil+BC* respiration. We also found that biocrusts can contribute substantially (up to 60%) to the total soil respiration depending on its moisture content. This contribution persists even in periods in which deeper soil layers are inactive, as small water pulses can activate the metabolism of carbon in soils through lichens, mosses and cyanobacteria associated with the biocrust, while deeper soil layers remain dormant. The important differences observed in CO_2 efflux between *Soil* and *Soil+BC* suggest that carbon models and budgets may underestimate soil CO_2 efflux in spatially heterogeneous Mediterranean areas. Our results highlight the importance of accounting for the biocrust contribution to soil respiration and its response to environmental drivers. We provide an accurate estimation of this key component of the carbon cycle at the ecosystem level in water limited ecosystems.

Keywords: Soil temperature; Soil moisture; Semiarid ecosystems; Soil respiration; Spatial and temporal heterogeneity; Carbon cycling

Introduction

Soil CO_2 efflux is the sum of autotrophic respiration by plant roots, heterotrophic respiration by decomposition of organic carbon (C) substrates operated by soil microbes, catabolic processes of mycorrhizas, respiration by arthropods, soil worms and other animals occupying different trophic niches (Hanson et al., 2000) and carbonate weathering (Rey, 2015). Soil CO_2 efflux is an important component of the C cycle (Fernandez et al., 2006) and is estimated to account for over 25% of global CO_2 emissions (Bouwmann and Germon, 1998). Despite its relevance and the determined efforts to studying this process, present knowledge still lacks reliable field data to enable better estimates of soil-atmosphere fluxes. Importantly, such knowledge gaps hinder our potential to accurately predict the effects of global environmental change such as rising atmospheric CO_2 and climate change on the C cycle.

The uncertainty surrounding the response of soil CO_2 efflux to environmental factors is greater for water-limited environments such as Mediterranean ecosystems. There are relatively fewer articles where CO_2 efflux has been quantified and the controlling factors established in dry areas compared to more humid environments (Castillo-Monroy et al., 2011). Mediterranean ecosystems are often characterized by complex spatial vegetation patterns, with patches devoid of vegetation that vary in size and shape depending on water availability and role of engineering plants (Gilad et al., 2007). Differences in the distribution of soil organic matter content (Reynolds et al., 2007), biological activity (Stubbs and Pyke, 2005) and microbial abundance (Gallardo and Schlesinger, 1992), are typically found between plant patches and bare soils and are largely responsible for the high spatial variation in soil respiration (R_s) in these areas (Maestre and Cortina 2003). Despite its recognized relevance for ecosystem functioning, the effects of this small-scale spatial variation in CO_2 efflux remain scarcely understood.

One key aspect of the spatial heterogeneity in Mediterranean ecosystems is the presence of the biological soil crusts (hereafter biocrust), which can encompass up to 70% of the cover in absence of vascular plants (Belnap and Lange, 2003). Biocrusts are specialized communities that cover the first millimeters of the soil surface in water-limited ecosystems worldwide. Such communities are composed of cyanobacteria, lichens, liverworts, fungi, eukaryotic algae and mosses in various proportions (Belnap and Lange, 2001; Eldridge and Greene 1994). They largely affect the ecosystem functioning by influencing soil nutrient cycling (Castillo-Monroy et al. 2010; Morillas and Gallardo, 2015), soil stability and water infiltration (Eldridge et al., 2010; Kidron, 2014; Reynolds et al. 2001), local hydrological cycle (Belnap et al., 2005), nitrogen fixation and transformations (Belnap, 2002; Evans and Lange, 2003; Hu et al., 2015), and C cycling (Maestre and Cortina, 2003; Thomas and Hoon, 2010; Zhao et al., 2014). Thus, the need to improve our estimation of soil-atmosphere fluxes is more acute in crusted soils due to the increased spatial heterogeneity of fluxes.

In Mediterranean ecosystems, the metabolic activity of microbes and roots is restricted by low temperatures in the humid winter and limited soil moisture during the warm summer (de Dato et al., 2010; Morillas et al., 2013). Thomas et al. (2008) showed that R_s does not respond to a large range of temperatures unless moisture levels are above a critical threshold, whereas temperature responses are much greater when moisture is sufficient to allow microbial respiration of organic matter. These findings highlight the pivotal role played by the interaction between temperature, moisture and CO_2 efflux in Mediterranean ecosystems. Indeed, soil CO_2 efflux models for water limited environments must account for the exponential effect of soil temperature (T_s) on the metabolism of soil organisms and roots, and the limiting effects of soil moisture (de Dato et al., 2010; Lloyd and Taylor, 1994; Reichstein, 2002; Vargas et al., 2011). Although the importance of the biocrust for the ecosystems biogeochemistry is widely recognized (Belnap, 2006, 2003; Belnap and Lange, 2003), its contribution to soil CO_2 efflux dynamics, which could be decoupled from the respiration of the deeper soil layers, has been neglected. Indeed, biocrust organisms can be activated by dew formation (Herrnstadt and Kidron, 2005; Kidron et al., 2002; Veste et al., 2008) that does not affect the deeper soil layers, and this can occur at any time of the year (Moro et al., 2007). There are a number of unique and subtle processes affecting soil CO_2 efflux in crusted soils, such as changes in diffusion gradients of CO_2 in soil pore spaces due to the biocrust cover (Belnap et al., 2003; Thomas et al., 2008), or CO_2 uptake by autotrophic biocrust components, which increases the complexity of assessing the biocrust role on soil CO_2 efflux.

In these circumstances, soil CO_2 efflux models may lose accuracy and neglect the contribution of the biocrust to this flux. Recently, some research efforts have been made to bridge this gap. Zhang et al. (2013) reported that the biocrust contributed up to 2/3 to total R_s in a temperate desert. Accordingly, Castillo-Monrroy et al. (2011) found that biocrust-dominated areas are the main contributor to the total R_s in a semiarid ecosystem. On the other hand, Wilske et al. (2008) reported that the highest biocrust-related net CO_2 uptake occurred during the wet winter, whereas low soil water content (SWC) resulted in low uptake rates which can hardly compensate for biocrust respiration. As this contribution depends on the biocrust cover and type (Zhao et al., 2014), it is of paramount importance to collect data from a broad range of environments. Therefore, understanding the biocrust contribution to soil CO_2 efflux in water-limited environments is crucial to better comprehend the functioning of these ecosystems and improve prognostic modeling.

In this article, we present the results of a manipulative experiment in a Mediterranean shrubland ecosystem in Sardinia, Italy. The objectives were to quantify the biocrust contribution to R_s and identify the main environmental factors affecting it. The objectives were addressed by measuring *in situ* soil CO_2 efflux over two different surfaces: soil deprived of biocrust (hereafter *Soil*), and intact soil (hereafter *Soil+BC*), and estimating by subtraction the CO_2 efflux associated with the biocrust (hereafter *BC*). Three hypothesis were tested: (i) the contribution of the biocrust to soil CO_2 efflux varies over the seasons being minimum during the dry months due to its large dependence on superficial moisture, (ii) CO_2 efflux of the studied surfaces (*Soil*, *BC* and *Soil+BC*) would respond differently to SWC and T_s as a consequence of the very different exposure to environmental factors regimes experienced by the biocrust and deep soil layers, (iii) since SWC is a main driver for respiration and since small rain pulses can increase the moisture content of the upper soil layers but have a minor effect on the soil moisture of the deeper layers, a two layer model would disentangle how the biocrust contribution to R_s occurs in periods when deeper soil layers are inactive and estimate its contribution on an annual base.

Materials and methods

Study site

This study was carried out in a maquis shrubland coastal ecosystem at the National reserve Portoconte – Capo Caccia in north-western Sardinia (40° 36' 18" N, 08° 09' 07" E; mean altitude 74 m asl), located 326 m from a deep sea cliff. The climate is sub-humid Mediterranean, with mild winter season and warm dry summer (usually from May to September). Strong predominant north-west (Mistral) and south-west winds blow in this area. Average annual air temperature (1970-2012) is 16.5 ± 5.0 °C ranging from a minimum of 6.2 ± 4.9 °C to a maximum of 32.7 ± 5.6 °C. The coldest month is February with an average temperature of 10.4 ± 1.1 °C, whereas the hottest month is August with an average temperature of 23.8 ± 1.5 °C. Spring and autumn are the rainy seasons, and mean annual rainfall is 494 ± 153 mm.

The underlying substrate is a Mesozoic limestone, and the main soil types are Lithic Xerorthent and Typic Rhodoxerarfs (USDA 1993). Soil texture is a clay-silt, with 55 – 60 % clay, 20 – 30 % silt and 10 – 25 % sand. Soil depth is 30 cm – 40 cm and because of its texture is highly erodible (Spano et al., 2009). Soil water holding capacity is 24 % and total Leaf Area Index ranges between 2.7 – 3.0 m^2 m^{-2} (Marras et al., 2011). Maximum canopy height is around 3 m, and the discontinuous vascular vegetation cover is 80 % on average. In this area, Mediterranean maquis appears as a shrubland of different species, mainly *Juniperus phoenicea* L. (53 % of total cover) but also *Pistacia lentiscus* L., *Phyllirea angustifolia* L.*,* *Smilax aspera* L. and *Chamerops humilis* L. (27 % of total cover). The remaining 20 % are the inter spaces between shrubs, colonized by a well-developed lichen-dominated biocrust with a depth of about 0.5 cm. The dominant lichens are *Cladonia* sp. (23.8 % of the inter spaces between shrubs), *Squamarina cartilaginea, Diploschistes* sp. and *Collema* sp. (10.4 % all together). Green mosses cover 14.4 %, whereas litter accumulation and bare soil areas cover 26.3 and 10.0 % of these patches, respectively.

Sampling design, field measurements and laboratory analyses

In April 2012, 16 permanent polyvinyl chloride (PVC) collars of 20 cm diameter were installed on the soil surface covered by biocrust (*Soil+BC)*. In March 2013, eight additional PCV collars were installed in areas where the biocrust was physically removed using a spatula i.e. the *Soil* surface. All the 24 collars were placed at random within eight inter spaces between shrubs: patches were between 10 m and 50 m from each other and collars within each patch were at a minimum of 2 m distance. Soil CO_2 efflux was measured from June 2013 to January 2015. Measurements did not have a specific frequency, but they were rather performed in order to measure over the widest range of combinations of T_s and SWC. All measurements were taken at least three days after the last precipitation event to avoid measuring at moments when the SWC was above its maximum field condition. In doing so, we attempted to minimize the disturbance generated in our long-term experimental site by trampling into the plots while soil was muddy. Thus, we avoided the CO_2 pulses that typically follow wetting of dry soils (Birch effect; Birch, 1964) and the physical displacement of CO_2-enriched pore space gases by water during rainfall, as we were interested in the effect of T_s and SWC on R_s rather than on the effect of rewetting on R_s. In any case, preliminary results showed that R_s was inhibited within 24 hours after a rain event in this study site, most likely due to the impeded diffusion of gas through the saturated clay soil. Direct measurements were collected on *Soil+BC* and *Soil* surfaces, whereas the CO_2 efflux emitted by *BC* surface was estimated by subtracting the soil CO_2 efflux of *Soil* surfaces from that of *Soil+BC*.

Soil CO_2 efflux was measured *in situ* using a portable LI-COR 8100 (Lincoln, Nebraska – USA) containing an infrared gas analyser system equipped with a flow-through closed chamber LI-COR 8100-103 connected to the central unit. The chamber of 20 cm of diameter was inserted 5 cm into the collar, and measurements were preformed in a dark condition. Measurements took 105 s per collar, and were performed between 8:00 and 9:00 a.m. (local time, GMT + 1) to avoid direct radiation from reaching the collars, which ensures minimization of variability in T_s within the sampling day. However, this experimental design could have underestimated daily efflux as a result of the time lags involved in diurnal variations in T_s, i.e. the soil surface will heat very quickly and there will be a short time lag between peak air and soil temperatures, whereas at deeper depths this lag could be of several hours. The offset of each collar was measured and taken into account to calculate the headspace of each collar and quantify the CO_2 emission. Soil temperature at 3 cm depth adjacent to the collars was measured at the same time as CO_2 efflux measurements using a probe connected to the LI-COR 8100.

Since 2004, a meteorological and radiometric station (2 m and 3.5 m above the ground, respectively) are located within the experimental site. Air temperature and relative humidity were measured hourly using a HMP45C probe (Campbell Scientific Inc., USA) and an aerodynamic rain gauge (ARG100, Environmental Measurements Limited, UK) was used to record precipitation data every hour. SWC was monitored at 20 cm depth using two Time-Domain Reflectometry (TDR) probes (Model CS616-L, Campbell Scientific Inc., Logan, UT, USA). In January 2012, two additional T_s and SWC sensors (EC-5, Decagon Devices Inc., Pullman, WA, USA) were placed at 5 cm depth. All sensors acquired data every minute and were stored as half-hourly averages.

In April 2014, 8 soil cores (2 cm in diameter) between 0 cm and 5 cm depth and between 5 cm and 10 cm depth below the mineral soil were collected. The samples were dried at 40 °C for 72 h and sieved using a < 2 mm mesh. Sieved samples were ground using Brinkmann Retsch Mortar Grinder (Model: RMO Polished), then 0.15 g analysed with an Elemental analyser (CHN 628, Leco, St. Joseph, USA), to determinate the total C and nitrogen (N) soil content. Soil pH was determined in a water slurry (1:5 w/v in H_2O). The effective cation concentration, Ca, Mg and Na, was extracted by cationic exchange capacity method, in solution of $BaCl_2$ according to ISO 11260, then concentration was determined by atomic absorption spectroscopy analyser (Analyst 200, Perkin Elmer, Waltham, USA). Available phosphorus (P_2O_5) was extracted as in Olsen et al. (1954) and quantified by spectrophotometric analysis (Cary 60 UV-Vis, Agilent, Santa Clara, USA). Calcium carbonate ($CaCO_3$) was quantified using a "Dietrich-Fruhling" calcimeter.

Statistical analyses

ANOVA for repeated measurements was used to test for differences among *Soil+BC, Soil*, and *BC* in soil CO_2 efflux measurements. Prior to ANOVA analysis, data were tested for assumptions of normality and homogeneity of variances, and were log-transformed when necessary. Partial correlations were performed to account for collinearity and thus to test the effects of T_s on soil CO_2 efflux of *Soil+BC, Soil* and *BC* while controlling for SWC at 5 cm and 20 cm depth (SWC_{-5cm} and SWC_{-20cm}, respectively) and *vice versa.* All statistical analyses were performed by using the IBM SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Different functions can be used to simulate soil CO_2 efflux response to environmental conditions. In this study, the dependency of R_s on T_s and SWC was investigated for the three studied surfaces (*Soil+BC*, *Soil* and *BC*). Three different approaches were used: a simple linear regression accounting for both T_s, and SWC_{-5cm} and SWC_{-20cm}, a multi-linear equation to account for the combined effect of these variables, and a modified version of the most common used Lloyd and Taylor (LLT) model (Lloyd and Taylor, 1994). The LLT function is an Arrhenius type equation which is temperature-driven and given by:

$$R_{s_{LLT}} = R_{ref} \cdot exp\left[E_0 \cdot \left(\frac{1}{T_{ref}-T_0} - \frac{1}{T_s-T_0}\right)\right] \tag{1}$$

where R_s is soil respiration, R_{ref} is the ecosystem respiration rate at a reference temperature (T_{ref}) and E_0 is an activation energy parameter that determines the temperature sensitivity. In the original study (Lloyd and Taylor, 1994), T_{ref} and T_0 are set to 10°C and -46°C respectively, and T_s is used as predictor. Although the Lloyd and Taylor equation fits well to the experimental data during standard water availability conditions, in semi-arid ecosystems the dependence of ecosystem respiration on SWC must also be considered. Here we used the modified Lloyd and Taylor equation (LLT_{mod}) proposed by Reichstein et al. (2002), which multiplies the original temperature-driven model (equation 1) by a moisture function:

$$R_s = R_{s_{LLT}} \cdot \frac{SWC - SWC_0}{(SWC_{1/2} - SWC_0) + (SWC - SWC_0)} \tag{2}$$

where SWC_0 is the residual SWC at which respiration is absent, and $SWC_{1/2}$ is the SWC at 50% of the maximal respiration (Reichstein et al., 2002). Equation 2 is the third approach used in this study to simulate R_s as function of T_s and SWC_{-5cm} and SWC_{-20cm}. Models performances were evaluated using the following statistical indices: the Pearson's correlation coefficient (r), the root mean square error (RMSE), the index of agreement (d) and the Akaike's information criterion (AIC test, Akaike, 1974).

