



Universitat de Lleida

## Assessing the effect of global change on plant functional structure, greenhouse gases, and soil functions in grasslands

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<http://hdl.handle.net/10803/436894>

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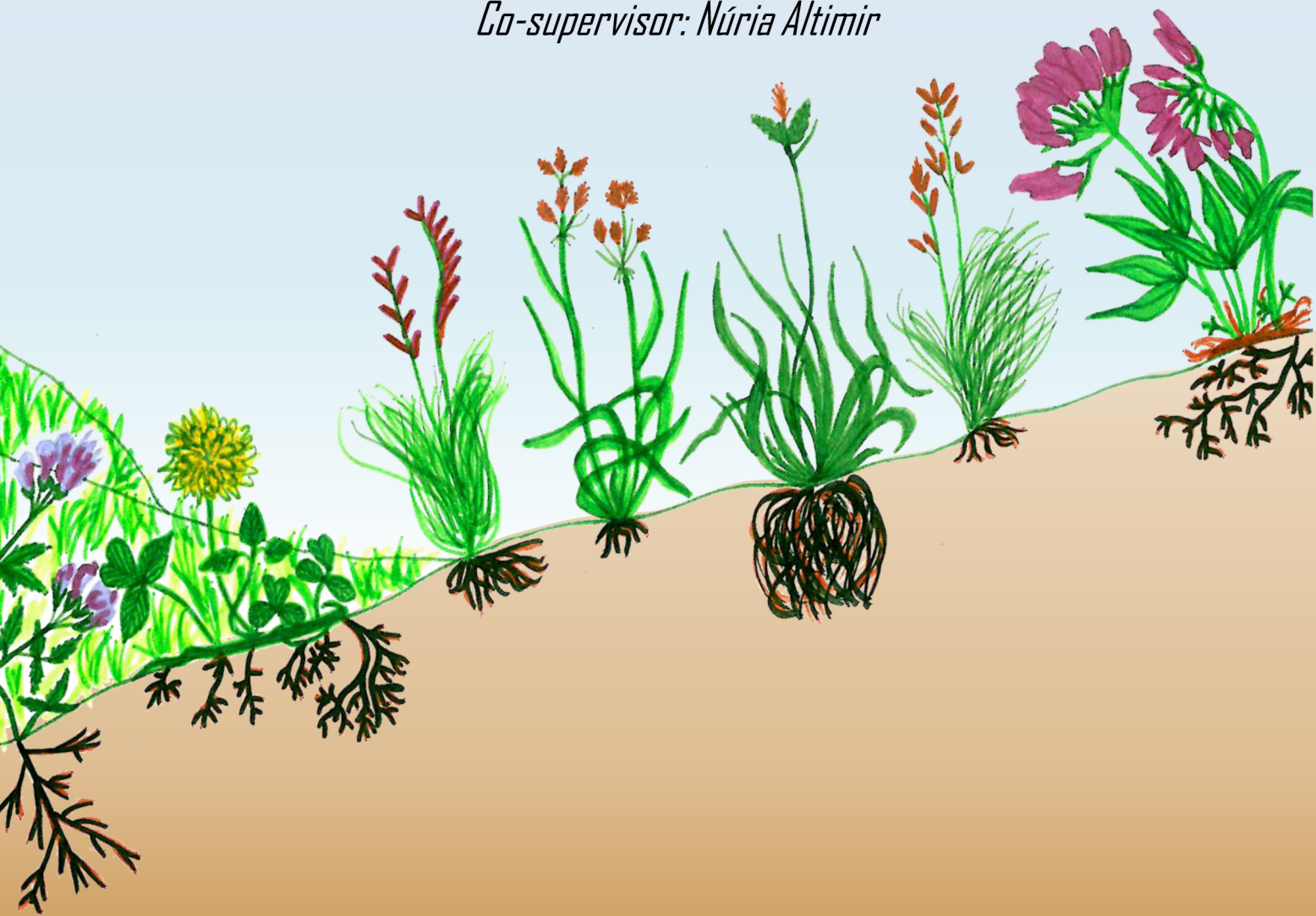
# Assessing the effect of global change on plant functional structure, greenhouse gases, and soil functions in grasslands

Doctoral Thesis  
by **Haifa Debouk**

*June, 2017*

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*Co-supervisor: Núria Altimir*





## **TESI DOCTORAL**

# **Assessing the effect of global change on plant functional structure, greenhouse gases, and soil functions in grasslands**

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Memòria presentada per optar al grau de Doctor per la Universitat de Lleida  
Programa de Doctorat en (Gestió Multifuncional de Superfícies Forestals)

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2017

*Cover and thesis design by*

*Haifa Debouk & Dominik Sperlich*

*To Dominik*  
*To my family*



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# Acknowledgments / Agraïments

First, I would like to thank my main supervisor Dr. Maria-Teresa Sebastià for giving me the opportunity to pursue this PhD and for guiding me through this journey. Thank you for your constructive criticism and encouragement. I would also like to thank my co-supervisor Dr. Núria Altimir for her supervision, valuable suggestions and support.

I would like to thank the Spanish ministry of economy and competitiveness for the FPI scholarship (BES-2011-047009) through the CAPAS project (CGL2010-22378-C03-01), which allowed me to pursue my doctoral research at the Forest Sciences Centre of Catalonia and the University of Lleida.

I am very thankful to Dr. Francesco de Bello for hosting me during two months at the Czech Academy of Sciences in České Budějovice in Czech Republic, where I learned a lot about plant functional traits. I also want to thank Dr. Jaana Bäck for hosting me at the group of ecosystem processes at the University of Helsinki in Finland, for a two-month period. I am also grateful to Rosa, Angi, and Leticia for their collaboration, for their work, and for the very fruitful discussions. I want to particularly thank the secretary of our department Núria Llop for being always there to help in the best way she could. Núria, our department and we are very lucky to have you!

The field work in this thesis would not have been possible without the technical assistance of my colleagues in the ECOFUN group, particularly Helena Sarri, Esther Iñiguez, Mercedes Ibañez, and Teresa Mari. Many thanks also go to people who were part of the ECOFUN group and made field and laboratory work easier, more efficient, and more fun: Riccardo, Antonio, Carla, Dafne, Luisa, Tullio, Juliette, Fabrice, David, and Cristina. Beside my friends and colleagues in the ECOFUN group, I would like to thank my friends in Lleida who made this PhD journey more fun and much more interesting: Sergi, Gonzalo, Harry, Martin, Reza, Roger, Salva, Ravi, and Xavi. I want to particularly thank Esther and Merche for helping me with the translation of the summary to Spanish and Catalan, and to Reza for helping me with printing the thesis. Muchas gracias!!

Special thanks go to my parents for their continuous support. Merci maman et papa pour tout ce que vous avez fait pour moi jusqu'à maintenant. Je ne serais pas ce que je suis aujourd'hui sans vous, sans votre amour inconditionnel et votre soutien. I am also very thankful to my siblings Hiba and Fouad for their encouragement and for being there when I needed them.

Barbara & Harald ich bin wirklich dankbar, dass Ihr da seid!

Dominik, without you I wouldn't have made it that far. I want to thank you for all the love, support, understanding and patience that you have showed me and that helped me continue and never give up. You were and are a main reason for this accomplishment! Ich bin froh und dankbar, dass ich Dich habe!

# Acronyms

Acronym	Unit	Variable name
<i>AGB<sub>L</sub></i>	<i>g</i>	Live aboveground biomass
<i>AGB<sub>T</sub></i>	<i>g</i>	Total aboveground biomass
<i>AIC</i>	<i>unitless</i>	Akaike information criterion
<i>CH<sub>4</sub></i>	<i>nmol m<sup>-2</sup> s<sup>-1</sup></i>	Methane flux
<i>CIS</i>	<i>° C</i>	Continental index of Sebastià (MST – MAT)
<i>C:N</i>	<i>unitless</i>	Carbon-to-nitrogen ratio
<i>CO<sub>2</sub></i>	<i>μmol m<sup>-2</sup> s<sup>-1</sup></i>	Carbon dioxide flux
<i>CWM</i>	<i>unitless</i>	Community weighted mean
<i>DI</i>	<i>unitless</i>	Diversity-Interaction model
<i>DL</i>	<i>unitless</i>	Detection limit
<i>DOC</i>	<i>mg C/kg</i>	Dissolved organic carbon
<i>DON</i>	<i>mg N/Kg</i>	Dissolved organic nitrogen
<i>DOY</i>	<i>unitless</i>	Day of the year
<i>E</i>	<i>unitless</i>	Evenness
<i>F</i>	<i>unitless</i>	Forbs
<i>G</i>	<i>unitless</i>	Grasses
<i>GHG</i>	<i>unitless</i>	Greenhouse gases
<i>L</i>	<i>unitless</i>	Legumes
<i>Lang</i>	<i>unitless</i>	Lang's rain factor index (1920) = MAP/MAT
<i>LDMC</i>	<i>mg g<sup>-1</sup></i>	Leaf dry matter content
<i>MAP</i>	<i>mm</i>	Mean annual precipitation
<i>MAT</i>	<i>° C</i>	Mean annual temperature
<i>MBC</i>	<i>mg C/kg</i>	Microbial biomass carbon

*Table continued*

Acronym	Unit	Variable name
MBN	Mg N/kg	Microbial biomass nitrogen
MSP	mm	Mean summer precipitation
MST	° C	Mean summer temperature
MTmax	° C	Mean maximum temperature
MTmin	° C	Mean minimum temperature
<i>NEE</i>	$\mu\text{mol m}^{-2} \text{s}^{-1}$	Net ecosystem exchange
<i>N<sub>2</sub>O</i>	$\text{nmol m}^{-2} \text{s}^{-1}$	Nitrous oxide flux
<i>PAR</i>	$\mu\text{mol m}^{-2} \text{s}^{-1}$	Photosynthetically active radiation
PAS	unitless	Photoacoustic spectroscopy analyzer
PFT	unitless	Plant functional type
RDA	unitless	Redundancy analysis
<i>R<sub>eco</sub></i>	$\mu\text{mol m}^{-2} \text{s}^{-1}$	Ecosystem respiration
<i>R<sub>soil</sub></i>	$\mu\text{mol m}^{-2} \text{s}^{-1}$	Soil respiration
<i>S</i>	unitless	Sedges
SD	unitless	Standard deviation
SLA	$\text{m}^2/\text{kg}$	Specific leaf area
SON	% s.m.s.	Soil organic nitrogen
SWC	fraction	Soil water content
<i>T<sub>a</sub></i>	° C	Air temperature
<i>T<sub>s</sub></i>	° C	Soil temperature
VP	unitless	Variation partitioning
W	unitless	Woody