To calculate the contribution of *BC* to R_s, the best models were run at a 30 minute timestep for two years (2013-2014) thus accounting for the variations of both T_s and SWC. Daily amounts of *BC* and *Soil* CO_2 efflux were first computed separately and then summed to obtain *Soil+BC* respiration rate. *BC* contribution to *Soil+BC* respiration rate was thus calculated as the ratio of the daily *BC* and the estimated *Soil+BC* respiration rates.

Results

Average annual rainfall during the study period was 430 mm, slightly under the 42-year average (494 mm), while annual mean T_{air} was 16.8 °C (Figure 1). SWC_{-20cm} was generally higher than SWC_{-5cm} during the entire study period, ranging from 19.0 % to 43.1 % for SWC_{-20cm}, and from 12.2 % to 33.6 % for SWC_{-5cm} (Figure 2a). At both depths, SWC followed the same overall pattern of seasonal changes showing higher values in spring, autumn, and winter (average values of 26.9 % and 17.7 % for SWC_{-20cm} and SWC_{-5cm} respectively, Figure 2a) and lower during summer (20.6 % and 13.3 % for SWC_{-20cm} and SWC_{-5cm} respectively, Figure 2a). Daily averages of T_s followed a sinusoidal pattern with maximum daily values of 33.3 °C and 31.1 °C recorded between June and August 2013 and 2014 respectively, and minimum values of 5.2 °C and 3.6 °C recorded during the cold season of 2013 and 2014, respectively (Figure 2b). We did not find any significant differences in soil chemical properties between 5 cm and 10 cm depth (Table 1).

Significant differences in CO_2 efflux were found among *Soil*, *BC* and *Soil+BC* (Figure 3a, b, c, Repeated Measures [RM] ANOVA; F = 1916, df = 2, P <0.001). Respiration rates for *Soil* ranged from 0.80 to 2.17 μmol CO_2 m^{-2} s^{-1} whereas that for *Soil+BC* ranged from 1.36 to 2.59 μmol CO_2 m^{-2} s^{-1} (Figure 3), which was always significantly higher than that of *Soil* (RM ANOVA; P <0.001). Soil respiration rates estimated for *BC* ranged from -0.12 to 1.32 μmol CO_2 m^{-2} s^{-1}, and were lower than *Soil* (RM ANOVA; P <0.001) except for one event in autumn 2014. The *BC* contribution to *Soil+BC* respiration rate was on average 6.9 %, ranging from -16.5 % in the driest and hottest period to a peak of 60.1 % recorded in autumn 2014 (Figure 3b). This event took place when the soil was rewetted after the rainless summer period. The minimum *BC* contribution to *Soil+BC* respiration (-1.6 to -16.5 %) was found during the dry months (i.e. July, August and September) in both 2013 and 2014, whereas higher values of contribution (-0.3 to 60.1 %) were found during the rest of the year (Figure 3b).

Partial correlations revealed a significant effect of T_s, SWC_{-5cm} and SWC_{-20cm} on the *Soil* CO_2 efflux (Table 2). The CO_2 efflux of *Soil+BC* was significantly correlated with

SWC_{-5cm} ($p<0.001$) and SWC_{-20cm} ($p=0.005$), but not with T_s, although the probability level was almost significant ($p_{5cm}=0.059$; $p_{20cm}=0.056$, Table 2). Finally, the respiration rate of *BC* was only significantly correlated with SWC_{-5cm} ($p=0.001$, Table 2).

When simulating *Soil* CO_2 efflux, the Lloyd and Taylor (1994) model modified according to Reichstein et al. (2002) to also account for SWC_{-20cm}, outperformed the other models as it is shown by r (0.81, $p<0.001$), RMSE (0.13 μmol m^{-2} s^{-1}), d (0.89),and the lowest AIC test value (=-8.1) (Table 3). The simple linear model which uses SWC_{-5cm} as independent variable, was the best model predicting estimated *BC* respiration rates according to all the statistical indexes (r=0.75, p=0.002, RMSE=0.15 μmol m^{-2} s^{-1}, d=0.84, AIC= -7.00). Also the multilinear model with T_s and SWC_{-5cm} showed good performances with a slightly higher AIC test result (AIC=-5.5) (Table 3).

Soil+BC CO_2 efflux was best explained by the multilinear model accounting for both T_s and SWC_{-5cm} (r=0.75, p=0.002, RMSE=0.19, d=0.85, AIC=1.7). However, the modified Lloyd and Taylor model accounting for SWC_{-20cm}, and the linear model using SWC_{-5cm} as independent variable, performed similarly but showed higher AIC test results (AIC>3.6) (Table 3). In agreement with these models, the CO_2 efflux of all the three surfaces increased with soil moisture (Figures 2 and 3). For *Soil* and *Soil+BC*, T_s and CO_2 efflux were positively correlated until the soil dried (see Figures 2 and 3) whereas for *BC* the metrics suggests that respiration was mainly controlled by SWC_{-5cm} with a seasonal pattern decoupled from that of *Soil* or *Soil+BC* (Figure 3).

Table 4 reports the coefficients of the best performing models for *BC, Soil* and *Soil+BC*. The daily respiration rate for the two layer model ranged from 89.8 to 406.7 mol CO_2 m^{-2} d^{-1} , with the daily modeled values for the *Soil* layer ranged from 87.0 to 181.1 mol CO_2 m^{-2} d^{-1} for *Soil*, and those for the *BC* layer between 0.2 and 193.2 mol CO_2 m^{-2} d^{-1} (Figure 4a). Modeled respiration rates showed the greater temporal variability found in *BC* CO_2 efflux compared to *Soil* (Figure 4a), highlighting the key role of the biocrust in *Soil+BC* respiration rates through the seasons. The modeled *BC* contribution to *Soil+BC* respiration rate was on average 23.6 %, ranging from 0.1 % in June 2012 to a peak of 61.9

% in February 2012 (Figure 4b). Following the trend found in estimated *BC* contribution to *Soil+BC* respiration, modeled *BC* contribution was minimum during the rainless season and became a relevant CO_2 source in the wet months (Figure 4b).

Discussion

The importance of biocrust for ecosystems functions has received increasing attention from the scientific community in recent years (Grote et al. 2010; Ladron de Guevara et al. 2014; Maestre and Cortina 2003; Thomas and Hoon, 2010). However, despite previous attempts to assess CO_2 efflux from biocrust in dryland ecosystems (Castillo-Monroy et al., 2011; Thomas, 2012; Thomas et al., 2008, 2011, 2014; Wilske et al., 2008, 2009; Zhang et al., 2013; Zhao et al., 2014), there is still a large uncertainty on the estimation and controlling factors of biocrust contribution to soil CO_2 efflux.

Wilske et al. (2008) followed an experimental design similar to ours to assess biocrust CO_2 efflux in a Mediterranean shrubland, but measuring net CO_2 exchange instead of R_s. Regardless of this difference, Wilske et al. (2008) also pointed out the key role of biocrust moisture in regulating CO_2 exchange, reporting high net uptake rates under wet conditions and decreased net uptake in rainless periods. Accordingly, Wilske et al. (2009) assessed the annual biocrust net C fluxes in the same study site using a model where SWC had a central role. Zhang et al. (2013) modeled the effect of moisture at different soil depths on both biocrust and rhizosphere respiration in a temperate desert, and they found that the former was determined by superficial water content, whereas the latter was driven by deep SWC. However, the soil depths considered in this study were rather coarse compared to those in our study, as superficial water content accounted from 0 cm to -20 cm soil layer, and deep SWC referred from -30 cm to -210 cm soil layer. Thomas et al. (2014) applied two different techniques to isolate biocrust efflux on a salt pan: by subtraction (same approach applied in this article) and by removing the biocrust and sitting in on a bed of inert substrate. Although both approaches have shortcomings, the second method is very manipulative and consequently, the CO_2 efflux measurements are performed in a highly artificial environment, which may make the subtraction method the most suitable for estimating the biocrust contribution to R_s. In their study, they found that the biocrust was a small contributor to R_s in the dry season and reported a net uptake of CO_2 to the biocrust when following the subtraction method in the wet season. The major short coming of the subtraction method is that the removal of the biocrust from the soil surface as part of the experiment may also have changed the natural soil to air diffusion gradient and may have increased the measured CO_2 efflux, without any biological processes being involved. That is, if by removing the biocrust the natural diffusion gradient between the soil and atmosphere has been increased, the subsoil CO_2 efflux would be enhanced to without any increased respiration, which would lessen the difference between R_s on *Soil+BC* and *Soil*. In this likely situation, the biocrust contribution to R_s would have been even greater than that reported. The relatively small and negative estimated values for *BS* respiration found in summer suggest that the possible increase in CO_2 fluxes due to the biocrust removal was not large and partially attributable also to spatial variation. It is well known that soil CO_2 efflux may change greatly even over short distances and that the relative differences in space also change in time as consequence e.g. of the mosaic pattern of clumps of vegetation and patches of soil typical of Mediterranean ecosystems. In fact, Qi et al. (2010) proved that CO_2 fluxes from the soil under the canopy of shrubs were significantly higher than those from the soil covered with biocrusts and the bare soil in the interplant spaces of a semiarid ecosystem. It is thus likely that our negative values for *BC* are partially due to an artefact created by both the subtraction method and spatial variability. However, these two components are difficult to separate and would require *ad-hoc* experiments to do so.

Zhao et al. (2014) and Thomas et al. (2011, 2008) reported that soil moisture was the primary limiting factor to biocrust CO_2 efflux in the Kalahari and Tengger deserts, respectively, but these studies did not estimate the contribution of biocrust to total R_s. In agreement with our results, both Zhang et al. (2013) and Castillo-Monroy et al. (2011) proved that the biocrust respiration is a major player in CO_2 efflux, contributing ca. 66 % and 42 % to total R_s in a temperate desert and a semiarid ecosystem, respectively. In the light of these findings and our own results which show a biocrust contribution of ca. 23.6 % in a sub-humid Mediterranean ecosystem, it could be inferred that the biocrust contribution to R_s increases with the aridity of the site. A higher contribution of deeper soil layers in the less arid ecosystems can be due to a number of factors promoting both root and microbial respiration such as higher root density, microbial biomass and soil organic C.

It is worth noting that the interpretation of CO_2 efflux data is complex, since it involves several different processes, such as soil microbial, root, arthropods and mycorrhizal respiration, and therefore changes occurring in any of these processes can be masked by opposite changes in other ones (Buyanovsky and Wagner, 1995). Consequently, it has been pointed out that soil CO_2 efflux data are of moderate value in differentiating soil processes (Raich and Tufekcioglu, 2000). However, as we always waited for soil to drain for three days after rainfall before measuring CO_2 efflux to minimize the plot disturbance while soil was muddy and to focus on the effect of T_s and SWC on R_s rather than on the effect of rewetting on R_s, we can exclude the possibility of a contribution from two typically important processes in this type of ecosystems. First, the physical displacement of higher concentrations of CO_2 following rainfall that would be accumulated in pore spaces between rainfall pulses (Huxman et al., 2004). Second, the rewetting of dry soils which frequently produces a R_s peak, also known as Birch effect (Birch, 1964). This process is particularly relevant in Mediterranean ecosystems, as it is known to constitute a large proportion of the total R_s in water limited ecosystems (Jarvis et al., 2007; Unger et al., 2010). On the other hand, our experimental design, which accounted for R_s in dark conditions, was conceived to exclude the contribution of the biocrust autotrophic components because we were interested in assessing R_s instead of soil net CO_2 exchange. Determining the length of the dark period required before the biocrust autotrophic activity stops is a controversial issue. Ladron de Guevara et al. (2014) measured R_s for 120 s leaving no time in dark conditions before measurement in a semiarid ecosystem, whereas Grote et al. (2010) darkened crusted soils for 10 minutes before measuring dark R_s in a desert ecosystem. In general, respiration rate appears to change over time so that CO_2 measurements are in part relative to the protocol used.

In agreement with other authors, respiration rates of *Soil* decreased as the soil dried (Inglima et al, 2009; Unger et al, 2012) and increased with T_s (de Dato et al., 2010; Poll et al., 2013). These results are in line with those from many previous studies that reported that T_s is a strong predictor of R_s, as rising temperatures increase soil decomposition rates, producing a proportionate increase in CO_2 efflux (Lloyd and Taylor, 1994). The Lloyd and

Taylor (1994) model has been reported to give good results for a variety of soil types (Lloyd and Taylor, 1994) and has been successfully used in a number of soil and ecosystem models (Adair et al., 2008; Kucharik et al., 2000; Thornton et al., 2002). However, as proposed by Reichstein et al. (2002), it was necessary to include soil moisture as a predictor to have an improved fit, confirming the importance of the interaction between T_s and SWC for R_s in Mediterranean ecosystems. According to this, the Lloyd and Taylor (1994) model modified by Reichstein (2002) was the best model for *Soil* respiration in our experimental site.

As microbial activity in drylands is limited by the availability of moisture and organic substrates (Zak et al., 1994), increased water content in the subsoil is likely to produce an augmented population and activity of heterotrophs, which is reflected by an increased soil CO_2 efflux. In line with these findings, Conant et al. (2000) found that soil moisture was the main factor determining R_s in three semiarid ecosystems in Arizona. However, although increased soil moisture generally leads to a rise of CO_2 efflux, respiration rates in *Soil* reached its minimum peak under the highest recorded SWC (35%) that was found in November 2014. Decreased respiration under high soil moisture has been previously reported (Grote et al., 2010; Maestre and Cortina, 2003), and is likely driven by the CO_2 affinity for water thus dissolving in soil water during wet periods, or alternatively by impeded diffusion of CO_2 through soil when the pore spaces are filled with water.

According to previous research (Ladron de Guevara et al., 2014; Thomas and Hoon, 2010; Thomas et al., 2011; Wilske et al., 2008), our results reveal that the respiration rate of the *BC* is strongly influenced by its moisture content and less by T_s. The biocrust can contribute to the R_s even in periods in which the deeper soil layers are inactive; consistently respiration rates of *Soil* depend on SWC_{-20cm}, whereas the correlation of respiration of the *BC* with SWC is strongest at 5 cm depth. The SWC at 20 cm and 5 cm depths strongly co-vary, however small water pulses can increase the moisture only of the top layer, and the top layer may dry much faster than the deeper ones. Thus, small water pulses can activate lichens, mosses and cyanobacteria associated to the biocrust, and the metabolism of C in soils, while deeper soil layers remain dormant. Likewise, respiration in the deeper soil layers may persist in periods when the biocrust is inactive. In line with these findings, Cable and Huxman (2004) found that the relative contributions of biocrust and deep soil layers to CO_2 efflux in Arizona varied with rainfall magnitude. Using δ13C and CO_2 data, they reported that over 80 % of the respiration originated from biocrust during small rainfall events, whereas 98 % was originated from deep soil layers after intense rainfall events. Consequently, the proportional contribution of the biocrust changes over the seasons, playing an important role when the soil is wet and being minimum during the dry months (i.e. July, August and September).

We found that both the modeled and estimated *BC* contribution to *Soil+BC* respiration rate peaked following the soil rewetting after the rainless summer period. Similar results have been found by Zhao et al. (2014) when studying mixed biocrust (composed of mosses, algae and lichens) and algae-dominated biocrust respiration responding to an extreme rainfall event in a mesocosm experiment. Zhao et al. (2014) reported that CO_2 efflux was inhibited for several days after the rainfall event in both biocrust types, and then it increased. However, less influence of extreme rainfall events on R_s was found for moss-dominated biocrusts. The authors attributed these findings to the increased soil hydrophobicity due to the biocrust extracellular polysaccharide (EPS) secretions (Mager and Thomas, 2011). EPS can block superficial soil pores (Avnimelech and Nevo, 1964) leading to water accumulation in the surface of mixed and algae-dominated biocrust after heavy rainfall. As EPS are decomposed by microbes (Fischer 2009), soil pores will reopen producing a burst in CO_2 efflux in mixed and algae-dominated biocrust. In contrast, the higher porosity and infiltration rates that characterizes moss tissue allows water to percolate deep into the soil avoiding water accumulation on the soil surface in moss-dominated biocrusts (Frey and Kürschner, 1991; Chamizo et al., 2012). An alternative explanation for these findings is that a relatively thick film of water for the first days after the rainfall may restrict the exchange of gases, impeding in turn photosynthesis and respiration until water evaporation (Lange et al., 1992). The higher infiltration on moss-dominated biocrust could also be attributed to the low amount of EPS at these crusts due to the fact that mosses do not excrete EPS (Kidron et al., 2003). Given that the soil rewetting dynamics have been proved to affect differently to the contributions of distinct biocrust types to R_s (Zhao et al., 2014), especially after large rainfall pulses (>10 mm), it is crucial to account for the biocrust composition in this type of studies.