# Summary

Grasslands are highly sensitive to global change. Climate change is expected to have a great impact on grasslands through changes in species and functional diversity, greenhouse gas (GHG) emissions, and soil functions. There is however an incomplete knowledge of how grasslands will respond to future climate change scenarios, and to which extent they will contribute to global warming. Therefore, there is a need to investigate how plant functional types (PFT) affect vegetation stability, GHG fluxes and soil functions, and how these interactions are regulated by climatic conditions. Our objectives were: a) to determine how short-term warming can affect productivity, species and functional diversity, and vegetation stability; b) to assess the magnitude and range of variability of CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O fluxes along an altitudinal gradient, and determine their main drivers; c) to study the effect of PFT on GHG fluxes and soil function, and whether changes in climatic and soil conditions modify this effect in semi-natural grasslands. A short-term warming experiment greatly altered vegetation functional structure and its relation to productivity. Warming led to the dominance of acquisitive fast growing species over conservative species; thus reducing species richness. The functional traits structure in grasslands had greater influence on the productivity and stability of the community under short-term warming, compared to diversity effects. Using chambers, we found mainly negative Net Ecosystem Exchange (NEE) fluxes indicating CO<sub>2</sub> uptake, and mostly positive CH<sub>4</sub> and N<sub>2</sub>O fluxes indicating sources. GHG fluxes decreased with altitude- the colder the grassland site the lower the fluxes-, and increased during summer. Aboveground biomass and temperature were the main drivers for all GHG fluxes. PFT composition strongly influenced GHG fluxes and soil activity and fertility; however PFT effects were regulated by environmental variables. The interaction between PFTs enhanced CH<sub>4</sub> and N<sub>2</sub>O uptake compared to single PFTs. PFT evenness and pairwise interactions between PFTs enhanced microbial biomass nitrogen (MBN), dissolved organic nitrogen (DON), total N, urease and phosphatase, and nitrification potential. The total variability of soil activity and fertility was best explained by environmental variables (73%), followed by the combined effects of environmental and diversity variables (23.5%). Overall, our results show that plant functional structure strongly influences vegetation stability, GHG fluxes, and soil activity and fertility in grassland, but this relationship is regulated by climate. Under warming, plant functional structure was greatly changed, and thus any alteration in functional diversity in grassland ecosystems under future climate change scenarios will have serious implications on the stability and soil activity of such ecosystems, and on their contribution to GHGs and warming.

# Resumen

Los pastos presentan una alta vulnerabilidad frente al cambio global; se prevé que el cambio climático afecte a su diversidad específica y funcional, las emisiones de gases de efecto invernadero (GEI) y las funciones del suelo. Aunque se desconoce cuál será la respuesta de los pastos a futuros escenarios de cambio climático y en qué medida contribuirán al calentamiento global es imprescindible investigar el efecto de los grupos funcionales de plantas a la estabilidad de la vegetación, los flujos de GEI y las funciones del suelo, y cómo las condiciones climáticas regulan sus interacciones. Los objetivos fueron: a) determinar el efecto que el calentamiento global ejerce a corto plazo en la productividad, la diversidad específica y funcional, y la estabilidad de la vegetación; b) cuantificar y evaluar la escala de variabilidad de los flujos de  $\text{CO}_2$ ,  $\text{CH}_4$  y  $\text{N}_2\text{O}$  en un gradiente altitudinal y determinar sus principales promotores; c) estudiar el efecto de los grupos funcionales sobre los flujos de GEI y las funciones del suelo, y si las condiciones climáticas y edáficas alteran dicho efecto. Un experimento de calentamiento mostró a corto plazo una gran alteración en la estructura funcional de la vegetación y su productividad. El calentamiento causó la dominancia de especies oportunistas sobre las más conservadoras; reduciendo así la riqueza específica. En condiciones de calentamiento, los rasgos funcionales tuvieron una mayor influencia en la productividad y estabilidad de las comunidades frente al efecto de la diversidad. Mediante cámaras se detectaron flujos negativos de intercambio neto del ecosistema, indicando asimilación de  $\text{CO}_2$ , y flujos positivos de  $\text{CH}_4$  y  $\text{N}_2\text{O}$ , indicando emisión. Los flujos de GEI aumentaron en verano y disminuyeron con la altitud, siendo inferiores cuanto más frío era el prado. Los principales promotores de todos los flujos de GEI fueron la temperatura, regulando los efectos de los grupos funcionales, y la biomasa aérea cuya composición vegetal influyó significativamente en los flujos de GEI y en la actividad y fertilidad del suelo. La interacción entre grupos funcionales incrementó la absorción de  $\text{CH}_4$  y  $\text{N}_2\text{O}$  respecto a grupos individuales. La equidad funcional y las interacciones entre grupos funcionales favorecieron el N de biomasa microbiana, el N orgánico disuelto, el N total, la ureasa, la fosfatasa y el potencial de nitrificación. La variabilidad total de la actividad y fertilidad del suelo se explicó mejor mediante variables ambientales (73%), seguidas de los efectos combinados de variables ambientales y de diversidad (23,5%). Concluyendo, la estructura funcional de las plantas en pastos influye la estabilidad de la vegetación, los flujos de GEI, la actividad y fertilidad del suelo, y ese efecto está regulado por el clima. Con el calentamiento, la estructura de la vegetación se modificó y, por tanto, cualquier alteración en la diversidad funcional de los pastos en futuros escenarios de cambio climático afectará la estabilidad y actividad de los mismos y su contribución a los GEIs y el calentamiento.

# Resum

Les pastures són ecosistemes altament vulnerables al canvi climàtic, essent susceptibles de patir impactes sobre la diversitat específica i funcional, les emissions de gasos d'efecte hivernacle (GEH) i les funcions del sòl. Malgrat les previsions, el coneixement sobre com les pastures respondran als futurs escenaris climàtics és encara incomplet. Així, es posa de manifest la necessitat d'investigar el paper dels grups funcionals de plantes (GFP) en relació a l'estabilitat de la comunitat vegetal, els fluxos de GEH i les funcions del sòl, i com aquestes interaccions es veuen regulades pel clima. En aquest context, els objectius de la present tesi es defineixen com: a) determinar l'efecte de l'escalfament sobre la productivitat, la diversitat funcional i específica i l'estabilitat de la vegetació; b) avaluar la magnitud, el rang de variabilitat i els factors reguladors dels fluxos de  $\text{CO}_2$ ,  $\text{CH}_4$  i  $\text{N}_2\text{O}$  al llarg d'un gradient altitudinal i c) estudiar l'efecte dels GFP sobre els fluxos de GEH i les funcions del sòl, i com aquest efecte es pot veure afectat pels canvis climàtics i les condicions del sòl. Mitjançant un experiment d'escalfament a curt termini es van observar modificacions en l'estructura i la productivitat. L'escalfament va afavorir la dominància d'espècies oportunistes i de creixement ràpid en detriment d'espècies més conservatives, causant una reducció en la diversitat específica. A més, la composició i l'estructura funcional van tenir una major influència en la productivitat i l'estabilitat de la comunitat que la diversitat específica. Mitjançant l'ús de cambres de sòl, es van enregistrar valors negatius d'intercanvi net del ecosistema, indicant assimilació de  $\text{CO}_2$ , mentre que de  $\text{CH}_4$  i  $\text{N}_2\text{O}$  es van enregistrar emissions. Els fluxos de GEH es van reduir amb l'altitud, essent les pastures més altes i fredes les que varen presentar fluxos més baixos, i incrementar durant l'estiu. La biomassa aèria i la temperatura van ser els principals factors moduladors dels fluxos de GEH. La composició de GFP també va influenciar fortament els GEH i les funcions del sòl però aquest efecte es va veure regulat per les variables ambientals. La interacció entre GFP va afavorir l'assimilació de  $\text{CH}_4$  i  $\text{N}_2\text{O}$  en comparació a la dominància d'un sòl GFP. La uniformitat en la abundància de GFP i les interaccions entre GFP van afavorir la biomassa microbiana, el nitrogen orgànic dissolt, el nitrogen total, la nitrificació potencial i la ureasa i la fosfatasa. La variabilitat en l'activitat de sòl i va ser explicada per les variables ambientals (73%) i pels efectes combinats de les variables ambientals i la diversitat (23.5%). En general, els resultats mostren com les l'estructura funcional influencia l'estabilitat de la vegetació, els fluxos de GEH i l'activitat del sòl, però aquest efecte està modulats pel clima. Així, sota condicions d'escalfament els canvis enregistrats en l'estructura funcional de la vegetació indiquen que qualsevol alteració en la diversitat funcional de les pastures tindrà serioses implicacions en l'estabilitat i l'activitat del sòl d'aquests ecosistemes i en la seva contribució a les emissions de GEH i l'escalfament global.







# Chapter 1

# General Introduction



## 1.1. Grasslands under global change

**G**rasslands, including sown pasture and rangeland, are among the largest ecosystems in the world (Suttie *et al.*, 2005) representing 26% of the global land surface area (FAO, 2006). They provide a wide range of ecosystem services: soil carbon sequestration, livestock feed, preservation and enhancement of water quality, soil protection, biodiversity conservation and regulation, etc. (Lemaire *et al.*, 2011). Grasslands – together with shrublands – also store 60.5 to 82.8 billion metric tons of carbon in their biomass and soils (Ward *et al.*, 2014). **Global change, however, poses a threat to the functioning of grasslands through land use changes (intensification or abandonment) and climate change (warming and/or drought).** Plant communities of high mountain grasslands are particularly vulnerable to these changes (Sanz-Elorza *et al.*, 2003; Kohler & Maselli, 2009; Spehn *et al.*, 2010; Gartzia *et al.*, 2016) because of the limiting growth conditions in these ecosystems, such as temperature extremes, and nutrient availability, etc. Future climate change scenarios predict that greenhouse gas (GHG) emissions and global warming are going to increase further in the near future, even under the most optimistic implementation scenarios of climate change mitigation policies (Barker *et al.*, 2007; Dumont *et al.*, 2015). As a consequence, community structure may be altered.

## 1.2. Warming effects on community stability

**I**ndeed, it has been shown that warming strongly modified biodiversity and vegetation composition in grasslands (e.g. Sebastià *et al.*, 2004, 2008; Sebastià, 2007; Cantarel *et al.*, 2013). This has led to a lively debate on the stability of these ecosystems and which factors determine the resistance and resilience of plant communities. The “diversity begets stability” theory, for instance, hypothesizes that species diversity determines the stability of plant communities (MacArthur, 1955; Elton, 1958; Tilman & Downing, 1994; Palmer & Maurer, 1997) because the presence of more species in the system provides a sort of insurance towards different environmental changes. However, this theory falls short to explain **the importance of plant functional traits in ecosystem stability**, which could simply depend on the type of dominant species in the system (Goodman, 1975; Lepš *et al.*, 1982; Naeem, 2002). The positive correlation between stability and diversity can be explained by the fact that higher diversity increases the probability of

including species with functional traits better adapted to the new environmental conditions (Huston, 1997; Lepš *et al.*, 2001; Loreau *et al.*, 2001). Plant functional traits are defined as any characteristics of organisms which impact their fitness, and which allow determining general community responses to environmental changes across regions and control ecosystem processes (vegetative growth, photosynthesis, decomposition, etc. (Carbognani *et al.*, 2013)). Species with distinct functional traits are expected to respond differently to environmental conditions, and hence influence ecosystem processes accordingly (Lavorel & Garnier, 2002; de Bello *et al.*, 2005, 2006; Klumpp & Soussana, 2009). **Therefore investigating warming impacts should focus not only on species diversity, but also on the plant functional traits of the community, their influence on the community resistance and the extent to which they contribute to its resilience under warming.** Overall, maintaining community stability in grasslands is important because any shifts in vegetation composition and species diversity can modify soil biological, physical and chemical processes such as: nitrogen (N) and carbon (C) cycling and storage (Niklaus *et al.*, 2006; De Deyn *et al.*, 2009); litter decomposition and soil fertility (Dybzinski *et al.*, 2008; Sylvain & Wall, 2011); soil respiration (Dias *et al.*, 2010; Metcalfe *et al.*, 2011); and soil microbial communities (Bartelt-Ryser *et al.*, 2005; Steinauer *et al.*, 2015) and consequently their feedback to the atmosphere.

### 1.3. Main drivers of soil functions

**S**oils are the livelihood for a wealth of organisms providing many important functions, such as water, carbon and nutrient stores, thus making them the life support system of our planet. Soil functions in grasslands, particularly in mountain areas, are expected to be highly affected by climate change (Mills *et al.*, 2014), manifested by significant inter- and intra-annual variability in precipitation and temperature (Craine *et al.*, 2012). Such fluctuations could have drastic effects on soil activity – including microbial biomass, nitrification and enzyme activity – and microbial communities, through changes in organic matter quality and supply from biomass, soil temperature, and soil hydrology (Brinkman & Sombroek, 1996). Microbial biomass converts soil organic matter into nutrients available for plant uptake, and structures the soil (Brookes, 2001; Bot & Benites, 2005). Since microbial biomass C and N are regulated by soil moisture and temperature (Garten *et al.*, 2009; Castro *et al.*, 2010), any changes in these abiotic factors (warming and/or drought) can alter nutrient release as well as soil structure and fertility. Nitrification, in turn, is one of the key microbiological processes in the soil N cycle which, in part, controls the form and availability of inorganic N, and hence determines ecosystem productivity (Le Roux *et al.*, 2003). It is also involved in nitrous oxide (N<sub>2</sub>O) emissions from soils (Lu *et al.*, 2012). Nitrification processes are regulated mainly by temperature, soil moisture, input rates and quality of plant residues, as well as C availability (Emmett *et al.*, 2004; Niklaus *et al.*, 2006). Subsequently,

any alteration of nitrification processes can have serious consequences on N<sub>2</sub>O emissions and thus on the feedback of the soil to the atmosphere, which is why estimating nitrification potential and rates, as well as understanding the processes behind is essential. Enzyme activity (e.g. phosphatase, glucosidase, urease) is another important soil activity because it controls the availability of nutrients such as carbon, nitrogen and phosphorus, and the rate at which they are produced in the soil for microbial assimilation, which eventually also controls plant growth (Chróst, 1991; Burns & Dick, 2002; Gianfreda, 2015). Because enzyme activity is highly sensitive to temperature and soil moisture, and because it is controlled by the quantity and activity of microbial biomass (Sardans *et al.*, 2008; Steinweg *et al.*, 2013), any changes in abiotic factors and microbial biomass can have major consequences on nutrient availability and plant growth.

**B**eside climatic factors, soil functions are affected also by plant diversity (Niklaus *et al.*, 2006; Castro *et al.*, 2010), because plants determine the quantity and the quality of residues, soil organic matter, as well as soil structure (Wardle *et al.*, 2004). For example, increased productivity as a result of increased diversity may lead to greater plant-derived carbon inputs, which may in turn alter soil microbial communities (Castro *et al.*, 2010). **Therefore, any changes in plant diversity, community structure and composition may imply changes in soil function and structure** (Kardol *et al.*, 2010; Cregger *et al.*, 2012; Thakur & Eisenhauer, 2015). Climate change can induce such changes, because the role of diversity on soil processes is suggested to be driven by climate (Neilson *et al.*, 2005; Pold & DeAngelis, 2013; Classen *et al.*, 2015). However, the relative contribution and the interactions of the regional climatic conditions, the local soil properties and the local biodiversity on soil activity (phosphatase, urease, glucosidase, microbial biomass, and potential nitrification activity) are poorly understood. **It is thus essential to investigate their exact involvement in greenhouse gas exchange because any changes may modify the soil function as being either a sink or a source** (Jungkunst & Fiedler, 2007; Oertel *et al.*, 2016).

## 1.4. Greenhouse gas emissions in grasslands

**G**reenhouse gas emissions – carbon dioxide (CO<sub>2</sub>), nitrous oxide (N<sub>2</sub>O) and methane (CH<sub>4</sub>) – mainly originate from soil processes (Bremner, 1997; Topp & Pattey, 1997; Le Mer & Roger, 2001; Butterbach-Bahl *et al.*, 2013) and are driven by temperature, humidity, nutrient availability, vegetation, land cover and land use, and are regulated by regional and local environmental conditions (Fig. 1.1, Oertel *et al.*, 2016). As a result, any alteration of these factors can have direct effects on GHG exchange and its feedback from the soil to the atmosphere.

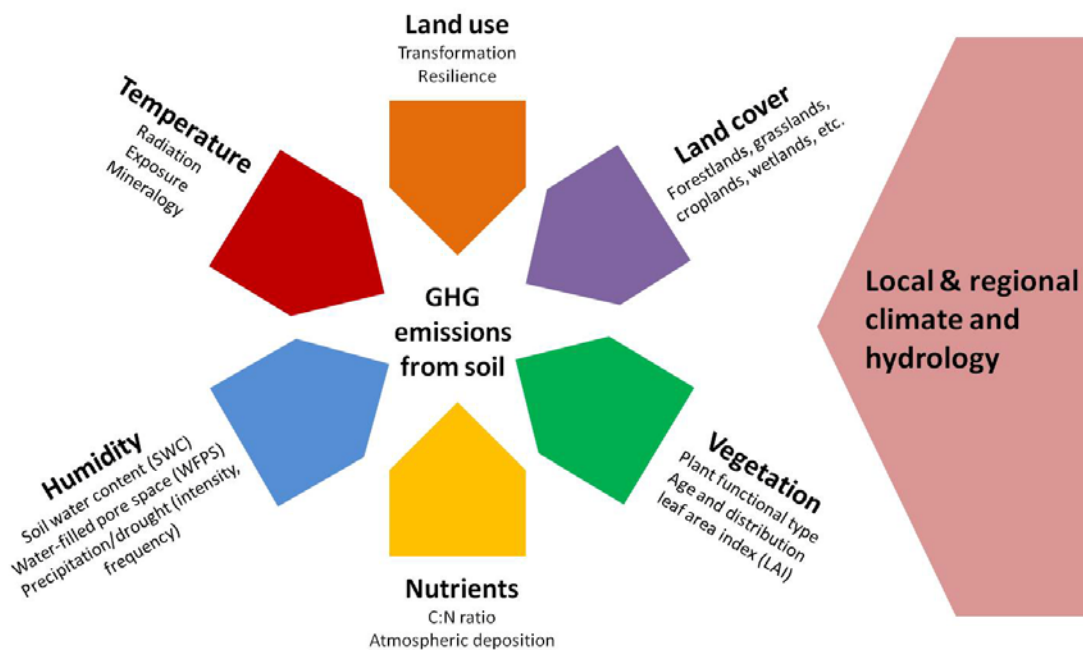
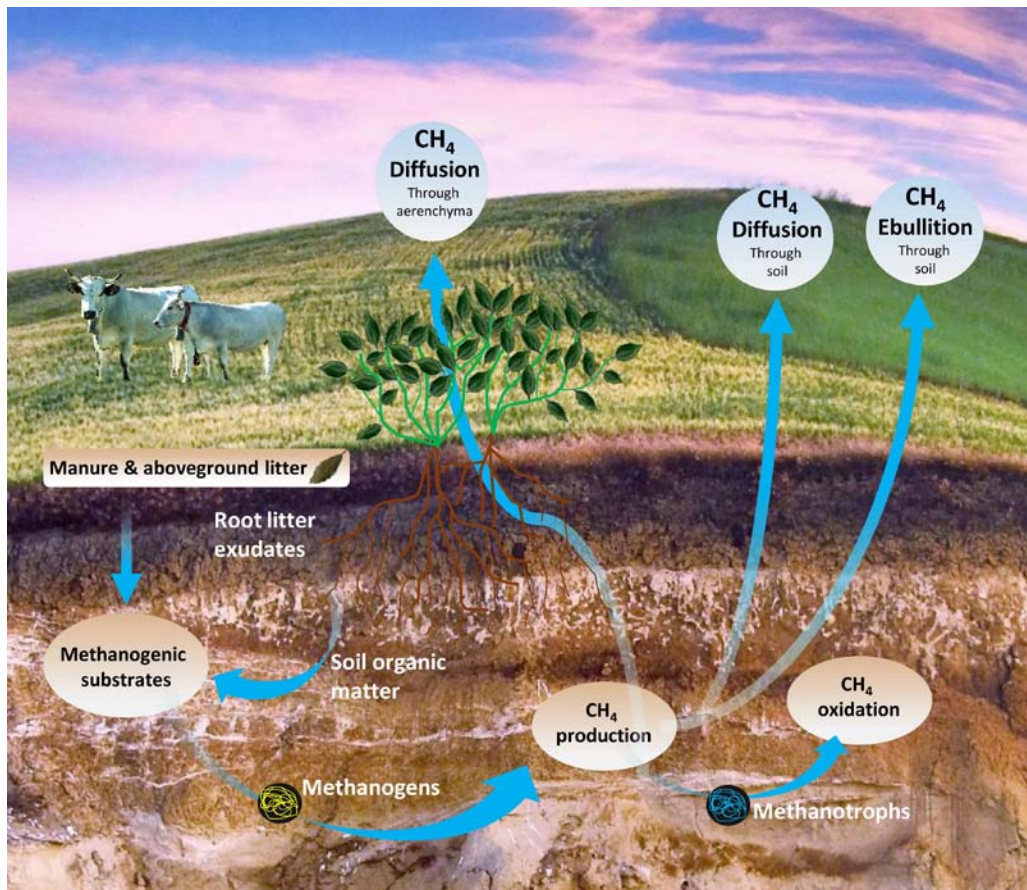