Dew events in water limited ecosystems are common throughout the year, and typically range from 0.15 mm to 0.30 mm per day (Jacobs et al., 2000; Moro et al., 2007). Previous studies reported that biocrust organisms can utilize the morning dew as a window of opportunity for activating their metabolism (Delgado-Baquerizo et al., 2013; Thomas and Hoon, 2010; Veste et al., 2008). However, other authors have found that while dew can wet rock and stones in a dew desert such as the Negev, its capability to wet the soil is very limited, since the activation threshold of 0.1 mm (Lange et al., 1992) is rarely reached on the soil surface (Agam and Berliner, 2006; Kidron et al., 2002). Our experimental design was focused on understanding the general effect of SWC and T_s and not of rain pulses nor of dew on biocrust respiration or the limiting effect of over saturated soil on diffusion. The estimation of superficial soil moisture is most likely an even stronger predictor for biocrust respiration than SWC_{-5cm}. Perhaps, if the superficial SWC had been monitored, an even greater decoupling of *BC* respiration from that of *Soil* would have been reported. However the isolation of superficial moisture from the background soil under field conditions is quite challenging.

This high dependence of *BC* respiration on SWC is not surprising given previous findings that linked SWC to soil microbial biomass (Van Gestel et al. 1992) and to biocrust biomass (Kidron et al., 2009), biocrust respiration to biocrust biomass (Kidron et al., 2015; Yu et al., 2012), and superficial and deep soil layers moisture to biocrust activity (Kidron and Benenson, 2014). Thus, the biocrust biomass, soil microflora and biocrust type are all shaped by the soil superficial moisture (Kidron et al., 2010). As biocrust respiration is highly dependent on biocrust type, our findings support that lichen-dominated biocrusts performance depends to a large degree on soil moisture conditions, contributing to total R_s mainly during the wet months. These findings complement those of Adebayo and Harris (1971) and Austin et al. (2004), which found that fungal-dominated microbial communities are more desiccation-tolerant than those dominated by cyanobacteria. Interestingly, although mosses generally dry during the dry season ceasing their metabolic activity and regrow in the wet season, Deltoro et al. (1998) found that mosses adapted to xeric conditions can maintain maximal levels of quantum efficiency when drying down to about 5 % soil moisture.

According to both the modeled and estimated *BC* contribution to *Soil+BC* respiration rate, *BC* represents a significant source of CO_2 during humid seasons, showing the highest peaks in autumn and winter coinciding with the maximum values of SWC_{-5cm} and SWC_{-20cm}. On the other hand, although in water limited ecosystems the biocrust is wet and metabolically active less than 10 % of the time (Lange et al., 1994), and high soil moisture likely does not occur regularly, the results of this study reveal the potential for the biocrust respiration rates under a broad range of SWC.

We found no effect of T_s on *BC* respiration, suggesting that in Mediterranean ecosystems T_s is not as an important driver of biocrust respiration as SWC. Johnson et al. (2012) and Zelikova et al. (2012) also found very limited sensitivity of the metabolic activity of biocrust communities dominated by cyanobacteria, cyanolichens, and mosses to T_s. Similar results were reported by Grote et al. (2010) in cyanobacteria- and lichen-dominated biocrust from the Chihuahuan desert and the Colorado Plateau.

We found higher R_s rates in *Soil+BC* than in *Soil*. Such difference can be explained by the fact that *Soil* has been devoid of habitats for a dependent foodweb of arthropods, lichens, fungi, bacteria, and other soil organisms typically found in the biocrust (Belnap and Lange, 2003), whose activity significantly contribute to soil CO_2 efflux (Castillo–Monroy et al., 2011). Given the decoupling of the respiration rates of *BC* and *Soil*, it was not possible to identify a model clearly outperforming the others for *Soil+BC*. The resulting model can be seen as a mix of the model for *Soil* and for *BC* as it responds to T_s and to SWC_{-5cm} instead of SWC_{-20cm}. Additionally, the model is multilinear losing the physiological mechanism accounted for by the Lloyd and Taylor model. The highly contrasting seasonal pattern of respiration observed in *Soil* is not visible in *Soil+BC*, suggesting that the biocrust might be masking the seasonal trend showed by deeper soil layers in *Soil+BC*, since the biocrust exhibits an independent dynamic driven by moisture in the first soil centimeters.

Conclusions

Our findings show that CO_2 effluxes emitted by *Soil, BC* and *Soil+BC* are differently driven by T_s and SWC: *BC* respiration is mainly controlled by superficial SWC, whereas T_s and SWC_{-20cm} mostly control *Soil* respiration, and T_s and SWC_{-5cm} drive *Soil+BC* respiration. Our results complement those of previous studies highlighting the key role of the biocrust as modulator of R_s in dryland ecosystems, and indicate that the biocrust has the ability to contribute to R_s responding to small water pulses in periods when deeper soil layers are inactive. Thus, our results suggest that accounting for the biocrust contribution to R_s and its responses to environmental drivers is highly relevant in providing accurate estimates of this key component of the C cycle at the ecosystem level. Projections based solely on bare ground and vegetated areas have been used to predict C budgets in semiarid (Rey et al., 2011) and temperate (Kim et al., 1992) grasslands. Nevertheless, the important differences observed in CO_2 efflux between *Soil* and *Soil+BC* suggest that these estimations may underestimate soil CO_2 efflux in spatially heterogeneous Mediterranean ecosystems. Given the large areas covered by biocrust, not only in drylands, but also in temperate, alpine and polar ecosystems (Belnap and Lange 2003), taking into account soil surface covered by biocrust in future modeling studies can significantly contribute to improve our understanding of the global C cycle and our ability to project the effects of global environmental change on soil CO_2 efflux.

Acknowledgments

Financial support was provided by the Regione Autonoma della Sardegna through the project Ecoscale Lg.7. (CRP- 60646). Special thanks are extended to Giovanni Battista Neri and Mario Antonello Deroma for helping us with field and laboratory assistance, respectively. We are also grateful to two anonymous reviewers for their detailed comments on an earlier draft.

Figures

Figure 1. Monthly precipitation and average, minimum, andmaximumair temperature at the study site from January 2012 to December 2014.

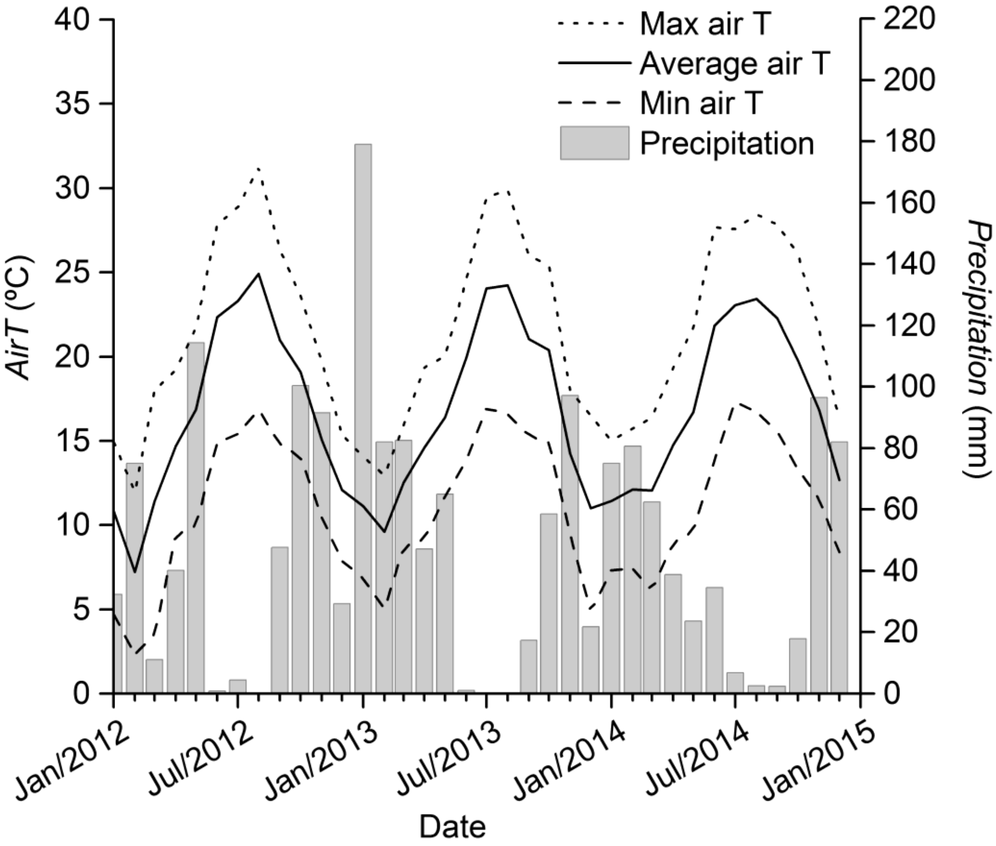


Figure. 2. (A) Daily sum of precipitation and soil water content (SWC) at 5 and 20 cm at the experimental site. (B) Daily average, minimum and maximum soil temperature (Ts) measured at 5 cm depth.

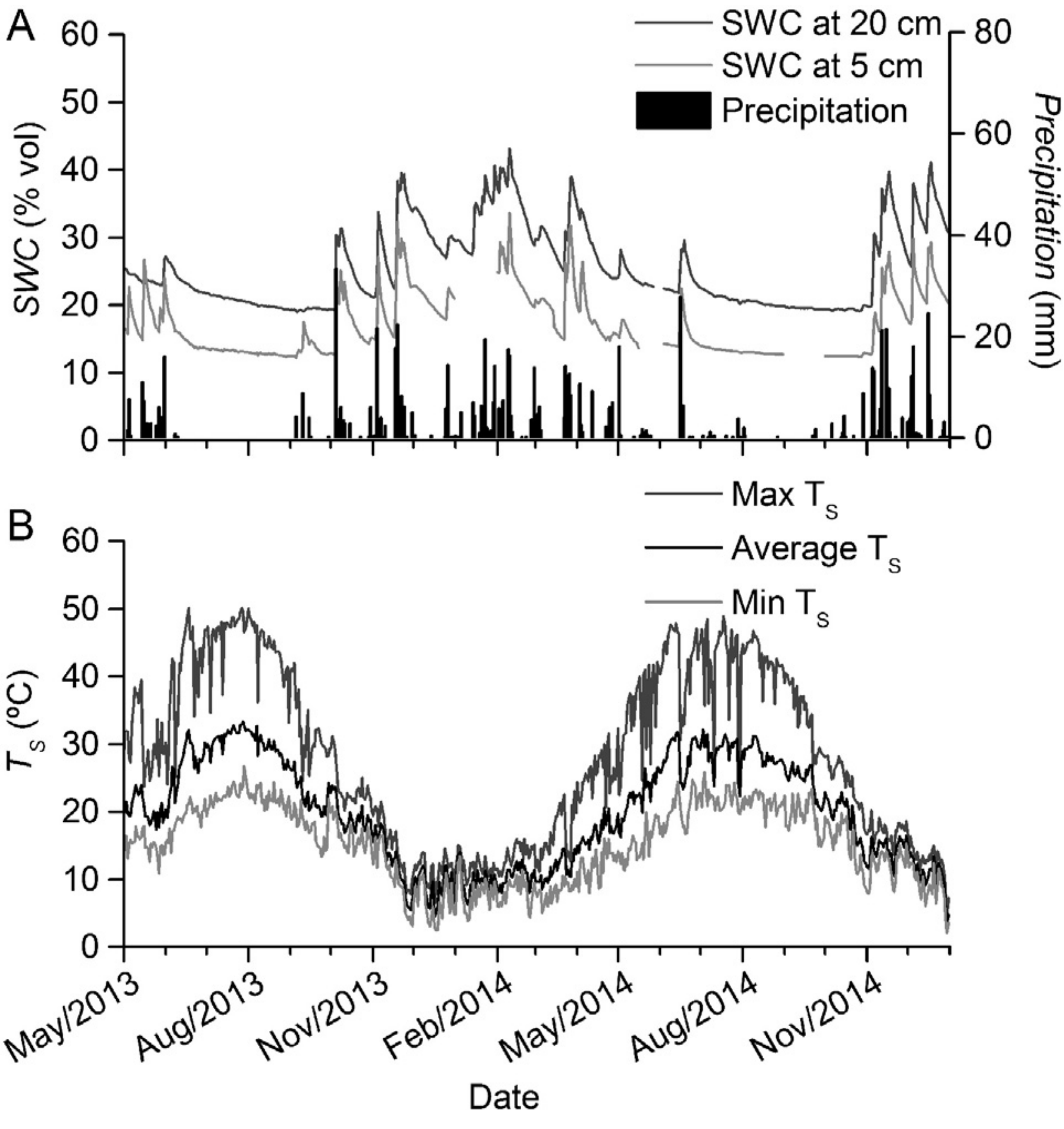


Figure. 3. CO_2 efflux emitted by (A) soil deprived of biocrust (Soil), (B) biocrust surface (BC), and (C) intact soil (Soil + BC). The BC contribution to Soil + BC respiration rate is also shown (gray line). Error bars are ±SE, n=16 for BC and Soil + BC, n = 8 for Soil.