Figure 1.1 | Key drivers of GHG emissions from soils (modified from Oertel et al. 2016).

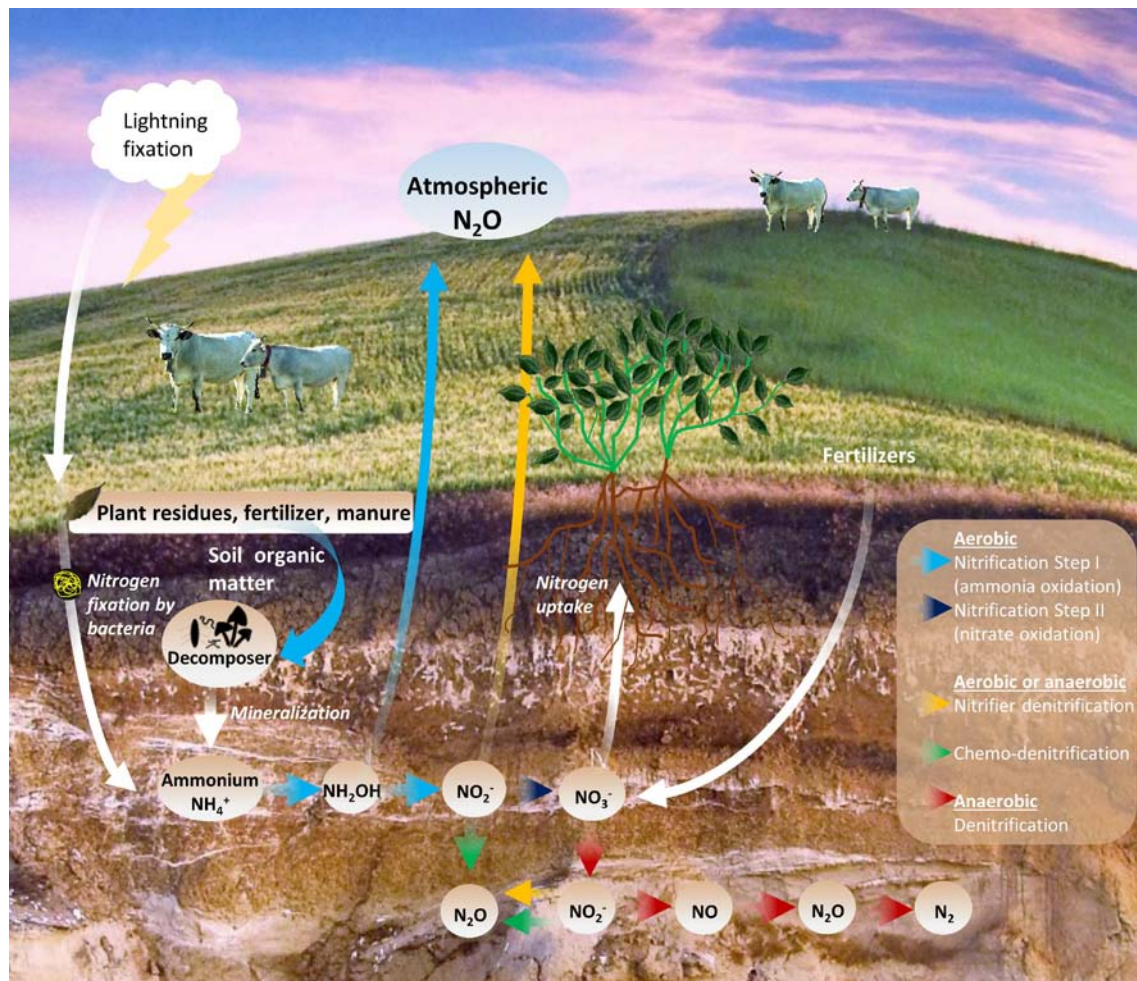
**A**tmospheric CO<sub>2</sub> is utilized by the plants during photosynthesis and transformed into plant biomass. Once the biomass decays, part of it returns to the atmosphere as CO<sub>2</sub>, while the rest is transferred into soil organic matter, which in turn decays and releases CO<sub>2</sub> to the atmosphere (Brevik, 2012). Methane, the second most potent GHG after CO<sub>2</sub> (IPCC, 2007), is produced by soil organisms under aerobic and anaerobic conditions (Fig. 1.2). Under anaerobic conditions, such as in peatlands and wetlands, methanogens utilize easily degradable carbon sources to produce methane – a process known as methanogenesis. In grasslands, where aerobic conditions dominate, methanotrophic bacteria oxidize CH<sub>4</sub> into CO<sub>2</sub> (Le Mer & Roger, 2001). When more methane is produced by methanogenic bacteria than is consumed by methanotrophic bacteria and thus more CH<sub>4</sub> is emitted to the atmosphere, an ecosystem is considered to be a CH<sub>4</sub> source, and vice versa (Le Mer & Roger, 2001). As for N<sub>2</sub>O, it is formed in the soil by a series of complex microbial metabolic pathways (Fig. 1.3), known mainly as nitrification and denitrification processes (Venterea *et al.*, 2012; Butterbach-Bahl *et al.*, 2013).



**Figure 1.2** | Methane production and oxidation in the soil, and the different pathways of methane to the atmosphere. Background image of the soil profile taken from Jim Richardson (Blöschl *et al.*, 2015).

Whereas the main drivers of CO<sub>2</sub> fluxes are well established (e.g. Raich & Schlesinger, 1992; Ruimy *et al.*, 1995; Reverter *et al.*, 2010; Leiber-Sauheidl *et al.*, 2014), **in the case of CH<sub>4</sub> and N<sub>2</sub>O establishing strong predictive relationships between their fluxes and environmental parameters remains complex** - despite the great number of studies investigating these gases (Luo *et al.*, 2013). This is due to the high spatial irregularity of CH<sub>4</sub> and N<sub>2</sub>O fluxes which are controlled by soil properties and/or site management (Dalal & Allen, 2008). European grasslands are likely to be a net sink of CO<sub>2</sub>, but a source of CH<sub>4</sub> and N<sub>2</sub>O originating mainly from the enteric fermentation of ruminants and from fertilization and manure deposition/addition (Soussana *et al.*, 2004). While most studies have focused on CO<sub>2</sub> fluxes (e.g. Byrne *et al.*, 2005; Fu *et al.*, 2009; Leiber-Sauheidl *et al.*, 2014; Merbold *et al.*, 2012; Wohlfahrt *et al.*, 2009), **the knowledge of the temporal and spatial variability of methane (CH<sub>4</sub>) and nitrous oxide (N<sub>2</sub>O) fluxes in grasslands, particularly in mountain areas is scarce** (Allard *et al.*, 2007; Soussana *et al.*, 2007; Imer *et al.*, 2013), **and even more so in extensively-managed semi-natural grasslands**. Although atmospheric concentrations and fluxes of CH<sub>4</sub> and N<sub>2</sub>O are small, their increased emissions can offset potential CO<sub>2</sub> sequestration (Leahy *et al.*, 2004; Schulze *et al.*, 2009; Ciais *et al.*, 2010), and enhance global warming due to their high global warming potential (GWP). Thus, a major concern addresses the question of how management in mountain

grasslands can contribute to enhance soil carbon sequestration and prevent carbon release to the atmosphere. **Understanding better the main drivers of GHG emissions – especially for CH<sub>4</sub> and N<sub>2</sub>O – is hereby a prerequisite for determining adaptive management strategies.**



**Figure 1.3** | Potential pathways of N<sub>2</sub>O production in the soil (Scheme of N<sub>2</sub>O production in the soil modified from Venterea *et al.*, 2012). Background image of the soil profile taken from Jim Richardson (Blöschl *et al.*, 2015).

## 1.5. Plant-soil-atmosphere feedback

**S**ince GHG emissions mainly originate from soils, and soil processes and microbial community structure are regulated by plant vegetation composition through composition and quantity of root exudates (De Deyn *et al.*, 2008), it is suggested that changes in land cover and plant community composition in grasslands, can play an important role in regulating gas exchange and carbon balance (Epstein *et al.*, 1998; Canadell *et al.*, 2007; Klumpp *et al.*, 2011; Smith *et al.*, 2014). Indeed, strong interactions between the functional composition of plant communities and nutrient inputs for soil processes, which consequently affect GHG exchange rates, were found (Niklaus *et al.*, 2006, 2016). However, **the effects of different PFTs and their interactions with environmental conditions on GHG emissions are not fully clear and understood, due to the complexity of the processes involved and the high variability of CH<sub>4</sub> and N<sub>2</sub>O.** Most

studies have focused on legumes due to their obvious role in nitrogen fixation (e.g. Jensen et al., 2012; Lüscher et al., 2014 and references herein), and less attention has been paid to other PFTs in grassland ecosystems, such as grasses, non-legume forbs, sedges, and subshrubs (e.g. Pugnaire & Luque, 2001; Armas & Pugnaire, 2005; Blumenthal *et al.*, 2013; Resco de Dios *et al.*, 2014). Also, not much is known about the effects and interactions of PFTs on GHG fluxes in semi-natural grassland ecosystems compared to wetlands and peatlands (e.g. Kuiper et al., 2014; Robroek et al., 2015; Ward et al., 2013), and sown pastures and croplands (e.g. Rochette & Janzen, 2005; Dusenbury *et al.*, 2008; Ribas *et al.*, 2015).

## 1.6 Major objectives of the thesis

This thesis is divided into 7 chapters and covers the effects of global change on plant functional and species diversity, greenhouse gas fluxes, and soil functions (Table 1.1). Chapter 1 introduces the general research topics and questions. Chapters 2-5 address the key research questions. Chapter 6 provides an overall discussion of all the findings of this study, and chapter 7 draws the main conclusions from the findings.

**Table 1.1** | Overview of the topics approached in the different chapters of the study

Aspects	Chapters			
	II	III	IV	V
<i>Plant functional diversity</i>	x	x		x
<i>Species diversity</i>	x			
<i>GHG fluxes</i>			x	x
<i>Soil functions</i>		x		

In the following sections, we define the main objectives of this study, and their corresponding chapters.

### Objective 1 (chapter 2)

In chapter 2, we investigated the effect of warming on productivity, plant traits composition and functional diversity during one growing season through transplanting grassland turves from highland (humid and cold) to lowland (dry and warm) locations. We aimed to analyze: (1) the resistance of the grassland community to warming, (2) the effect of diversity and functional traits on biomass production, and (3) the effect of species and functional diversity and functional traits composition on community stability in terms of vegetation composition.

### Objective 2 (chapter 3)

For a thorough understanding of the effects of climate change on plant-soil interactions, we investigated in the third chapter: 1) The effects of PFT composition, including identity effects,



pairwise interactions, and evenness, on soil activity and fertility in grasslands; 2) if environmental variables (regional and local) modify the relationship between PFTs and soil activity and fertility.

### Objective 3 (chapter 4)

Chapter 4 presents chamber measurements of CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O fluxes in four semi-natural grasslands in the Pyrenees across an altitudinal gradient and along different seasons covering the grazing and the non-grazing periods. The measurements were carried out under both light and dark conditions, as well as on vegetation and on bare soil, using static chambers connected to a field gas photoacoustic monitor (PAS). The study aimed to: 1) describe the magnitude and range of variability of the fluxes of CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O from semi-natural grasslands in the Pyrenees at different altitudes and along different seasons, 2) identify the main biotic (e.g. grazing animals, aboveground biomass) and abiotic (e.g. SWC, PAR, T<sub>a</sub>) drivers of the recorded CH<sub>4</sub> and N<sub>2</sub>O fluxes in those grasslands, and 3) determine the effect of vegetation and light presence on the measured CH<sub>4</sub> and N<sub>2</sub>O fluxes.

### Objective 4 (chapter 5)

The research interests of chapter 5 were to investigate the role of plant functional type composition and climatic conditions, in combination with their interactions on fluxes of CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O. The specific aims of the study were to investigate: 1) The effects of PFT composition, including both identity effects and pairwise interactions, on greenhouse gas fluxes: CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O; 2) if the climatic conditions modify the relationship between PFTs and GHG fluxes.

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# Chapter 2



# Functional trait changes, productivity shifts and vegetation stability in mountain grasslands during a short-term warming



An edited version of this chapter was published in *PLOS ONE* (2015), DOI: 10.1371/journal.pone.0141899  
Debouk H., de Bello F., Sebastià, M-T. 2015.

## 2.1 Abstract

**P**lant functional traits underlie vegetation responses to environmental changes such as global warming, and consequently influence ecosystem processes. While most of the existing studies focus on the effect of warming only on species diversity and productivity, we further investigated (i) how the structure of community plant functional traits in temperate grasslands respond to experimental warming, and (ii) whether species and functional diversity contribute to a greater stability of grasslands, in terms of vegetation composition and productivity. Intact vegetation turves were extracted from temperate subalpine grassland (highland) in the Eastern Pyrenees and transplanted into a warm continental, experimental site in Lleida, in Western Catalonia (lowland). The impacts of simulated warming on plant production and diversity, functional trait structure, and vegetation compositional stability were assessed. We observed an increase in biomass and a reduction in species and functional diversity under short-term warming. The functional structure of the grassland communities changed significantly, in terms of functional diversity and community-weighted means (CWM) for several traits. Acquisitive and fast-growing species with higher SLA, early flowering, erect growth habit, and rhizomatous strategy became dominant in the lowland. Productivity was significantly positively related to species, and to a lower extent, functional diversity, but productivity and stability after warming were more dependent on trait composition (CWM) than on diversity. The turves with more acquisitive species before warming changed less in composition after warming. Results suggest that (i) the short-term warming can lead to the dominance of acquisitive fast growing species over conservative species, thus reducing species richness, and (ii) the functional traits structure in grassland communities had a greater influence on the productivity and stability of the community under short-term warming, compared to diversity effects. In summary, short-term climate warming can greatly alter vegetation functional structure and its relation to productivity.



## 2.2 Introduction

High mountain ecosystems are considered to be particularly vulnerable to global warming (Price & Waser, 2000; Pauli *et al.*, 2007). Under climate change conditions, floristic biodiversity in European semi-natural, species-rich and subalpine grasslands is expected to be highly affected (Sala *et al.*, 2000; Pauli *et al.*, 2012; Singh *et al.*, 2013). A number of studies have observed that warming enhances productivity (Rustad *et al.*, 2001; Sebastià *et al.*, 2004; Sebastià, 2007; Lin *et al.*, 2010; Baldwin *et al.*, 2014), alters species composition and reduces species diversity (Sebastià *et al.*, 2008; Baldwin *et al.*, 2014), at least in a short term. The increase in biomass productivity has been attributed to the enhanced plant growth as a transient result of increased soil organic matter mineralization (Epstein *et al.*, 2000; Shaver *et al.*, 2000) and nutrient availability with warming (Rustad *et al.*, 2001; Sebastià, 2007). These resource enriched environmental conditions are considered favorable to species characterized by fast growth and high returns on resources investment (Crick & Grime, 1987; Endara & Coley, 2011). This means that conservative species, with slower nutrient acquisition and slower growth (as opposed to acquisitive species, Diaz *et al.* (Diaz *et al.*, 2004)) could be out-competed as a result of nutrient depletion by fast-growing species (Aerts, 1999) or could disappear as a result of low adaptation ability to the new conditions. Increased productivity can, therefore, either increase or reduce diversity depending on the relationship between productivity and diversity (Grime, 1973; Pärtel *et al.*, 2007).

The impact of warming should also depend on the resistance and resilience, altogether defining stability, of plant communities. In many studies, the stability of the community has been related to diversity – with an a priori known as the “diversity begets stability” hypothesis. The theory suggests a positive stability-diversity relationship because the system could be more stable when more species are available, thus providing a sort of insurance towards different environmental changes (MacArthur, 1955; Elton, 1958; Tilman & Downing, 1994; Palmer & Maurer, 1997). However, other studies question this hypothesis (Goodman, 1975; Lepš *et al.*, 1982; Naeem, 2002) because ecosystem stability could simply depend on the type of dominant species in the system. These studies advocate that the positive correlation between stability and diversity can be explained by the fact that higher diversity simply increases the probability of including species with traits better adapted to the new environmental conditions (Huston, 1997; Lepš *et al.*, 2001; Loreau *et al.*, 2001). They also argue that looking only at species composition and diversity does not give a mechanistic view on the ecosystem and does not allow generalizations beyond specific sites.

While several studies have assessed the effect of global warming on species diversity (Hughes, 2000; Sala *et al.*, 2000; Bassler & Angeringer, 2011; Pauli *et al.*, 2012; Singh *et al.*, 2013), the response of the community plant functional traits to increasing temperatures and their role in the vegetation stability have been generally overlooked. The importance of plant functional traits (any characteristics of organisms which impact its fitness) lies in the fact that they consistently reveal community responses to environmental changes across regions and control ecosystem processes (vegetative growth, photosynthesis, decomposition, etc. (Carbognani *et al.*, 2013)). Species with distinct functional traits are expected to respond differently to environmental conditions, and hence influence ecosystem processes accordingly (Lavorel & Garnier, 2002). In warming experiments, it remains unclear how diversity and plant functional traits influence the community resistance and to which extent they contribute to its resilience under warming. It is also unclear how ecosystem processes such as productivity and stability will be affected by changes in vegetation structure and what type of species will become more dominant. Therefore, we expect that the study of functional traits can improve our understanding of the mechanisms related to the stability of the ecosystem, under warming.

In this study, we investigated the effect of warming on productivity, plant traits composition and functional diversity during one growing season through transplanting grassland turves from the highland (humid and cold) to the lowland (dry and warm) areas. We focus on semi-natural subalpine grasslands in the Eastern Pyrenees, which are considered to be vulnerable to climate change due to their position at the south-western edge of the semi-natural grassland biome in Europe (Sebastià, 2007). Additionally, climate change scenarios in the region predict an increase in mean annual temperature of 0.5 °C and a decrease in mean annual precipitation of 10% (GECCC, 2010). Particularly in the Spanish Pyrenees, effects of warming are believed to be particularly pronounced (López-Moreno, 2008; López-Moreno & Beniston, 2009).