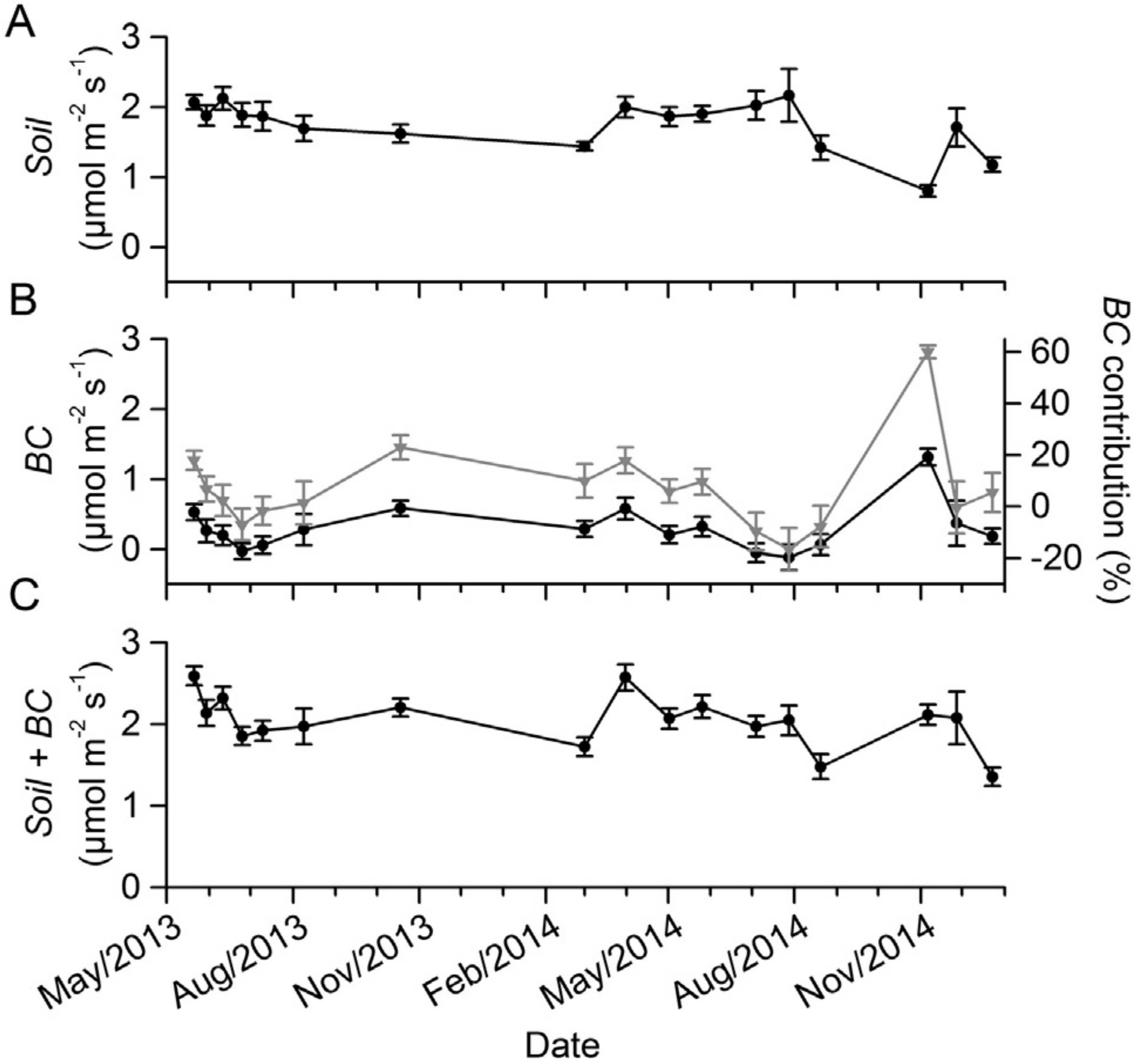
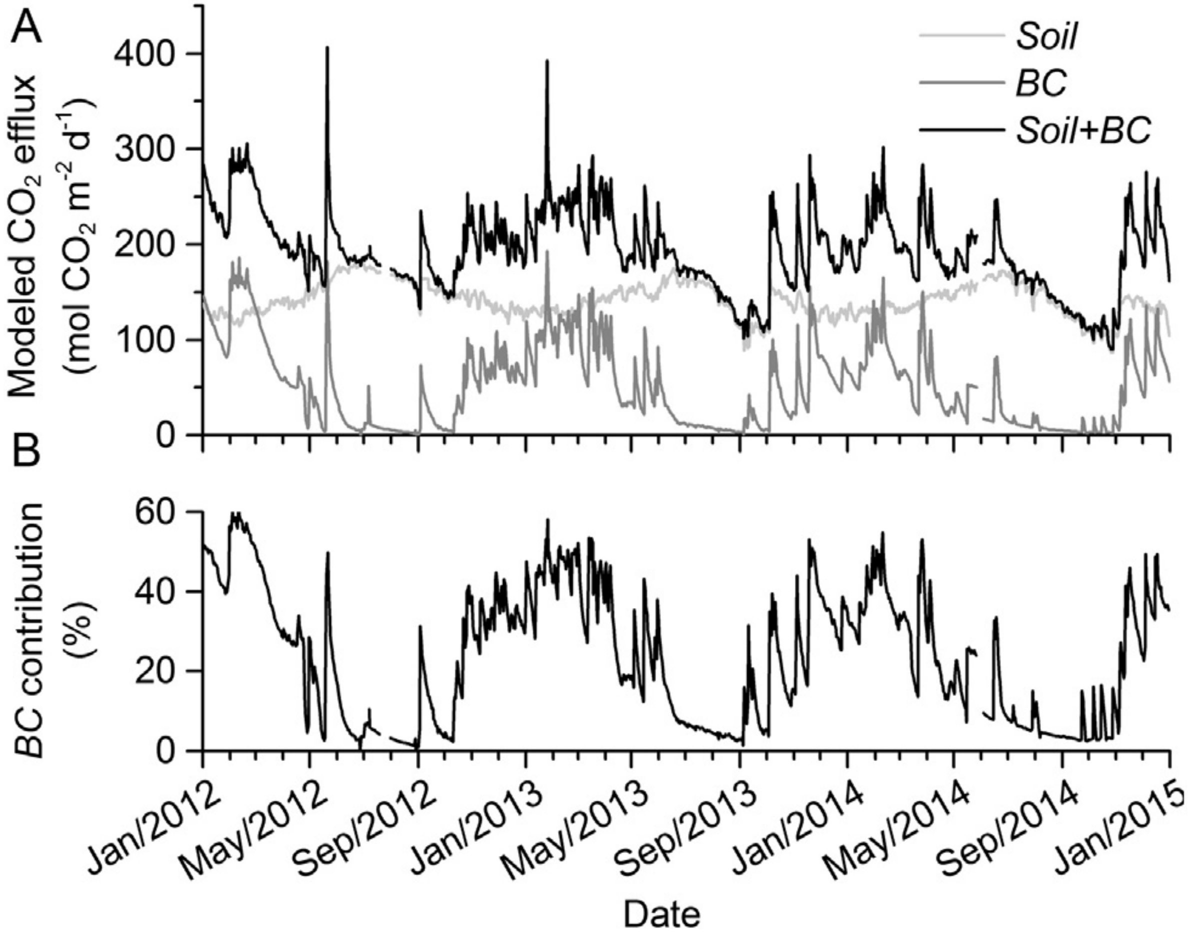


Figure. 4. (A) Daily values of modeled respiration rates emitted by soil deprived of biocrust (Soil), biocrust surface (BC), and intact soil (Soil + BC). (B) Modeled BC contribution to Soil + BC respiration rate.



Tables

Table 1. Soil chemical properties in the top 5 and 10 cm of the soil profile at the experimental site. No significant differences between depths were found. Soil sampling was performed in April 2014. SE means Standard Error (n = 8).

Soil properties	**Soil depth**			
	5 cm		10 cm	
	Mean	SE	Mean	SE
pH	7.25	0.12	7.26	0.11
Total C (%)	3.56	0.57	2.77	0.40
Total N (%)	0.194	0.03	0.162	0.02
C/N	19.25	2.34	16.62	1.31
Phosphate (P_2O_5, mg/kg)	1.75	0.45	1.12	0.30
$CaCO_3$ (g/kg)	8.62	2.53	7.37	2.07
Organic matter (g/kg)	61.03	9.44	48.33	6.63
Ca (meq 100 g^{-1})	12.80	3.09	11.77	2.23
Mg (meq 100 g^{-1})	5.88	0.65	6.01	0.51
Na (meq 100 g^{-1})	0.99	0.10	1.11	0.13
K (meq 100 g^{-1})	1.32	0.10	1.53	0.14

Table 2. Summary of partial correlations among CO$_2$ efflux, soil temperature (T$_s$) and soil water content (SWC) at 5 and 20 cm depth respectively for soil deprived of biocrust *(Soil)*, biocrust surface *(BC)* and intact soil (*Soil+BC*). Statistical differences are indicated as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). P values below 0.05 are indicated in bold.

		T$_s$	SWC$_{-5cm}$		T$_s$	SWC$_{-20cm}$
Soil	CO$_2$ efflux	**0.594***	**0.546***	CO$_2$ efflux	**0.632****	**0.563***
	T$_s$		**-0.589***	T$_s$		**-0.700****
BC	CO$_2$ efflux	-0.178	**0.710*****	CO$_2$ efflux	-0.093	0.502
	T$_s$		-0.111	T$_s$		-0.396
Soil+BC	CO$_2$ efflux	0.495	**0.749*****	CO$_2$ efflux	0.499	**0.643****
	T$_s$		**-0.568***	T$_s$		**-0.661****

Table 3. Summary of models simulating CO_2 efflux for soil deprived of biocrust (*Soil*), biocrust surface (*BC*) and intact soil (*Soil+BC*). Soil temperature (T_s) and water content (SWC), at both 5 and 20 cm depth, are used as main predictor in linear, multi-linear, and Lloyd and Taylor (LLT) equations. The best model predicting soil respiration rates for each surface is indicated in bold. Statistical differences are indicated as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). The Pearson's correlation coefficient (r), the root mean squared error (RMSE, $\mu mol\ m^{-2}\ s^{-1}$), the index of agreement (d), and the Akaike's information criterion (AIC) test are calculated for each model.

		r	RMSE	d	AIC
Soil	T_s	0.403	0,199	0,534	0.57
	SWC_{-5cm}	0.302	0.208	0.434	1.7
	SWC_{-20cm}	0.218	0.212	0.327	2.4
	T_s+SWC_{-5cm}	0.642^{*}	0.167	0.763	-2.4
	T_s+SWC_{-20cm}	0.654^{*}	0.165	0.769	-2.8
	LLT_{mod} (SWC_{-5cm})	0.440	0.196	0.563	4.0
	LLT_{mod} (SWC_{-20cm})	**0.814^{***}**	**0.127**	**0.887**	**-8.1**
BC	T_s	0.367	0.212	0.488	2.3
	SWC_{-5cm}	**0.746^{**}**	**0.152**	**0.838**	**-7.0**
	SWC_{-20cm}	0.589^{*}	0.184	0.711	-1.6
	T_s+SWC_{-5cm}	0.755^{**}	0.149	0.847	-5.5
	T_s+SWC_{-20cm}	0.594^{*}	0.183	0.710	0.3
	LLT_{mod} (SWC_{-5cm})	0.616^{*}	0.180	0.726	1.7

	LLT_{mod} (SWC_{-20cm})	NA	NA	NA	NA
Soil+BC	T_s	0.128	0.291	0.189	11.2
	SWC_{-5cm}	0.654^{*}	0.222	0.783	3.6
	SWC_{-20cm}	0.482	0.258	0.631	7.7
	T_s+SWC_{-5cm}	**0.754^{**}**	**0.193**	**0.849**	**1.7**
	T_s+SWC_{-20cm}	0.650^{*}	0.223	0.775	5.7
	LLT_{mod} (SWC_{-5cm})	0.267	0.283	0.340	14.4
	LLT_{mod} (SWC_{-20cm})	0.723^{**}	0.203	0.823	5.1

Table 4. Summary of the best models simulating CO_2 efflux for soil deprived of biocrust (*Soil*), biocrust surface (*BC*) and intact soil (*Soil+BC*). Statistical differences are indicated as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). Depending on the model the intercept parameter (a), the T_s slope (b), the SWC slope (c), the ecosystem respiration rate (R_{ref}), the activation energy parameter (E_0), the residual SWC at zero repiration (SWC_0) and SWC at half of maximal respiration rate ($SWC_{1/2}$) were calculated.

	Model	R_{ref}	E_0	SWC_0	$SWC_{1/2}$	a	b	c
Soil	LLT $_{mod}$ (SWC_{-20cm})	1.54 (0.16) ***	84.62 (25.62) **	18.49 (1.01) ***	18.98 (0.54) ***			
BC	SWC_{-5cm}					-1.07 (0.33) **		0.09 (0.02) **
Soil+BC	T_s+SWC_{-5cm}					-0.29 (0.66)	0.02 (0.01) *	0.12 (0.03) **

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Chapter 4: Contrasting effects of nitrogen deposition on soil respiration in two Mediterranean ecosystems

Manuscript published*: Environmental Science and Pollution Research*
Special issue: "*(E)merging directions on air pollution and climate change research in Mediterranean Basin ecosystems*

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Abstract

Increased atmospheric nitrogen (N) deposition is known to alter ecosystem carbon source-sink dynamics through changes in soil CO_2 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 (Rs). To fill this gap, we assessed the effects of N supply on Rs dynamics in two Mediterranean sites: Capo Caccia (Italy), where 30 kg ha^{-1} yr^{-1} were supplied for four years, and El Regajal (Spain), where plots were treated with 10, 20 or 50 kg N ha^{-1} yr^{-1} for eight years. The effects of spatial variability and seasonality typical of Mediterranean ecosystems were also tested. At both sites, the addition of N altered soil chemistry and caused a complex, non-linear response in Rs, which depended on amount and duration of the treatments as well as the spatial and temporal heterogeneity of the sites. While the addition of N may initially stimulate Rs, at high doses or over long periods the accumulation of N may reduce Rs. Spatial heterogeneity in soil cover and seasonality of precipitations also played a key role in determining the spatial variation of Rs and the overall effects of N on Rs.

Keywords: Air pollution, C and N interaction, C and N cycling, CO_2 emission, fertilization experiment, global change, soil nutrients, spatial-temporal variability.

Introduction

The global carbon (C) and nitrogen (N) cycles have been drastically altered by humans as a result of the increasing demand for energy, raw material and food (Gruber and Galloway 2008; Lamarque 2005). Combustion of fossil fuels and increasing industrial activities are associated with the emission of carbon dioxide (CO_2), which is largely responsible for the rapid rise in atmospheric CO_2 concentration during the past 50 years (IPCC 2007). During the same period, there has also been a dramatic increase in the concentration of oxidized (NO_x) and reduced (NH_3) nitrogenous compounds in the atmosphere that is largely attributable to vehicular traffic and agricultural practices, respectively (Ciais et al. 2013). Reactive nitrogenous compounds are subsequently deposited back to the biosphere, where they can affect the structure and functioning of ecosystems (Galloway et al. 1995; Vitousek et al. 1997). Besides the direct effects of increasing atmospheric CO_2 concentration and eutrophication, the complex interactions and feedbacks between the C and N cycles can have important consequences for global climate change (Schimel 1995). Given that most terrestrial ecosystems are limited by N availability, increasing N deposition could result in increasing C sequestration (Janssens and Luyssaert 2009; de Vries et al. 2014), although positive feedbacks between N deposition and climate change can also be expected as a result of altered fire regimes or a general degradation of ecosystem health.

Soil CO_2 emissions, mostly derived from the microbial processing of organic matter, represent the second largest 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 soil respiration (Rs) can have large cascading effects on atmospheric CO_2 concentration and thus the global C balance. Nitrogen additions are known to strongly influence the abundance, composition and activity of soil microbial communities (Reich, Hungate & Luo 2006b; Ferretti *et al.* 2014), which are the main drivers of soil-atmosphere CO_2 fluxes. Nitrogen deposition can also affect organic matter decomposition through changes in soil faunal and microbial community structure (Hungate et al. 2003). The most widely reported pattern is a reduction of Rs in response to N addition, which could mitigate rising atmospheric CO_2 concentration and climate change (Janssens et al. 2010; Reich, Hobbie, et al. 2006). In line with this, a meta-analysis of experimental N manipulation studies showed a general reduction in Rs of *ca.* 15% in response to N additions (Janssens et al. 2010). However, this response can range from no effect at all to positive and negative depending on ecosystem type, age, dominant plant species, soil chemical characteristics (Pregitzer et al. 2008; Rodriguez et al. 2014), climate conditions and experimental duration (Zhou et al. 2014). In another study, (Zhang et al. 2014) reported a positive correlation between N addition and Rs in a semiarid ecosystem in China, whereas Bowden *et al.* (2004) showed that N addition suppressed Rs in a Californian grassland. In a recent study, He *et al.* (2015) found a significant reduction of Rs in response to high N doses (112.5 kg N ha^{-1} yr^{-1}) in a semiarid ecosystem, whereas lower N deposition (37.5 kg N ha^{-1} yr^{-1}) significantly increased Rs. Thus, despite the general assumption that N additions lead to a reduction in Rs, numerous studies have shown a complex and non-linear relationship between N additions and Rs. Moreover, most studies assessing the effect of N additions on Rs have been conducted in temperate (Thomas Quinn et al. 2010) and boreal forested ecosystems (de Vries et al. 2009), whereas little is known about how increasing N availability affects Rs in semiarid Mediterranean ecosystems, as research in these areas has been classically neglected.

Understanding the effect of N deposition in Mediterranean ecosystems is particularly relevant as these ecosystems experience high rates of N deposition and projections for 2050 estimate a further increase due to increased 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} yr^{-1} of mid-1990s to 12 kg N ha^{-1} yr^{-1} in 2050 (Phoenix et al. 2006). Semiarid ecosystems, such as those typically found in the Mediterranean Basin, are also regarded as particularly important controllers of between-year variations in the global C cycle due to extremely pronounced high intra and inter-annual climatic variations (Poulter et al. 2014), which makes it even more difficult to extrapolate findings from other ecosystem types (e.g., boreal, temperate) to Mediterranean ecosystems. In this sense, 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, Fisher, and Grimm 2005). Although this seasonal pattern has been recognized as an important driver of Rs in Mediterranean environments (Almagro et al. 2009; Curiel Yuste et al. 2003), manipulative experiments assessing the effects of N addition on Rs, 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 comparable N addition experiments in Mediterranean ecosystems; one in a coastal macchia in Sardinia (Italy) and one in a semiarid shrubland in (central Spain) in which the amount of N added mimicked both N deposition rates projected for the Mediterranean Basin in 2050 (Raúl Ochoa-Hueso, Maestre, et al. 2013) and N deposition rates measured in other Mediterranean-type ecosystems (Fenn, Haeuber, et al. 2003). The main objective of this study was to assess how the amount and duration of N additions affect soil chemistry and *in situ* Rs fluxes. Measurements of Rs were also taken directly beneath shrub canopies as well as in open bare soils (BS) to determine if Rs responses to N addition are similar among the landscape or if they depend on the spatial heterogeneity of aboveground vegetation. We hypothesized that i) Rs would be negatively influenced by N addition (Janssens et al. 2010), yet this response would strongly depend on the amount and duration of N additions, and that ii) the effect of N addition on Rs would strongly depend on the spatial variability and seasonality typical of Mediterranean ecosystems. We specifically predicted that Rs responses to N would be clearer and more clear and stronger in the spring growing season, when soil moisture and temperature conditions are optimal for microbial activity and plant growth.