Many studies suggest that biodiversity enhances ecosystem function (Finn *et al.*, 2013; Lefcheck *et al.*, 2015) and is indispensable for the stability of plant communities in terms of productivity and vegetation composition (MacArthur, 1955; Elton, 1958; Tilman & Downing, 1994). However stability does not only depend on species richness but also on the functional traits of the species and on the growth-related traits (Lepš *et al.*, 1982; Naeem, 2002). Therefore, we hypothesize that community traits associated to resource acquisition and competitiveness (specific leaf area, leaf dry matter content, start of first flowering, plant height, growth habit, rhizome-formation, etc.), together with species richness, will contribute to the stability of the community in the face of warming, in terms of vegetation composition. We also hypothesize that the increased productivity in the lowland is not exclusively the result of the initial species richness, but also of the functional trait changes under warming conditions. In our study, we analyze (1) the resistance of the grassland community to warming, (2) the effect of diversity and func-

tional traits on biomass production, (3) and the effect of species and functional diversity and functional traits composition on community stability in terms of vegetation composition.

## 2.3 Material and methods

### 2.3.1 Transplanting

A short-term (one growing season) climate change experiment was carried out through transplanting grassland turves from highland (moist and cold) to lowland (dry and warm) areas. The highland study system consisted of two cold temperate semi-natural subalpine grassland sites (p1 and p2) at around 2000 m a.s.l. with a distance of approximately 1 km from one another at the Pla de Rus in Cadí-Moixerò Natural Park (1·993°E, 42·276°N). These subalpine grasslands are characterized by cold temperate climate (mean annual temperature is 5.3°C and mean annual precipitation is 1183.4 mm; Atlas Climàtic Digital de Catalunya <http://www.opengis.uab.cat/acdc/>, accessed in July 2013). The vegetation was dominated, in both sites, by subalpine perennial mesic grasslands on limestone dominated by *Festuca nigrescens* Lam., in addition to *Carex caryophyllea* Latourr., *Anthoxanthum odoratum* L., *Potentilla neumanniana* Reichenb., *Galium verum* L., *Thymus pulegioides* L., and *Koeleria macrantha* (Ledeb.) Schultes (see Sebastià et al. (2004) for more details on original vegetation and environment).

The experiment addressed mostly a temperature and water change scenario (see below) during the vegetative period of the plants, representing an extreme warming event. At each of the two highland sites, 60 turves were extracted and each turf was put in plastic trays of 40 cm x 40 cm x 20 cm (depth). Half of the turves from each site were selected randomly and placed back in the grassland ('highland' treatment). The other half was placed in experimental fields of the School of Agrifood and Forestry Science and Engineering of the University of Lleida ('lowland'). The lowland site has a continental Mediterranean climate (mean annual temperature is 14.5°C and mean annual precipitation is 442 mm). Since the lowland site (Lleida) is exposed to very dry and warm conditions during summer, water was added weekly to approximate half of the natural rain frequency at the subalpine site and to prevent the mortality of the plants due to the high discrepancy in precipitation between the highland and the lowland. The short-term duration of the experiment implies that new colonizers did not have time to colonize the turves, and thus observed responses correspond only to internal responses of the original system. It should be noted that higher temperature and reduced water availability were the main stressors in the lowland compared to the highland. However, other factors could also differ between the highland and the lowland. Since we did not observe any obvious difference in pests and parasites

between highland and lowland, we can safely attribute the observed changes to increased temperature (see published work on this data for a more detailed discussion; [Sebastià et al., 2008; Sebastià, 2007b]). As revealed by this work, changes in mineralization could explain some of the patterns observed, but again these changes were triggered by the increase in temperature.

The turves were randomly distributed within the lowland, but kept separated between two sites as in the highland. A 20 cm x 20 cm quadrat was fixed in the centre of the 60 turves. This quadrat was divided into a grid of 100 subquadrats of 2 cm x 2 cm each. All the species present in each quadrat were recorded every two weeks. Frequency records and species counts were taken seven times throughout the growing season in the highland and eight times in the lowland (for more detailed information about the experiment and vegetation composition changes see Sebastià et al., 2008; Sebastià, 2007b). We did only seven measurements at the highland site because the seventh measurement had already covered the end of the growing season in the subalpine grassland.

At the end of the experiment, 17 weeks after the start, above-ground biomass was estimated by harvesting vegetation on a 20 x 20 cm quadrat placed in the middle of a subset of 28 out of the 60 turves used for species frequency. A previous harvest was carried out in 28 different turves 10 weeks after the start of the experiment. The biomass data at the two dates were used to analyze the diversity-productivity relationship.

### 2.3.2 Community functional structure

We considered various relevant functional traits that are linked to different species responses to biotic and abiotic factors. These were: specific leaf area (SLA, leaf area per unit dry mass), leaf dry matter content (LDMC, the ratio of leaf dry mass to fresh mass), vegetative reproduction (presence of rhizomes or stolons), plant growth habit (erect vs. prostrate), mean plant height ( $H_{\text{mean}}$ ), and start of first flowering (month). Trait values of the species present were obtained from two traitbases, i.e. the LEDA traitbase (Kleyer *et al.*, 2008), and the CLO-PLA traitbase (Klimešová & Klimeš, 2005) and complemented by specific standardized measurements for SLA (Cornelissen *et al.*, 2003) in the field. Only few trait values were acquired from de Bello *et al.* (de Bello *et al.*, 2009). One important factor in the response of species to warming could be trait plasticity (Jung *et al.*, 2014) which is considered one of the major means by which species can cope with new environmental conditions (Gratani, 2014). Unfortunately, we could not measure the traits directly in the field. As such, our analyses might only partially capture the full extent of plant community response to warming.

Two indices were considered to reveal the major characteristics of the community functional trait structure (Ricotta & Moretti, 2010). The first one is the community weighted means of the traits (CWM). The CWM for each trait is calculated as the mean of the trait values in the community, weighted by the relative abundance of each species (Díaz *et al.*, 2007). For categorical traits, this corresponds to the relative abundance of species bearing a certain type of traits. The second index is a commonly used measure of functional diversity expressed with the Rao quadratic entropy (Ricotta & Moretti, 2010). The Rao index expresses the sum of trait dissimilarity between each pair of species in a turf and it is weighted by species relative abundance. This index is mostly independent from the number of species. It was expressed as equivalent numbers (de Bello *et al.*, 2010) and for all traits combined. The numbers equivalent of a diversity index indicates the effective diversity, i.e. how many effectively different species in terms of abundance and, in case of traits, functional difference (Jost, 2007), thus providing a more intuitive concept of diversity comparable to species richness. Using Rao not in equivalent numbers produced strongly related results (as they are strongly related mathematically). Two additional indices of species diversity were calculated: species richness and Simpson's diversity, also expressed in terms of equivalent numbers (de Bello *et al.*, 2010).

### 2.3.3 Data analysis

The effects of the short-term climate change on the CWM of the traits (SLA, % of rhizomes, % of prostrate, start of first flowering, LDMC, and mean plant height) and the diversity indices (species richness, Simpson, and Rao) were analyzed using repeated measures ANOVA; where transplant (highland and lowland), extraction site (p1 and p2 in highland) and time (as quantitative value, 1 to 7 or 8) are the fixed factors. Results by Sebastià (Sebastià, 2007) suggested an increase in above-ground biomass in the lowland in comparison to the highland. Therefore, we conducted multiple regression models to examine the effect of each of the diversity indices and CWM on above-ground biomass in the two sites in both the highland and the lowland. In all the multiple regression analyses, we selected the best model based on the Akaike information criterion (Akaike, 1973) using stepwise regressions. Correlation tests between the traits and the diversity indices were carried out prior to including them in the regression models to minimize collinearity problems (Tables S1 and S2). The stronger correlation was between species richness and Simpson's diversity index ( $R = 0.864$ , table S2) but we decided to keep both variables because they cover different ecological implications.

Changes in vegetation composition between turves of the two treatments (the highland and the lowland) were already observed by Sebastià *et al.* (Sebastià *et al.*, 2008), and a higher dissimilarity in species composition was detected among the highland samples in comparison to the lowland, suggesting a change in the vegetation composition with warming. To

determine the dynamics of change in vegetation composition within the turves as a result of the transplant, we calculated the Bray-Curtis dissimilarity (Bray & Curtis, 1957) at the first and the second harvest (end of experiment) within each turve, using the CRAN package “vegan” in R software (Oksanen *et al.*, 2013). Multiple regression models were carried out to test the effect of each of the initial diversity indices and the initial CWM of traits on the change in vegetation composition (Bray-Curtis) in both the highland and the lowland (explanatory variables). We made additionally a stepwise regression model including all the variables of diversity and CWM traits with the Bray-Curtis dissimilarity. All statistical analyses were carried out using R software (R Core team, 2013).

## 2.4 Results

### 2.4.1 Functional structure

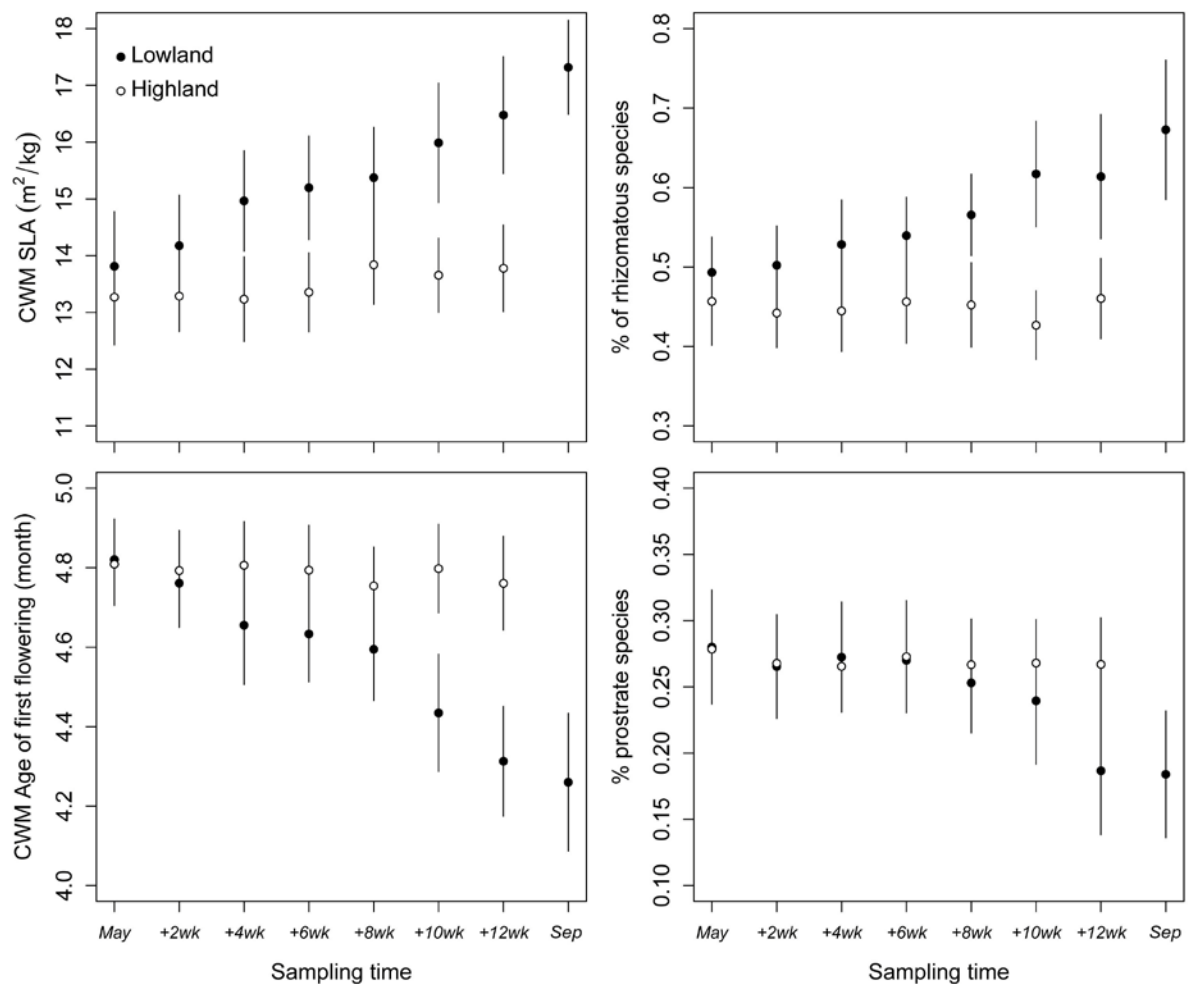
**A**fter the transplant, the functional structure of the grassland communities changed considerably (Table 2.1; Figs. 2.1 and 2.2). Few weeks after the transplant, the community weighted mean (CWM) of SLA, start of first flowering and proportion of rhizomatous and prostrate species started to diverge from the lowland to the highland. The functional structure in the highland remained relatively unchanged along the growing season (Fig. 2.1). Communities in the lowland appeared to be progressively more dominated by species with higher SLA, earlier flowering, erect growth habit and with rhizomes (Table 2.1; Fig. 2.1). However leaf dry matter content (LDMC) did not respond to the short-term warming (Table 2.1). All those responses occurred based on the original species composition in each turf (we observed no newcomer appearing in the turves). Such an increase in abundance by a certain type of species was accompanied by the disappearance of other species (lower species richness and Simpson diversity) in the lowland (Fig. 2.2). These two diversity components, in addition to functional diversity, decreased significantly in magnitude after the transplant (Table 2.1; Fig. 2.2).

**Table 2.1** | Results of repeated measures ANOVA to assess the effect of the transplant on diversity indices and CWM traits with transplant (highland and lowland), site (p1 and p2 in the highland) and time. See Figures 2.1 & 2.2 for a graphical representation.

	Transplant		Site		Time		Transp. x Time		Site x Time		Transp. x Site x Time	
	F	P	F	P	F	P	F	P	F	P	F	P
<b>Diversity</b>												
Species richness	25.502	<0.001	0.791	0.3776	156.37	<0.001	126.002	<0.001	5.613	0.0183	6.455	<b>0.0115</b>
Simpson	14.113	<0.001	0.041	0.8396	60.595	<0.001	134.248	<0.001	0.462	0.4971	18.931	<b>&lt;0.001</b>
Rao	22.137	<0.001	0.119	0.7312	87.449	<0.001	55.558	<b>&lt;0.001<sup>a</sup></b>	2.159	0.1426	1.473	0.2256
<b>CWM traits</b>												
SLA	32.091	<0.001	0.014	0.906	142.03	<0.001	52.828	<b>&lt;0.001</b>	1.483	0.224	0.031	0.859
% Rhizomes	27.521	<0.001	7.630	0.008	60.605	<0.001	53.480	<b>&lt;0.001</b>	0.014	0.907	1.570	0.211
% Prostrate	1.426	0.238	1.264	0.266	47.863	<0.001	27.925	<0.001	8.599	0.004	4.681	<b>0.031</b>
Start of first flowering	17.462	<0.001	2.464	0.122	132.52	<0.001	80.168	<b>&lt;0.001</b>	3.52	0.061	0.659	0.417
LDMC	1.079	0.303	0.005	0.942	4.211	<b>0.041</b>	3.428	0.065	0.42	0.517	0.033	0.856
Mean Height	0.03	0.863	0.901	0.347	4.648	0.032	1.134	0.288	1.069	0.302	9.826	<b>0.002</b>

<sup>a</sup>P-values in bold indicate significant relationships.

We tested the change in vegetation composition within turves in both the highland and the lowland with multiple regression models and found that neither the initial diversity nor the initial functional traits diversity, had an influence on the change in vegetation composition (Table 2.2 and S2.3 Table). On the contrary the communities with initially low CWM of SLA witnessed a significant greater change in vegetation composition after the transplant (Fig. 2.3; S2.3 and S2.5 Tables). Our results show that, in the lowland, turves originally dominated by species with higher SLA were more stable as they did not change considerably in terms of vegetation composition under warming. On the other hand, in plots originally dominated by species with lower SLA, species characterized with high SLA replaced those with low SLA as a result of warming (Fig. 2.3).



**Figure 2.1** | Effect of the transplant experiment on the Community weighted means (CWM) of SLA (upper left), % of rhizomatous species (upper right), start of first flowering (lower left), and % of prostrate plants (lower right) along time. The black points correspond to the lowland, and the white points correspond to the highland. The whiskers refer to standard deviation. The x axis indicates the dates of the repeated samplings (frequency) within each turf. The first sampling was done in mid May and the last one in the lowland at the beginning of September. For the corresponding statistical tests see Table 2.1.



## 2.4.2 Productivity

We observed that the productivity in the grassland community under warming increased with diversity (Table 2.2; Fig. 2.4), particularly in the last harvest. The results of the best multiple regression model showed a significant positive effect of diversity on biomass, except for Simpson’s diversity index (Table 2.2).

Plant functional trait composition (CWM) also seems to affect productivity, either negatively or positively (Table 2.3). Particularly, in the lowland, biomass production increased in communities with taller and less rhizomatous species (*estimate* = 44.85 for height, *estimate* = -1091.43 for rhizomatous propagation, S2.4 Table). Whereas in the highland, biomass increased with more rhizomatous species (*estimate* = 136.38, S2.4 Table). Other traits, such as SLA and prostrate growth presented a significant positive relationship with biomass, in both the highland and the lowland (*estimate* = 71.6,  $P < 0.001$  for SLA and *estimate* = 405.66,  $P < 0.05$  for prostrate growth form, Table 2.3). Our stepwise regression results also showed that the CWM traits, particularly the height and the proportion of rhizomes, have a greater effect on above-ground biomass than diversity (S2.4 Table).

**Table 2.2** | Results of multiple regression models to assess the effect of the diversity indices (species richness SR, Simpson’s diversity index, functional diversity FD) on above-ground biomass (final harvest) and changes in vegetation composition (Bray-Curtis), with transplant (highland and lowland) and site (p1 and p2 sites in the highland). See Fig. 2.4 for a graphical representation of the biomass models.

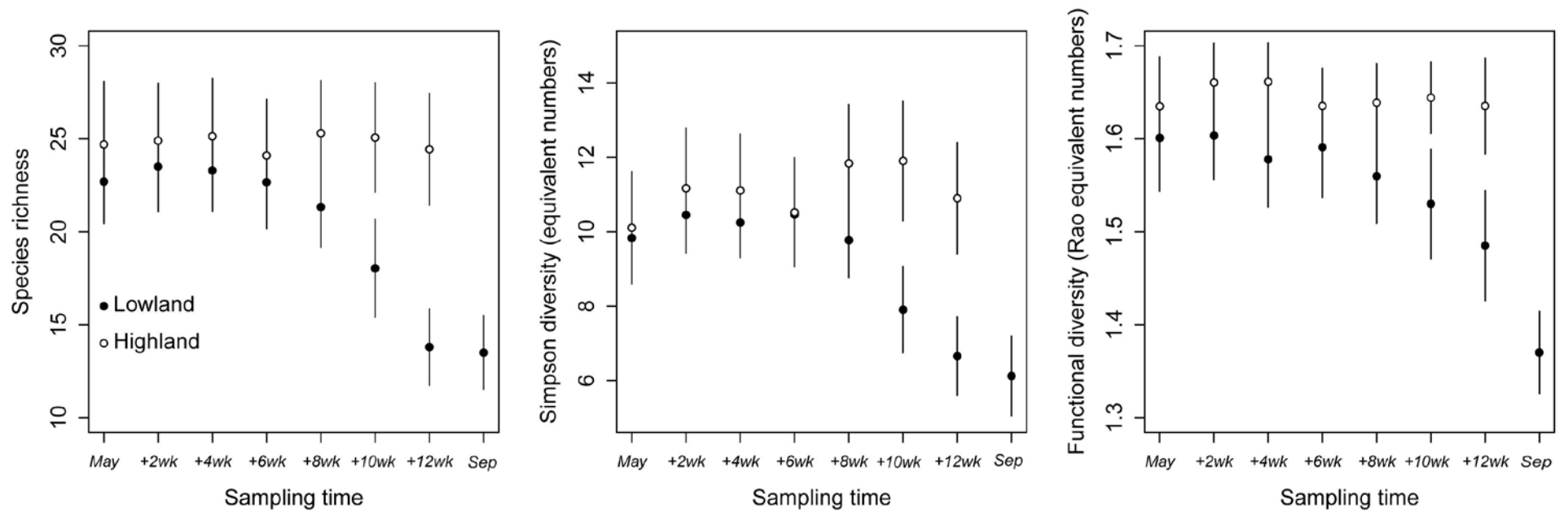
	Above-ground biomass			Bray-Curtis		
	Model	R <sup>2</sup> adj.	P	Model	R <sup>2</sup> adj.	P
	*** <sup>a</sup>	0.810		***	0.538	
Site			0.741			0.568
Transplant			0.989			0.102
SR			0.144			0.932
SR*transplant			<b>0.008</b>			0.852
	***	0.690		***	0.543	
Site			0.829			0.325
Transplant			0.803			0.406
Simpson			0.636			0.414
Simpson*transplant			0.405			0.570
	***	0.800		***	0.540	
Site			0.390			0.441
Transplant			0.328			0.592
FD			0.067			0.682
FD*transplant			<b>0.008</b>			0.685

<sup>a</sup>\*\*\*P < 0.001

<sup>b</sup>P-values in bold indicate significant relationships.