Material and methods

Sites description

Capo Caccia, Italy

Capo Caccia is a coastal ecosystem (74 m a.s.l.) 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 (Supplementary table 2a). 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 (Supplementary table 2a). The soil is Lithic Xerorthent and Typic Rhodoxerarfs (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% is BS colonized by a well-developed biological soil crust (BSC) community composed by common species in Mediterranean soils (Supplementary table 1) 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) (Simpson et al. 2012) natural NOx deposition from 2000 to 2013 was approximately 0.96 Kg NOx ha^{-1} yr^{-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 ha^{-1} yr^{-1}

A meteorological station, located 20 m away from the closest plot, has been acquiring data since 2004 and includes measurements of: air temperature (Tair) 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 m x 7.5 m) were randomly established. Each plot contained at least one fully developed individual of *Juniperus phoenicea* L. and a portion of BS. 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 BS microsite (n = 48 total PVC collars). Starting in April 2012, four plots were treated with 30 kg N ha^{-1} yr^{-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 ha^{-1} yr^{-1}, while the next applications were of 7.5 kg N ha^{-1} yr^{-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.

Soil CO_2 efflux measurements were performed *in situ* using a portable Li-8100 (LI-COR Inc., Lincoln, NE, USA) containing an infrared gas analyser 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 three days after a rainfall event to avoid measuring at times when soil water content (SWC) was abnormally above its normal field capacity. From August 2012 to October 2015, measurements of soil CO_2 efflux were always taken between 12:00 a.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 (Ts) and SWC.

In order to assess local environmental differences, six Ts 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 a.s.l.) 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 (Supplementary table 2b). Mean annual Tair 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 (Supplementary table 2b), (data provided by the AEMT for Getafe, the closest meteorological station at 45 km north from El Regajal). 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. (Supplementary table 1). 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 NOx deposition of approximately 1.05 Kg NOx ha^{-1} yr^{-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 ha^{-1} yr^{-1}.

In September 2007, six replicated blocks were established. Each block consisted of four 2.5 x 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} yr^{-1} respectively as described in Ochoa-Hueso and Manrique (2011) and compared to an unfertilized control plot. Two L of a 0, 19, 37 and 93 mM NH_4NO_3 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, Baron, et al. 2003), a three-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 CO_2 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, Rs 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 Rs measurements in each plot were averaged prior to data analysis. Soil CO_2 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 intercalibration between the two Licor-8100 instruments under controlled laboratory conditions.

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, Rs 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 Ts and moisture probe (EC-5, Decagon Devices Inc., Pullman, USA) placed at 5 cm depth between the blocks. Sensor was connected to external data logger (HOBO 4-chanel, 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 ºC 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 ($K_2Cr_2O_7$) 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 (H_2SO_4) and potassium sulfate (K_2SO_4) 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 K_2SO_4 at a ratio of 1:5, followed by shaking for 1 h at 200 rpm at 20 ºC and then filtered through a 0.45 mm Millipore filter (Jones and Willett 2006). The NH_4^+-N concentration was estimated directly via the indophenol blue method using a microplate reader (Sims, Ellsworth, and Mulvaney 1995). The NO_3^--N content was first reduced to NH_4^+-N with Devarda alloy and its concentration was determined as the difference between the Devarda-incubated and unincubated samples (where the NH_4^+-N concentration was estimated). Soil inorganic N concentration was calculated as the sum of NH_4^+-N and NO_3^--N. The DON content in the extracts was first oxidized to NO_3^--N with potassium persulfate ($K_2S_2O_8$) in an autoclave at 121 ◦C for 55 min and then reduced to NH_4^+-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 g of fresh soil were 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 K_2SO_4 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 [GLMM], 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 showed) 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 Rs 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 Rs.

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

To examine whether Rs 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 Ts effects were considered as covariates, whereas the time measurement and the experimental design were treated as random variables (Table 1). We also tested how CML.N affected Rs in the two microsites independently using the same GLMM but excluding the microsite effect (Table 1, Figure 1, inset and Figure 2, inset).

We tested how CML.N affected Rs in El Regajal using GLMM. Similar to the previous model, CML.N was the fixed factor and SWC, and Ts 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 (Figure 3, inset).

To identify time of year when N addition affected Rs, at both study a one-way ANOVA among the treatments was performed for each single date of measurements (Figures 2, 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 proprieties 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 in 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). Organic carbon in control BS resulted lower in treated UC.

Capo Caccia

During the three-year study, 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±0.3%, respectively) than in summer (21.0±0.1%, Figure 4). A similar SWC trend was found the UC microsite for spring, autumn and winter (26.1±0.4%, 28.1±0.5% and 37.7±0.5%; respectively) than in summer (18.8±0.1%, Figure 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 BS 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 UC microsite Ts ranged between 32.1 °C in mid-July 2015 and 2.5 °C mid-February 2012 (Figure 4). Although not significant, Ts was slightly higher in the BS microsite compared to the UC microsite, during the rainless period

Seasonal pattern in Rs at both microsites was dependent on SWC and Ts. In the UC microsite, Rs was negatively and significantly correlated with SWC ($r = -0.15$, $p = 0.01$), whereas Ts was not significantly correlated with Rs ($r= 0.09$, $p = 0.11$). In contrast, Rs at the Bs microsite was positively correlated with Ts ($r = 0.16$, $p = 0.01$) but not with SWC ($r = 0.06$ $p = 0.31$). In the UC microsite, Rs 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; Figure 2, inset). Consequently, when analyzing both microsites together, N addition resulted in marginally higher Rs (Table 1).

Soil pH in the BS microsite was the only variable within microsite significantly reduced by the addition of N (Table 2). We also found a significant difference in the Organic C soil content between control BS and fertilized UC (Table 2).

El Regajal

In El Regajal, SWC values were higher in spring and summer (6.7±0.7% and 8.8±0.4%, respectively) compared to early autumn (6.7±0.5%). Soil temperature at 5 cm depth ranged from 38.5 °C in early July 2015 to 3.8 °C at the early December 2015 (Figure 5).

In all treatments, Rs was correlated with change in both Ts ($r = -0.26$, $p = 0.01$) and SWC ($r = -0.54$, $p = 0.01$) which resulted in Rs reaching its maximum during the early growing season before decreasing to lower rates in August and late October (Figure 3). The treatment effect was not significant in early spring measurements, while all N addition levels (i.e., 10 and 20 kg N ha^{-1} yr^{-1}) resulted in a significant reduction of Rs ($p < 0.05$ 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 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} yr^{-1} (Table 2).

Discussion

Our results revealed contrasting responses of Rs to N addition (overall increase at Capo Caccia and reduction at El Regajal), suggesting that the amount and duration of N additions as well as the spatial and temporal heterogeneity typical of Mediterranean ecosystems can modulate the effects of atmospheric N deposition on Rs 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 low N addition doses reduced Rs. In contrast, in Capo Caccia, an increase in Rs was observed in both the UC and BS microsites, which suggests that the dose of N addition may not be the most important factor influencing Rs. Instead, the cumulative amount of N added may be more important and it is only after a certain threshold has been reach that, one or more ecosystem properties to shift to a new qualitative and quantitative state. Since El Regajal has received N additions for eight years compared to the four years at Capo Caccia, it is possible that N additions initially cause an increase in Rs and after a long period of N addition a threshold is reached, resulting in reduced Rs. 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 response to added N (Litton, Raich, and Ryan 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. The contrasting responses of Rs to N additions may also reflect soil N availability (Zhu et al. 2016) and cationic exchange. In N-limited ecosystems, N additions may cause an initial increase in soil microbial biomass and heterotrophic respiration. However, with further N additions a threshold will be reached where N is no longer limiting microbial and plant growth thereby reducing Rs. Opposite responses of Rs could be due to different initial conditions, e.g. additional N may inhibit respiration in already N rich systems while it could be enhanced in nutrient poor sites. 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 n El Regajal have an NO_3 concentration that is about 5 times higher than in Capo Caccia and are also richer in DON. These NO_3 and DON indeed represent two forms of N that are actually used by microbes and roots suggesting that Rs is inhibited in soils rich in these two N forms which is not reflected by a higher concentration in Total N.

The high spatial and temporal heterogeneity in plant cover and soil properties plays a key role in modulating Rs rates in Mediterranean ecosystems (Maestre and Cortina 2003) and could be affected differently by N additions. Our findings are in line with other studies in semiarid ecosystems that suggest that SWC and Ts are the main environmental drivers of Rs (Almagro et al. 2009; Morillas et al. 2017). Despite higher soil temperatures in the BS microsite at Capo Caccia, Rs 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 CO_2 efflux (Han et al. 2007; Hasselquist, Metcalfe, and Högberg 2012; Tang and Baldocchi 2005) 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 ecosystems found that autotrophic respiration accounts for roughly 25% of total soil Rs in a Mediterranean pine forest Matteucci et al. (2015).

Higher rates of Rs 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. 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 explaining relatively higher Rs 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 Rs. 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).

Interestingly, the positive effects of N addition on Rs at Capo Caccia are primarily found when soil moisture is high (above 30%). The lack of N addition effect in drier periods suggests that water availability may also be a limiting factor for Rs, which is consistent with other studies (Curiel Yuste et al. 2007), especially for heterotrophic respiration (Correia et al. 2012). However, the opposite mechanism takes place in the El Regajal, with most variability in Rs explained by soil properties in spring (more water available) while in summer the Rs of all N treated plots is inhibited compared to the control.

Conclusion

Results from this study are some of the first to show how increasing rates of N addition may influence soil C dynamics in semi-arid ecosystems from the Mediterranean Basin. This study clearly shows a complex, non-linear response of Rs to N additions, which is likely the result of the amount and duration of N additions as well as the spatial and temporal heterogeneity typical of Mediterranean ecosystems. The addition of N may initially stimulate Rs, whereas at high doses or over long-time periods the accumulation of N may reduce Rs. Spatial heterogeneity in soil cover and seasonality of precipitations also played a key role in determining the spatial variation of Rs and the effects of N on Rs.

Acknowledgments

This research was financially supported by the project “ECO-SCALE” through the Regional Administration of Sardinia, RAS, L.R. 7/2007 “Scientific Research and Technological Innovation in Sardinia”. MLC received funding by PhD grant from the Euro-Mediterranean Centre on Climate Change (CMCC) and received an Erasmus scholarship in collaboration with the Department of Biogeography and Global Change, Spanish National Research Council (CSIC), Madrid, Spain (2014-1-IT02-KA103-000341). This research was also financially supported by a research grant of the Spanish Ministerio de Economía y Competitividad (BIOMOD CGL-2013-44661-R). Comunidad de Madrid also funded this work through REMEDINAL-3 project (S2013/MAE-2719). The authors are very thankful to the owners of “Finca El Regajal” for their kind allowance to work in their property. Special thanks are extended to Iolanda Cocco for helping us with field and laboratory assistance. SM thanks the Fundação para a Ciência e Tecnologia (FCT) Investigador grant.

Figures

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

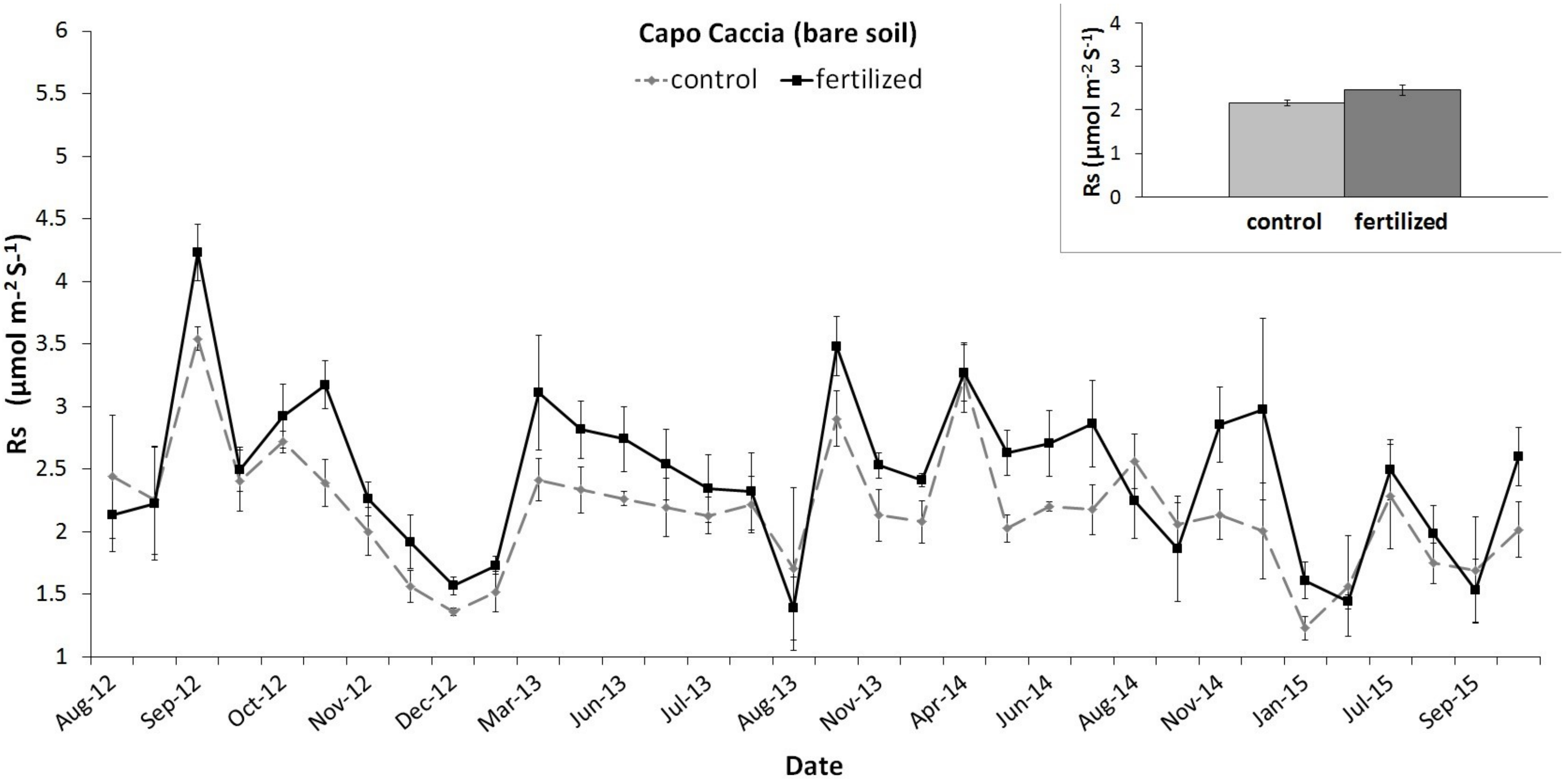


Figure 2. Effect of N addition (30 kg N ha^{-1} yr^{-1}) on soil respiration (Rs) under the canopy of *Juniperus phoenicea* L. at Capo Caccia (n=4). Asterisks indicate significant differences between fertilized and control plots ($p < 0.05$ ANOVA). Inset graph shows the grand mean Rs for the fertilized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

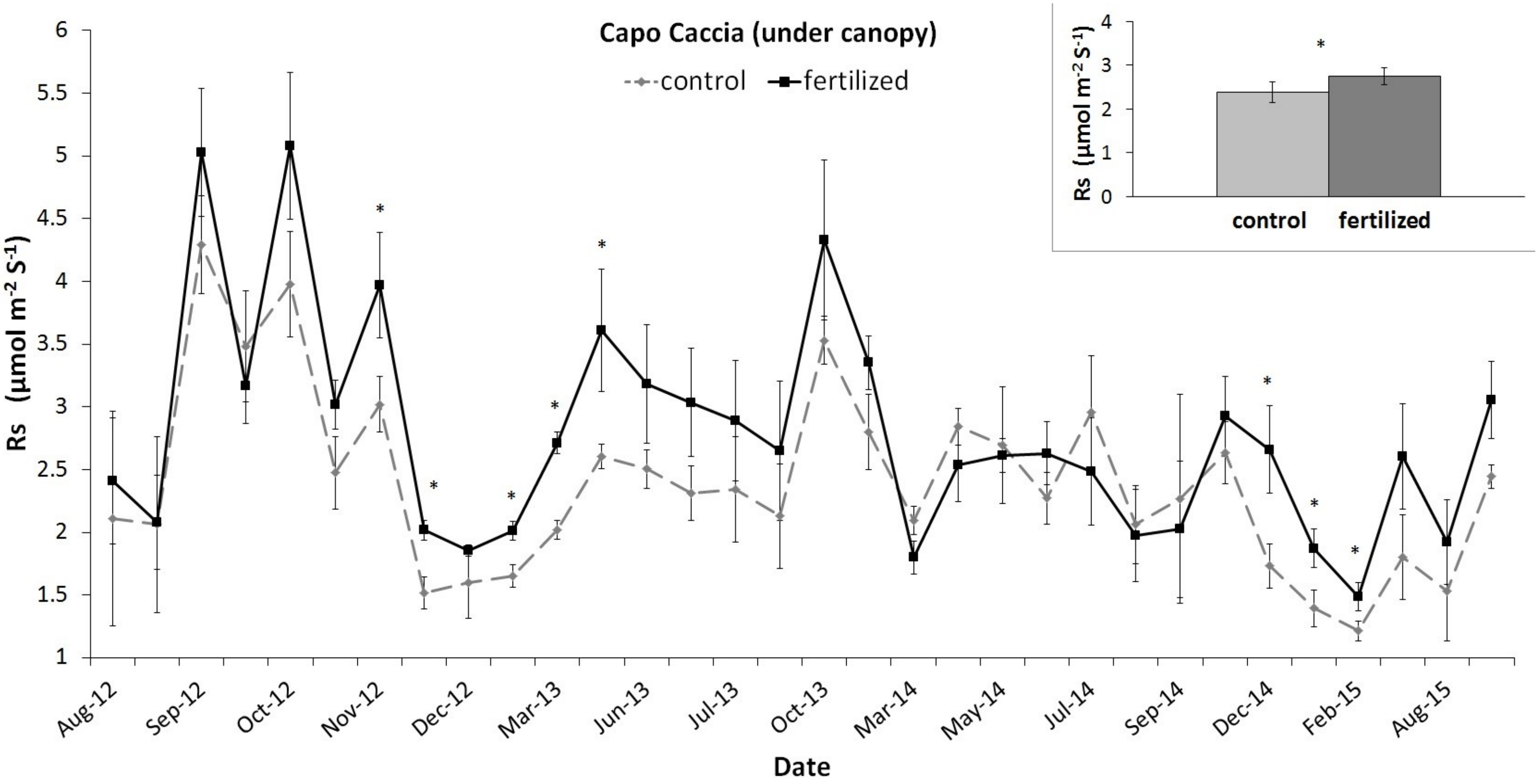


Figure 3. Mean (±SE) soil respiration (Rs) for all N addition treatments (0, 10, 20 and 50 kg N ha^{-1} yr^{-1}) at El Regajal (n=6). Different letters indicate significant differences among treatments ($p < 0.05$ ANOVA, post hoc LSD test). Inset graph shows the overall mean Rs average for control (±0.06) and N treatments, 10 (±0.04), 20 (±0.07) and 50 kg N ha^{-1} yr^{-1} (±0.10). Different letters in the inset indicate 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} yr^{-1}.

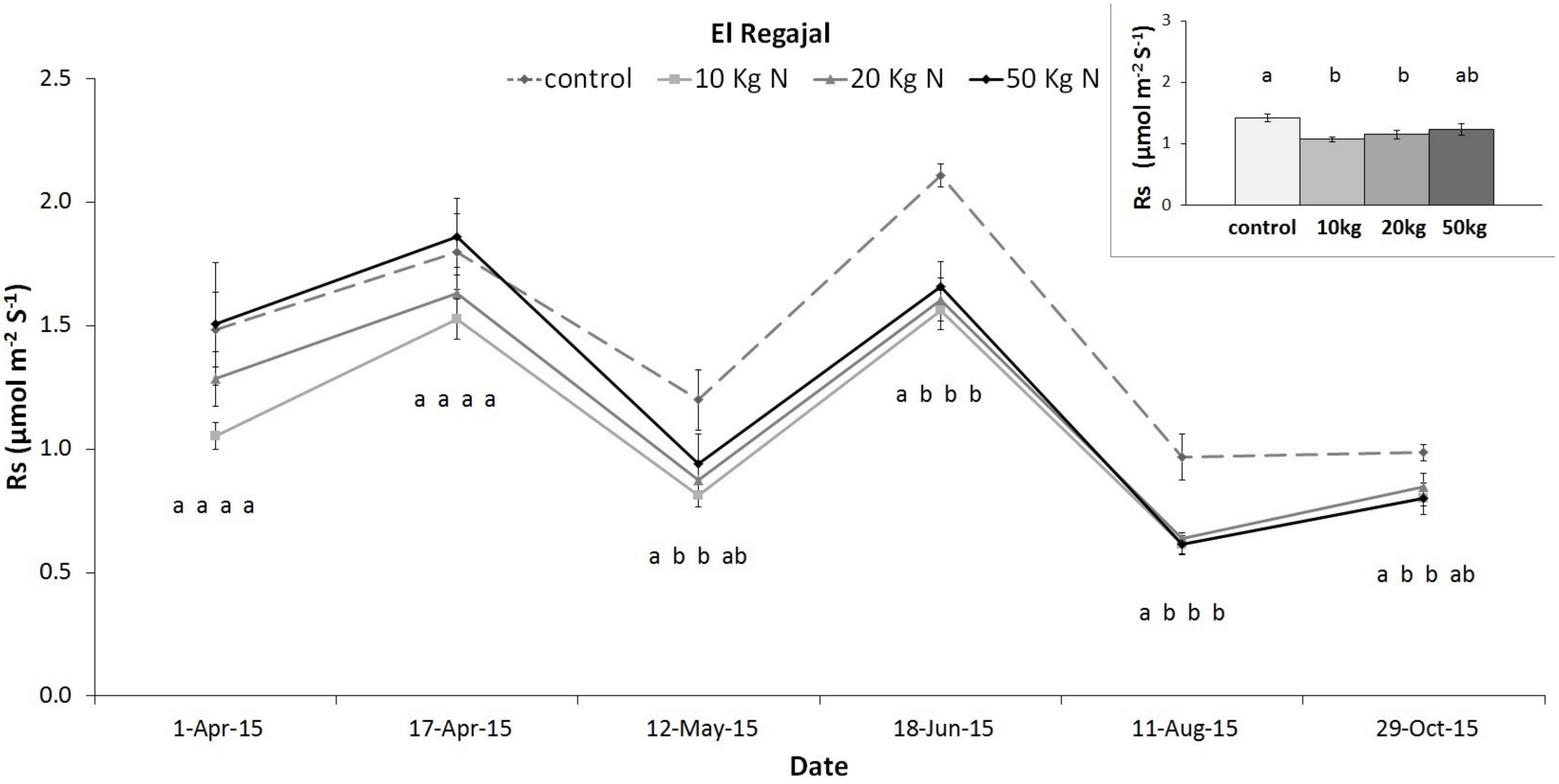


Figure 4. Daily mean soil temperature (Ts) (upper panel) at 5 cm depth measured for the bare soil (BS) and under canopy (UC) microsites and daily mean soil water content (SWC) (lower panel) at 20 cm depth for BS and UC microsites in Capo Caccia. Data collected from January 2012 to December 2015 (n=1460).

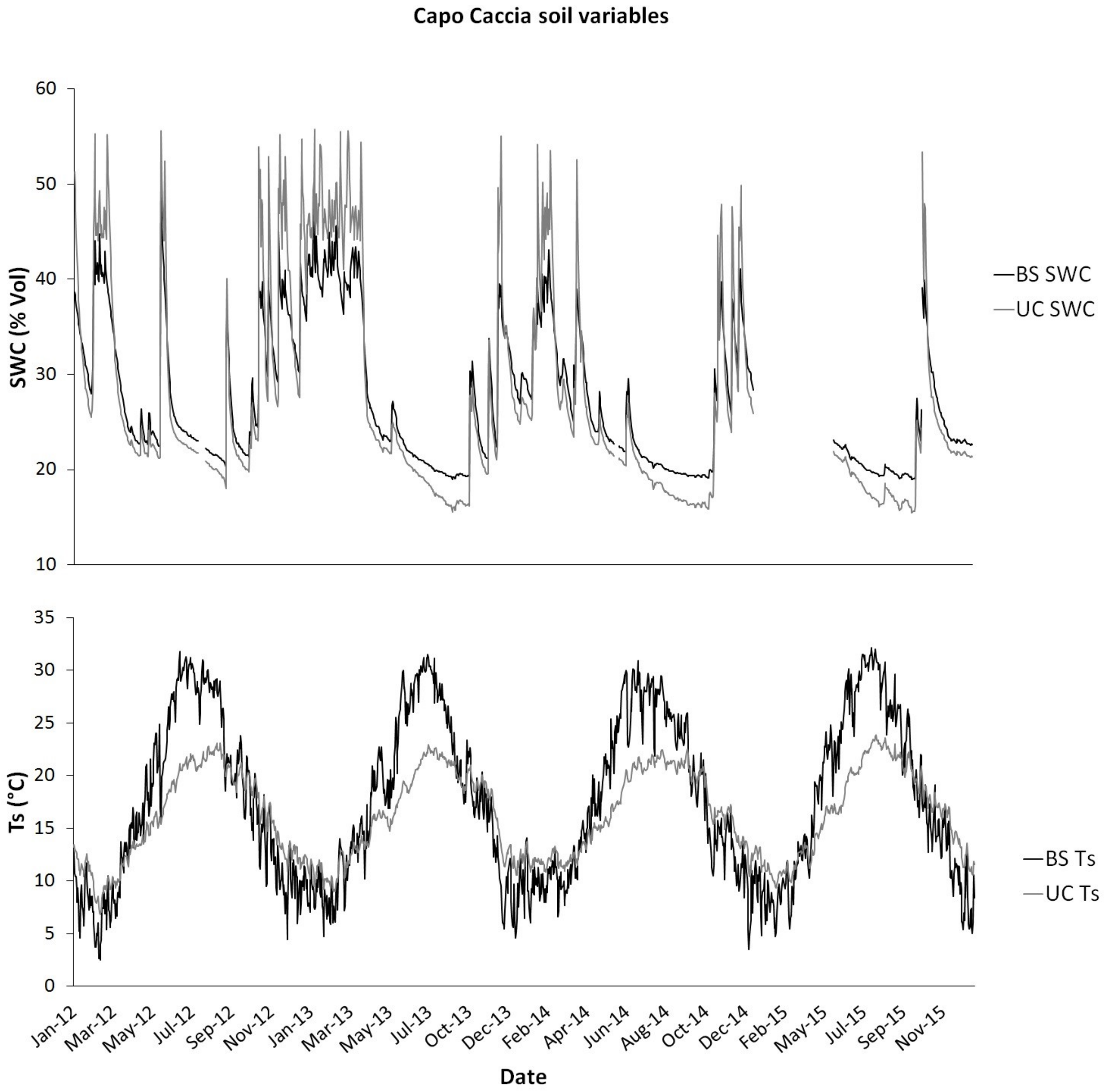
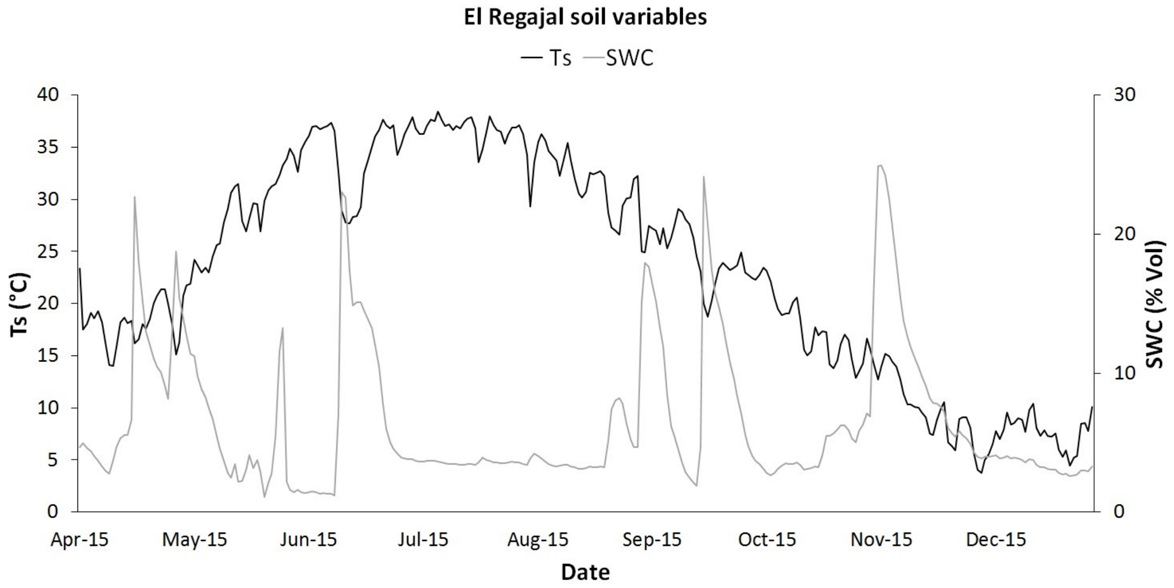


Figure 5. Daily soil temperature **(**Ts) and soil water content (SWC) at 5 cm depth measured in El Regajal. Data collected from April 2015 to November 2015 (n=275).



Tables

Table 1. Results of the generalized linear mixed model (GLMM) for Capo Caccia, including the two microsites (bare soil and under canopy) and for El Regajal. In Capo Caccia the GLMM accounts for the interaction between cumulative N (CML.N) and microsites (ms) as fixed factors and soil water content at 20 cm depth (SWC20) and soil temperature at 3 cm depth (Ts) as covariates on Rs response. In El Regajal the GLMM accounts for CML.N as fixed factor, soil water content at 5 cm depth (SWC5) and Ts as covariates, on Rs response. The measuring date (date) and the respective experimental designs were included in the GLMM as random factors. Statistical differences are indicated as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and are highlighted in bold. The F-value test (F-value) and the level of significance (p-value) are shown for each model.

Capo Caccia GLMM		
	F-value	p-value
CML.N	**2.026***	0.024
ms	1.659	0.219
SWC20	0.001	0.974
Ts	**6.701****	0.009
CML.N x ms	**2.472****	0.004
bare soil		
CML.N	1.271	0.261
SWC20	**5.457***	0.023
Ts	**14.858*****	<.0001
under canopy		
CML.N	**1.898***	0.045
SWC20	0.003	0.856
Ts	0.602	0.438
El Regajal GLMM		
CML.N	**3.94*****	<.0001
SWC5	**7.749***	0.050
Ts	**4.903***	0.028

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). Soil samples were collected in April 2015. Statistical differences are highlighted in bold, 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).

Capo Caccia	**bare soil**		**under canopy**		**EL Regajal**			
	Control	Treated	Control	Treated	Control	10N	20N	50N
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

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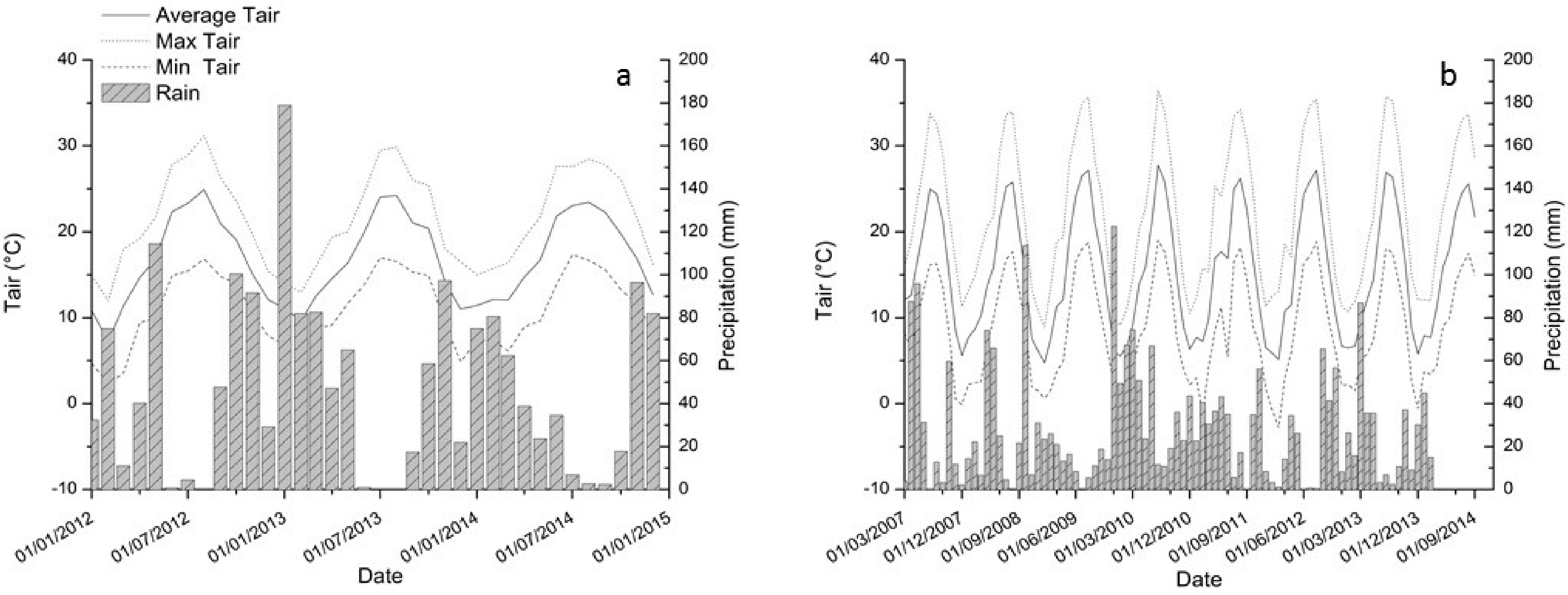
Supplementary Tables

Supplementary table 1. 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 a.s.l.)	Spain, Aranjuez 40°00'00.0"N 3°36'00.0"W (600 m a.s.l.)
Climate	Sub-humid Mediterranean	Semi-arid 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
max and min temperature (°C)	32.7 and 6.2	40 and 0
Mean annual rainfall (mm)	494	356
Canopy species cover (%)	*Juniperus phoenicia* L. (<80) *Pistacia lentiscus* L. (>12) *Phyllirea angustifolia* L. (>4) *Smilax aspera* L. (<3) *Chamaerops humilis* L. (<2)	*Quercus coccifera* L. (>85) *Rosmarinus officinalis* L. (<15) Therophytes : *Limonium echioides* L. *Asterolinon linum-stellatum* L.
Pedologic substrate	Mesozoic limestone	Cenozoic limestone
Soil type	Lithic Xerorthent Typic Rhodoxerarfs	Gypsum soil

Supplementary table 2 - Air temperature (Tair) 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).



Chapter 5: Nitrogen do affect soil microbial community abundance and activity with consequence for decomposition and potential carbon sequestration

Publish as conference paper: II CAPERmed 2016 - Brescia (IT) and XIV MEDECOS 2017 - Seville (SP)

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Abstract

The establishment of networks of experimental sites is a crucial strategy to answer global ecological questions and correctly address environmental issues. However, Mediterranean and semi-arid ecosystems are underrepresented in global networks, including those focusing on the effects of nitrogen (N) on ecosystem structure and processes. NitroMed is a recent network composed of three N manipulation experimental sites, namely Arrábida (Lisbon, Portugal), El Regajal (Madrid, Spain) and Capo Caccia (Alghero, Italy). Here we present a preliminary analysis performed on the three sites using Structural Equation Modeling (SEM) to understand the cause-effect mechanisms that determine changes in litter decomposition under different N loads. Our results suggest that N deposition increases soil N availability and reduces soil pH, which in turn has an effect on microbial community structure (to lower fungi/bacteria ratio) and overall enzymatic activity, responsible for decomposition rates. Our findings also show how site-specific heterogeneity can greatly influence ecosystem processes, highlighting the importance to increase the number of experimental sites in the NitroMed network.

Introduction

Over long time frames, the decomposition and humifaction of organic matter determines the carbon (C) accumulation in soil. Given the importance of CO_2 emission and C sequestration for climate change (CC), substantial research is focusing on how to avoid loss of C from soils or enhance C accumulation rates in both natural and agricultural lands. Microbial-mediated decomposition of Soil Organic Matter (SOM) gives a substantial contribution to soil respiration, which is typically the main source of CO_2 arising from terrestrial ecosystems (Yuste et al. 2011). Despite the central role played by decomposition, there is still a large uncertainty on the effect of increased N depositions and CC over the ecology of microbial communities and consequently on soil CO_2 emissions. Microbial communities responsible for the decomposition of SOM are known to be affected by numerous abiotic and biotic factors as water availability, soil acidity, plant community composition and nutrient availability.

The Mediterranean region has been identified as one of the most prominent "Hot-Spots" in future climate change projections (Giorgi 2006), consisting of a pronounced decrease in precipitation, especially in the warm season,(Giorgi and Lionello 2008). The structure and functioning of semi-arid ecosystems are strongly influenced by precipitation patterns (Miranda et al. 2011) and especially in Mediterranean- type ecosystems water availability is already the most important environmental constraint (Yuste et al. 2011)**.** Drought limits the physiological performance of microbes, inhibit the diffusion of extracellular enzymes, altering the diffusion of soluble nutrients in the soil pore (Yuste et al. 2011)**.** Among nutrients, nitrogen (N) covers a key role in different biological processes, but reactive N (Nr) has well-known deleterious effects at high concentrations (Shibata et al. 2015), and is well reported that the anthropic activities are strongly altering the N cycle in ways that impact the integrity, health and resilience of the ecosystems at multiple geographical scales (Galloway et al. 2004)

Increases of anthropogenic Nr emission and deposition substantially alter N pools, cycles, and transport among ecosystems, in addition the high seasonal variability specific of the

Mediterranean climate (García-Ruiz et al. 2011) and the future predicted altered rain events promotes an increase of the N dry deposition which becomes bioavailable with the first rain event (Fenn et al. 2003; Ochoa-Hueso and Manrique 2011). This nutrients overload is increasingly being found to influence structures and characteristics of ecosystems and particularly soil microbial processing (Stoddard 1994; Piatek et al. 2005; Curtis et al. 2012) (Allen et al. 2007).

Altered precipitation regimes, higher soil temperatures, and increasing atmospheric N deposition (Craine et al. 2007), can induce direct (i.e., alter physiological activity) and indirect (i.e., alter the soil pH and/or alters resource supply, which, in turn, alters physiological activity) influences on soil microbial communities, and therefore influence the C and N soil biogeochemistry. At present , there is an uncertainty regarding how increases in nutrient levels and CC will impact the decomposition processes and is becoming impelling to study and disclose the mechanism(s) underlying the cause/effect relationship of above described observations , to predict the magnitude and direction by which the multiple facets of environmental change will alter the future functioning of terrestrial ecosystems (Zak et al. 2011).

We based our study on a network of three long term N manipulation experiments located in the Mediterranean basin (NitroMed network) permanently established with the aim of monitoring long-term impacts of air pollution and CC. The main objective was to investigate the direct, indirect, and interacting effects of atmospheric N deposition and soil aridity on SOM mineralization rates. The general objective of the study was to determine the effects of N addition on the belowground decomposition rates in Mediterranean ecosystems. More precisely, we aimed at determining how the effect of N addition and soil water content (SWC) on the capacity of soil microbial communities to decompose organic matter is mediated by changes in microbial composition (fungi/bacteria ration), in soil External Enzymatic Activity (EEA), in soil chemistry and functional diversity of the microbial community.

Two main pathways were tested using Structural Equation Modeling (SEM). Specifically: 1) The effects of N on decomposition are mediated by the effects of N on the ratio between F/B and EEA but not by its effect on soil chemistry; 2) The F/B ratio and EEA are affected by changes in the chemical properties and by aridity, however aridity does not have a direct effect on decomposition; Our models considered a complete set of hypotheses showed in Fig. 1. These hypotheses were based on literature, previous exploratory analyses (anova, mix model, correlations), and our own previous experience (Lo Cascio et al. 2017).

Material and methods

Site description

Capo Caccia; Italy

A maquis shrubland coastal Mediterranean ecosystem located at the north-west coast of Sardinia within the Nature Reserve “Portoconte-Capocaccia” (Alghero; IT) (40°36'15.2"N 8°09'15.0"E; mean altitude 23 m MSL).

The vegetation covers the 80% of the area available, the dominant evergreen gymnosperm is represented by the species *Juniperus phoenicia* L. (53%), while the remaining species are *Pistacia lentiscus* L., *Phyllirea angustifolia* L., *Smilax aspera L.*, and *Chamerops humilis L*. Furthermore, the inner spaces between shrubs is composed by a well-developed biological soil crust(Table 5).

El Regajal-Mar de Ontígola; Spain

A semiarid Mediterranean shrubland (~50 km away from the city of Madrid) within the Nature Reserve “El Regajal-Mar de Ontígola” (central Spain,40°00'50.0"N 3°36'24.8"W; mean altitude 580 m MSL). Annual rainfall in the study site is 425 mm, mainly falling from October to May (Ochoa-Hueso et al. 2014).

The vegetation is dominated by sclerophyllous species represented by *Quercus coccifera* L., Fagaceae and *Rosmarinus officinalis* L. and small therophytes during the brief growing season. These therophytes are short-lived species such as *Limonium echioides* L. and *Asterolinonlinum-stellatum* L. Soils are rich in calcium carbonate and with a slightly basic pH (8.01). Nitrate is the dominant inorganic N form in soils, with mean (2008-2011) soil values ranging from 8.2 to 20.7 mg N kg soil^{-1}(Ochoa-Hueso et al. 2014) (Table 5).

Arrábida; Portugal

A maquis shrubland Mediterranean ecosystem which is located in the Arrábida Natural Park, south of Lisbon, Portugal (38°29'00.0"N 9°01'00.0"W; mean altitude 270 m MSL), was selected for this study. The climate is sub-humid, warm, with ~730 mm average annual precipitation, (1971-2000 e Instituto Nacional de Meteorologia e Geofísica).

The vegetation consists of a dense maquis dominated by *Cistus ladanifer* L. Other abundant plant species include *Erica scoparia* L., *Calluna vulgaris* (L.) Hull, *Genistatriacanthos* Brot., *Ulexdensus* Welw. ex Webb, *Dittrichia viscosa* L., and *Myrtus communis* L (Dias et al. 2011) (Table5).

The soils of the site have been classified as Calcic rhodo-chromic luvisoils and calcareous chromic cambiols.. Silt predominates in the soil (57%), while clay and sand contents are 28% and 15%, respectively. The typical Mediterranean "terra rossa" has accumulated in some places (Cruz et al. 2008).

Experimental design and N addition

Capo Caccia; Italy

There are eight plots, in which were installed collars, in order to obtain soilCO_2 fluxes. There are eight plots 36 m^2. Starting in April 2012, four plots were treated with 30 kg N ha^{-1} y^{-1} while the other four plots served as unfertilized controls. Nitrogen was applied by dissolving ammonium nitrate (NH_4NO_3) in 20 L of water and seasonally applying it to the soil surface using a backpack sprayer. At the same time N was applied to the N addition plots, 20 L of water was added to the unfertilized control plots.

El Regajal-Mar de Ontígola; Spain

In El Regajal, 24 plots were established following a 6-block design. Within each block, 4 plots of 2.5 m x 2.5 m were randomly selected: three plots were subjected to N additions of 10, 20 and 50 kg N ha^{-1} y^{-1}, respectively, over the background (~6.4kg N ha^{-1} yr^{-1},(Ochoa-Hueso et al. 2013) and compared to an unfertilized control plot. Starting in October 2007, N was added to individual plots by dissolving pellets of NH_4NO_3 in 2 L of water before applying it to the soil surface. The same amount of water was added to control plots. Buffer stripes of at least 1 m were set up between the plots.

Arrábida; Portugal

In January 2007, 12 plots (20 x 20 m) were established within of the Arrábida natural park in a 3-block design. N availability was modified within the Portuguese experimental site, by the addition of 40 and 80 kg N ha^{-1} y^{-1} in the form of NH4NO3 and 40 kg N ha^{-1} y^{-1} in the form of N-NH4+. The treatments are homogenously added, by hand, in three equal applications throughout the year: spring, summer and middle autumn/winter (Dias et al. 2013).

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 and 9 samples in Arrábida. 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 ºC 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 ($K_2Cr_2O_7$) 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 (H_2SO_4) and potassium sulfate (K_2SO_4) 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 K_2SO_4 at a ratio of 1:5, followed by shaking for 1 h at 200 rpm at 20 ºC and then filtered through a 0.45 mm Millipore filter (Jones and Willett 2006). The NH_4^+-N concentration was estimated directly via the indophenol blue method using a microplate reader (Sims et al. 1995). The NO_3^--N content was first reduced to NH_4^+-N with Devarda alloy and its concentration was determined as the difference between the Devarda-incubated and unincubated samples (where the NH_4^+-N concentration was estimated). Soil inorganic N concentration was calculated as the sum of NH_4^+-N and NO_3^--N. The DON content in the extracts was first oxidized to NO_3^--N with potassium persulfate ($K_2S_2O_8$) in an autoclave at

121 ◦C for 55 min and then reduced to NH_4^+-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 g of fresh soil were 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 K_2SO_4 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).

To quantify the abundance of total bacterial, fungal, AOB and AOA genes in the studied sites, soil DNA was extracted from 0.6 g of defrosted soil using the MoBio Powersoil DNA Isolation Kit (Carlsbad, USA) according to the instructions provided by the manufacturer. We performed quantitative PCR reactions in triplicate using 96-well plates on a AB 7300 Real-Time PCR (Life Sciences Technologies, Carlsbad, California, USA). The Bacterial 16S, fungal 18 s, AOB and AOA genes were amplified with the Eub 338-Eub 518, ITS 1-5.8S (Evans and Wallenstein 2011), amoA1F-amoA2R (Rotthauwe et al. 1997) and Arch-amoAF-ArchamoAR (Francis et al. 2005) primer sets, respectively. The 25 µl reaction mixture contained: 12.5 µL Fast Start Universal SYBR Green Master (Rox), 1.25 µL (10 mM) each primer, 1 µL BSA, 1-10 ng template DNA and ultraclean water to volume. The cycling conditions were 95 °C for 10 min, followed by 35 cycles of 95 °C 60 s; 53 °C 30 s and 72 °C 60 s, for both the fungal and bacterial primer sets, and 95 °C for 10 min, followed by 35 cycles of 95 °C 60 s; 55 °C 45 s and 72 °C 60 s, for both the AOB and AOA primer sets. Quantitative PCR was performed on an iCycler iQ5 thermocycler (Bio-Rad, Hercules, CA, USA). Amplification was conducted using the SYBR® Premix Ex Taq™ as described by the suppliers (TaKaRa, Dalian, China) using the primers and PCR conditions shown in Table S1. The amplification mixtures contained 12.5 µL of 2× SYBR®

Premix, 1 µL of bovine serum albumin (25 mg mL−1), 0.5 µL of each primer (10 µmol L−1), 1 µL of tenfold diluted DNA as template, and 9.5 µL of deionized water in a final volume of 25 µL.

Structural equation modelling (SEM)

The SEM models allow explanatory variables to be placed in 'series' rather than 'parallel', using sequential arrangements that reflect hypothetical causal chains linking the effects. Such models provide insights that are not possible with standard covariance analyses into the relative importance of different direct and indirect effects of N deposition. The model include causal relations among these biotic/abiotic variables, i.e. inorganic N influence C/N and pH, which in turn is also influence by C/N ratio. Additionally, we included in our model causal relations of AI hypnotizing that it will influence F/B, EEA activity and decomposition. Standardized path coefficients were estimated by using the maximum likelihood algorithm (Shipley, 2002). Prior to analyses, all variables were tested for normality, and log transformations were applied to meet variance homoscedasticity when required, except abundance matrices of microbial assemblage and metabolic profiles.

Results

SEM analysis

- the N cumulative, as expected is positive correlated with the soil N content and negatively correlate whit the soil pH. These result are confirmed by previous work.
- Soil C/N ration showed a negative significant correlation whit the pH and soil inorganic N. We expected an correlation between N cumulative and soil C: N but in this model resulted non-significant.
- The pH showed to be a good predictor of soil extra cellular enzyme activity (EEA) whit a high positive correlation. Almost 62 % of the variability is explained.
- pH is positively correlated with the fungi/bacteria ratio. This is consistent with other studies, demonstrating that fungi generally exhibit wider pH ranges for optimal growth.
- The EEA resulted be correlated with the tea bag index (TBI) stabilization factor (S) the proxy of the decomposition of label C.
- The soil aridity partially explained our second hypothesis confirming its significant and negative effect on soil microbial community composition but even showing and direct effect on mineralization processes.

The cumulative N resulted highly correlated to the soil chemistry, specifically higher inorganic soil N (r=0.42) and lower pH (r = -0.32) (Ste-Marie and Paré 1999). The soil acidity in turn induced a change of the microbial community (Treseder 2008) shifting to higher fungi/bacteria ratio (r = 0.31), confirming that fungi are more pH tolerant. In addition pH becomes the main predicted variable modulating the enzyme extracellular activity (r =0.62), considered as a proxy of the soil functionality (Fig. 1).

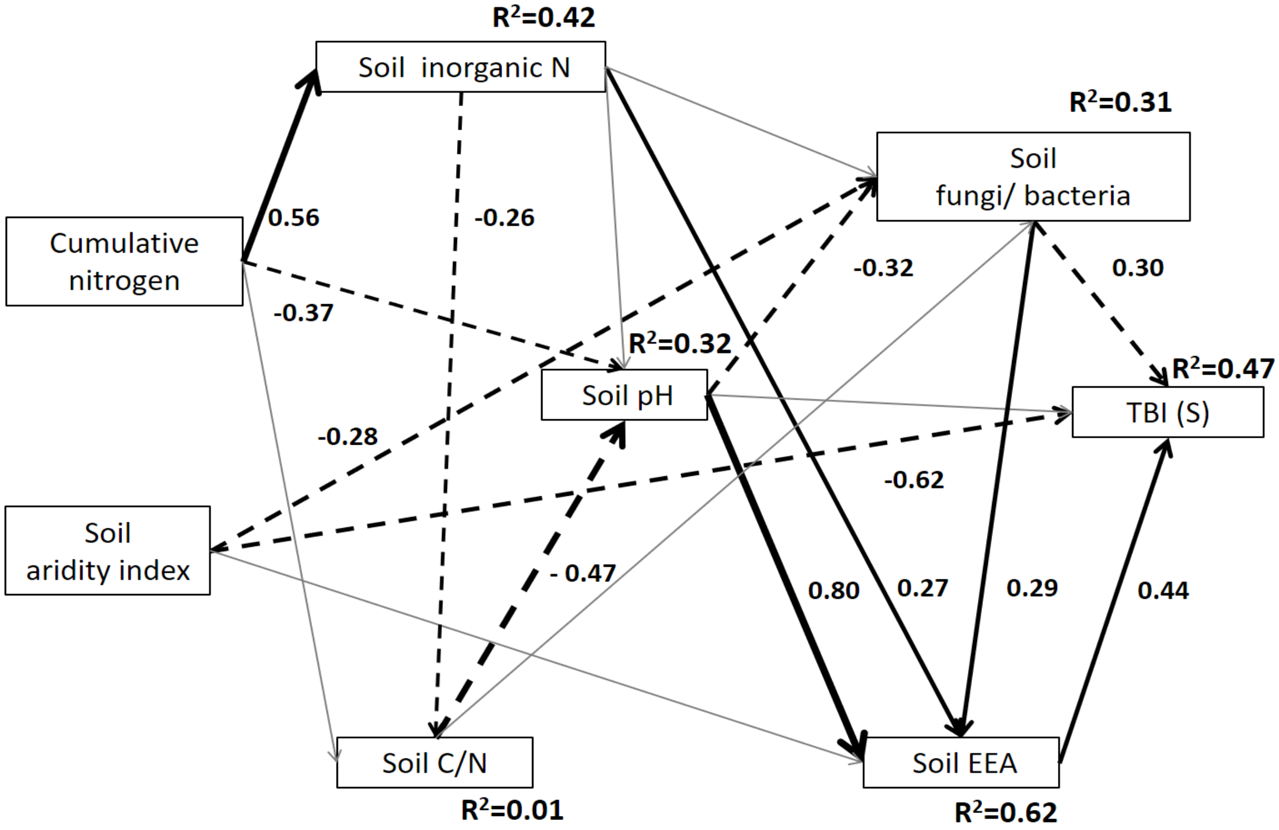
Discussion

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 the 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 microbial biomass growth in favor of soil C stabilization, while low CUE values indicate an increase in Rs rate (Manzoni et al. 2012). The CUE is sensitive to environmental changes. In particular, the future expected higher temperature and drought events could reduce the CUE values (Tucker et al. 2013), whereas increasing availability of N and nutrients are expected to increase the CUE (Ågren et al. 2001).

As we could see the biological systems are complex and any response is mediated by different component of the system itself. Aiming to disentangle this complexity, in chapter 5 through a powerful statistical approach we could test the multiple cause – effect relationship among some of the component involved in the C mineralization process, testing the response to the cumulative N effect. Overall the (preliminary) results underline a direct significant cause-effect relationship of the soil pH on EEA and microbial community composition, and at least for the observation period, the cumulative N reduces the decomposition of the labile carbon.

Figures

Figure 1 Structural equation model of the causal relationships among the study variables for lable C mineralization in the three experimental sites. *Solid arrows*: positive effects; *dashed:* negative effects and *grey* not significant effect; widths of arrows are proportional to the magnitude of the standardized path coefficients. Goodness-of-fit (chi-square/df) 0.297



Tables

Table 1. Soil chemical properties (means ± SE) for each N treatment in at Spain (n=6), Italy (n=4) and Portugal (n=3). Statistical differences are highlighted in bold and letters indicate significant differences ($p<0.05$) between/among N treatments within each site. (one-way ANOVA, post hoc LSD test).

	Spain				**Italy**		**Portugal**		
	Control	10N	20N	50N	Control	30N	Control	40N	80N
pH	8.8±0.08	7.91±0.11	7.87±0.12	7.76±0.13	**7.5±0.14**^{a}	**7±0.07**^{b}	**5.95±0.08**^{a}	**6.19±0.02**^{ab}	**6.43±0.13**^{b}
Organic C (%)	3.07±0.18	2.70±0.18	2.62±0.16	2.97±0.17	3.76±0.24	3.82±0.27	1.72±0.44	2.91±0.86	1.82±0.53
Total N (mg/g)	2.20±0.16	1.68±0.13	1.90±0.13	2.29±0.15	1.48±0.20	1.84±0.20	0.78±0.17	1.42±0.34	0.97±0.34
Inorganic N (µg/g)	**7.29±0.87**^{a}	**8.97±1.41**^{a}	**16.17±2.09**^{b}	**24.95±3.01**^{c}	5.82±0.41	5.65±0.45	9.06±3.37	15.02±1.62	16.57±5.14
Total P (mg/g)	0.25±0.04	0.23±0.03	0.25±0.04	0.26±0.04	0.22±0.08	0.27±0.06	0.10±0.01	0.13±0.02	0.10±0.03
C/N	15.05±2.22	16.15±2.5	13.81±0.49	14.62±1.26	30.36±12.39	21.12±8.62	21.6±1.12	20.12±1.94	19.97±5.04

Table 2. Soil microbial community (means ± SE) for each N treatment in at Spain (n=6), Italy (n=4) and Portugal (n=3). Statistical differences are highlighted in bold and letters indicate significant differences ($p<0.05$) between/among N treatments within each site. (one-way ANOVA, post hoc LSD test).

	Spain				**Italy**		**Portugal**		
(N. of gene copies gr^{-1} soil)	Control	10N	20N	50N	Control	30N	Control	40N	80N
Total_bacteria (log10)	11.51±0.21	11.36±0.74	12.68±1.06	11.43±0.67	12.48±1.04	10.98±0.15	11.24±0.22	11.06±0.62	10.70±0.89
Nitrifiers bacteria (log10)	**5.34±0.09^{ab}**	**5.22±0.18^{ab}**	**5.23±0.10^{a}**	**5.50±0.09^{b}**	**5.15±0.03^{a}**	**5.05±0.03^{b*}**	5.90±0.12	5.67±0.32	6.11±0.40
Nitrifiers archaea (log10)	7.27±0.12	7.50±0.16	7.10±0.40	7.06±0.30	6.78±0.13	6.80±0.14	5.21±0.08	5.23±0.18	5.17±0.03
Total_fungi (log10)	**6.71±0.34^{ab}**	**7.17±0.21^{ab}**	**7.00±0.50^{a}**	**6.48±0.95^{b}**	**7.27±0.13^{a}**	**6.43±0.24^{b}**	8.60±0.82	8.01±0.01	7.77±1.44

Table 3. Tea Bag Index (TBI), S (litter stabilization factor) and K (decomposition rate), (means ± SE) for each N treatment in at Spain (n=6), Italy (n=4) and Portugal (n=3). Statistical differences are highlighted in bold and letters indicate significant differences ($p<0.05$) between/among N treatments within each site. (one-way ANOVA, post hoc LSD test).

	Spain				**Italy**		**Portugal**		
	Control	10N	20N	50N	Control	30N	Control	40N	80N
TBI (S) 4 cm depth	0.36±0.02	0.34±0.02	0.38±0.02	0.35±0.02	**0.25±0.05^{a}**	**0.23±0.01^{b*}**	0.36±0.21	0.37±0.00	0.32±0.06
TBI (K) 4 cm depth	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
TBI (S) 8 cm depth	0.30±0.00	0.34±0.03	ND	0.34±0.03	0.18±0.02	0.20±0.04	0.32±0.00	0.38±0.03	0.34±0.03
TBI (K) 8 cm depth	0.01±0.00	0.01±0.00	ND	0.01±0.00	0.01±0.00	0.01±0.00	**0.01±0.00^{a}**	**0.02±0.00^{b}**	**0.01±0.00^{a}**

Table 4. Soil extracellular activity (means ± SE) for each N treatment in at Spain (n=6), Italy (n=4) and Portugal (n=3). Statistical differences are highlighted in bold and letters indicate significant differences ($p<0.05$) between/among N treatments within each site. (one-way ANOVA, post hoc LSD test).

(nanomol/h*g soil)	**Spain**				**Italy**		**Portugal**		
Variables	Control	10N	20N	50N	Control	30N	Control	40N	80N
Glucoronidase	**233.19±60.81**^{ab}	**201.08±93.21**^{a}	**183.91±42.95**^{ab}	**267.07±48.65**^{b}	87.13±26.44	89.56±13.82	64.38±14.37	77.52±15.12	66.67±14.26
Cellobiohidrolase	**208.21±43.08**^{a}	**178.38±69.97**^{a}	**175.87±33.87**^{a}	**252.41±31.93**^{b}	82.85±41.43	94.68±47.34	**70.21±14.30**^{a}	**123.25±18.43**^{b*}	**127.85±13.98**^{b}
Glucosidase	**1261.17±96.89**^{a}	**1028.55±221.97**^{b}	**1168.79±136.48**^{ab*}	**1469.70±167.79**^{ac}	527.16±33.47	536.73±140.86	410.89±60.73	618.13±141.72	535.30±98.53
Xilosidase	**293.75±64.29**^{ab}	**242.07±81.14**^{a}	**245.39±51.38**^{ab}	**317.50±53.11**^{b}	135.02±58.57	135.93±34.87	82.03±15.31	86.22±22.25	110.90±15.27
Leucine Aminopeptidase	175.45±25.77	143.29±31.04	141.88±23.67	160.18±20.01	81.86±23.34	114.26±42.89	84.03±13.69	67.66±28.61	61.02±22.51
N-acetil Glucosaminidase	**410.54±58.71**^{ab}	**380.18±107.55**^{a}	**394.48±70.74**^{ab}	**480.89±53.56**^{b}	349.50±58.38	364.90±124.55	234.84±75.61	343.01±101.37	367.51±70.98
Phosphatase	471.99±65.71	528.97±85.68	538.01±80.60	653.80±129.96	870.30±435.15	915.73±457.86	876.45±68.75	1292.49±254.94	1133.63±297.47

Table 5. Main characteristics of the study sites, Spain, Italy and Portugal

Location	Portugal	Spain	Italy
Experimental Site	Arrábida Natural Park	El Regajal-Mar de Ontígola	Capo caccia “Le prigionette”
Year	January 2007	October 2007	March 2014
Treatments NH_4NO_3 (kg ha^{-1} yr^{-1})	0, 40, 80 and (40 N-NH4$^+$)	0, 10, 20, 50	0, 30
Replicate	3	6	4
Soil	Terra Rossa_silt 57%	Gypsum _ calcium sulphate	Terra Rossa_ clay 60%
Soil pH	6.1	8.1	7.2
Climate	Sub-humid Mediterranean, warm variant	Semi-arid Mediterranean	Sub-humid Mediterranean
Mean annual rainfall (mm)	650	425	494
Mean annual temperature (°C)	16	15	16.5
Canopy species cover (%)	*Cistus ladanifer* L. *Erica scoparia* L., *Calluna vulgaris* L.	*Quercus coccifera* L. (>85) *Rosmarinus officinalis* L. (<15)	*Juniperus Phoenicea* L. (<80) *Pistacia lentiscus* L. (>12)

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Chapter 6: Conclusion

General conclusions

Terrestrial ecosystems from the Mediterranean Basin are currently at risk due to air pollution and climate change (CC), but only a limited number of isolated and geographically-restricted studies have addressed this topic. This thesis focused on two important ecosystem functions as soil CO_2 efflux and decomposition rates in Mediterranean sites drawing some light in the complex underlying processes behind them thus addressing some of the major knowledge gaps identified after a literature review and expert knowledge survey presented in chapter 2.

It was shown that the biocrust has a non-negligible role in ecosystem respiration highlighting its important role in Mediterranean ecosystems. The manipulative experiment described in chapter 3 quantified the contribution of the biocrust to the total CO_2 emitted to the atmosphere as 23.6%. However, only few articles have addressed this topic and as the work presented here helps to have a more complete picture of the importance of the biocrust it also suggests that its relative role in ecosystem respiration varies with the humidity of the system considered perhaps reaching the highest relative role in the most arid environments. Interestingly, the biocrust uses partially different resources from the surrounding woody vegetation and also responds differently to abiotic environmental inputs. This differential use of resources implies an efficient use of resources by the ecosystem and also helps stabilizing this ecosystem function through the seasons. Considering the well reported sensitivity of the biocrust to nitrogen (N) deposition, lets imagine that N deposition could strongly effect not only the mean ecosystem function but also its stability. In general, results suggest that the expected temperature increases due to the CC, will increase C losses through respiration.

Addition of N to ecosystem generally results in a decrease of respiration rates from the soil and a more positive C balance of ecosystem has been reported in many cases. Also over this issue the Mediterranean ecosystems are represented in the literature and even less experiments have used N doses comparable with those projects for 2050. It was shown (chapter 3) that the effects of N addition on soil respiration cannot be generalized as it may have inhibitory effects, no effects, or enhancing effects. This could possibly depend on the duration of the exposure but also on the preexisting N loads in the soil considered. Additionally, the effects of N could well vary depending on the composition and environmental characteristics of the many microsites present in Mediterranean ecosystems which are known for their high spatial variability. Indeed, N addition did not have an effect on microsites covered by the biocrust while it had an enhancing effect in the under-canopy of the dominant species.

While evidence is emerging on the effects of N deposition on ecosystem functions, there is still a scarce knowledge of the complex cause effect mechanism that ultimately determines the effect. In chapter 4 it was shown how the effects of N addition on the decomposition rates are mediated by its effects on the soil pH which decreases alters the microbial community composition and reduces the extra cellular enzymatic activity. Ultimately, these effects determines a general decrease of decomposition rates in the soil which is in agreement with the general decrease of the soil CO_2 observed in N manipulation experiments. It also suggests that the N addition effects are largely impacting on the heterotrophic respiration while its effects on autotrophic respiration still remains uncertain.
Summing up the results of this manuscript and literature it emerges that water availability remains the main driver of ecosystem functions in semiarid environments and that the effects of N are mostly visible during the humid seasons. This suggest that the projected reduction in precipitation in the Mediterranean could override the effects of N addition on ecosystem functions on one hand but also that the effects of N deposition could reduce the efficiency with which these ecosystem use resources. A lower efficiency and lower ability to use the added N could bring to a higher N leaching especially after intense rain events

during dry periods, thus posing a threat on the water quality of water systems (ground water, lake, rivers, and marine ecosystems).

Future studies are needed to assess impacts of N pollution in the Mediterranean Basin in order to determine the critical loads of atmospheric deposition and implement them in environmental policies and land management activities in particular.