## 2.5 Discussion

We found that short-term climate warming can have a great impact on the functional structure of grassland communities, both in terms of CWM of traits (Table 2.1; Fig. 2.1) and functional diversity (Table 2.1; Fig. 2.2). No major changes in the highland were observed along the growing season in terms of CWM of traits and diversity, suggesting that changes in the lowland are related to climatic stress and not seasonality. The significant interaction “transplant x site x time” suggests indeed that the pattern over time is different depending on which highland site the sample came from. While in some cases this interaction was significant, the estimates in Table 2.1 were often not very high and from visual inspection these effects were not strong. We therefore concluded that these effects, although existing were not very marked. Diversity variables and community traits showed different resistance in front of the new environmental conditions in the lowland (Figs. 2.1 and 2.2). CWM of SLA and start of first flowering started shifting already after only two weeks whereas most of the other variables were resistant for around six weeks (Figs. 2.1 and 2.2). Particularly, the proportion of prostrate species manifested a considerable resistance until the eighth week of the experiment before declining (Fig. 2.1). Our results also showed that traits which provide opportunistic use of increased resource availability (in our case species with higher SLA, early flowering, erect growth habit, and rhizomatous strategy) became dominant as a result of the short-term climate change, while conservative species became less abundant or disappeared from the system (Table 2.1; Fig. 2.1). Species with higher SLA, but not diversity components (species and functional diversity), contributed to the stability of the grassland community under warming conditions (Fig. 2.3; Table 2.2 and S2.3 Table). In both the highland and the lowland, there was a positive relationship between species richness and productivity. However, in the lowland the vegetation was more productive and less diverse (as found by Sebastià et al. (Sebastià *et al.*, 2008)). Overall the results suggest that plant functional traits, in terms of CWM, are the main drivers to the increased productivity in the lowland. We discuss these hypotheses and the patterns detected in the following sections.



**Figure 2.2** | Effect of the transplant experiment on diversity indices: Species richness (left), Simpson’s reciprocal diversity index with equivalent numbers (centre), and Functional diversity’s index Rao with equivalent numbers (left) along time. The black points correspond to the lowland, and the white points correspond to the highland. The whiskers refer to standard deviation. See Fig. 2.1 for more information on sampling dates and Table 1 for the corresponding statistical tests.

## 2.5.1 Community changes

**O**ur results suggest that the warming in the lowland favored opportunistic erect species with higher SLA, anticipated flowering period, and more rhizomes (Fig. 2.1). It is widely acknowledged that species from nutrient-rich habitats have a greater relative growth rate, hence greater SLA, manifested by fast resource capture and fast turnover of organs (Grime, 1977; Chapin, 1980; Poorter & De Jong, 1999). Mountain areas are characterized by low nutrient availability, where mineralization is often limited by cold temperatures (Schmidt *et al.*, 1999; Weintraub & Schimel, 2003). Removing the temperature limitation in the lowland caused an increase in fast growth and resource acquisition species (e.g. higher SLA and earlier flowering). Earlier flowering of the plants as a response to warming was already observed in previous studies (Menzel *et al.*, 2006; Amano *et al.*, 2010; Wolkovich *et al.*, 2012). Despite increases of SLA as a response to short-term warming, it seems to be unresponsive to long-term climate warming (Cantarel *et al.*, 2013). The dominance of species with rhizomes under warming conditions (Fig. 1) can be similarly explained by the fact that these species acquire the available nutrients faster compared with species that need to establish new stems (Grime, 1979). Additionally, these species can store resources in the rhizomes from previous years (storage effect; Chesson (Chesson, 2000)) and are therefore the fastest in reacting to improved environmental conditions (i.e. decrease in thermal constrain). Prostrate plants' proportion decreased with time in the lowland compared to the highland (Fig. 2.1). Increased abundance of erect plants in the lowland suggests that increased competition for light with enhanced biomass with warming (Sebastià, 2007; Sebastià *et al.*, 2008) must have lead to the exclusion of prostrate plants (Zimdahl, 2013).

**S**urprisingly, in spite of this increase of biomass and erect species proportion, plant height was not responsive to warming, and increased similarly in both locations throughout time. This was thus a plant growth effect, which likely increased competition for light during the growing season (Westoby *et al.*, 2002). It could have also been the effect of using database traits, and not actual measurements. We also expected a change in LDMC, which is often related to SLA and that was reported to increase due to climate warming in Cantarel *et al.* (Cantarel *et al.*, 2013). However this variable did not change in our experiment, which could also be related to the high sensitivity of this trait to local conditions, not captured by database information. LDMC may vary considerably between different plants in the same site within the same day (Cornelissen *et al.*, 2003) and within the same species (De Bello *et al.*, 2011). This variability could have been revealed if field measurements had been available. Nonetheless, for other traits mostly retrieved from databases, the observed patterns were however responsive, suggesting that trait information measured in other sites can provide valuable information on vegetation responses to environmental changes.

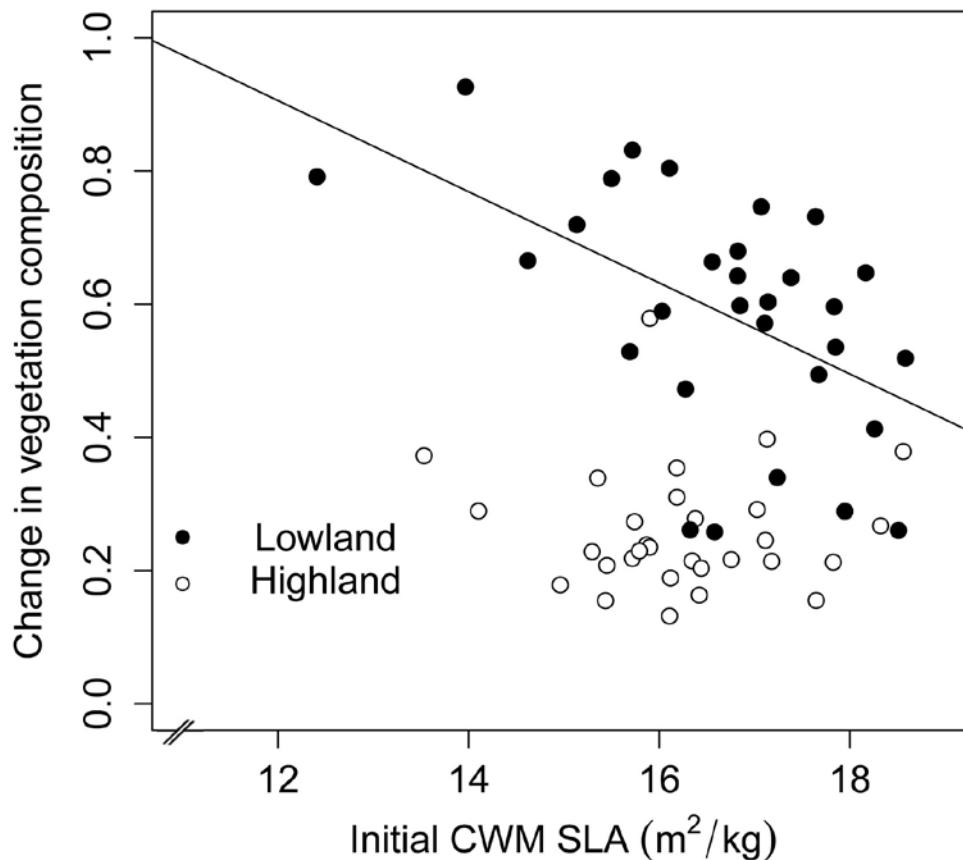
**Table 2.3** | Results of multiple regression models to assess the effect of the plant functional traits (SLA, LDMC, height, start of first flowering, % prostrate, % rhizomes) on above-ground biomass, with transplant (highland and lowland) and site (p1 and p2 sites in the highland).

	Above-ground biomass					
	Model	R <sup>2</sup> adj.	Estimate	Std. Error	T value	P
Site	***a	0.984	-181.98	28.54	-6.38	<0.001
Transplant			-1683.23	426.60	-3.95	0.001
LDMC			1.49	0.88	1.69	0.111
SLA			71.60	13.48	5.31	<0.001
Prostrate			405.66	181.42	2.24	<b>0.040</b>
Rhizomes			2653.65	265.88	9.98	<0.001
Height			-16.46	10.87	-1.51	0.150
Start of first flowering			-995.52	193.78	-5.14	<0.001
Transplant*rhizomes			-2156.80	175.02	-12.32	<0.001
Transplant*height			21.37	9.34	2.29	<b>0.036</b>
Transplant*start of first flowering			693.08	137.38	5.05	<0.001

a\*\*\*P < 0.001

<sup>b</sup>P-values in bold indicate significant relationships.

The shifts in CWM of traits were accompanied by a loss of species and a decrease in functional diversity, although our results show that the community-level traits (Fig. 2.1) responded more rapidly to warming than species diversity (Fig. 2.2). This was also observed by Suding *et al.* (Suding *et al.*, 2008) and Cantarel *et al.* (Cantarel *et al.*, 2013). The reduction of species in the temporarily nutrient-rich habitat (lowland) may be the outcome of environmental filtering which excludes species that are less adapted to the warming period. It may also be the result of the out-competition of conservative species by opportunistic species with a faster growth rate (competitive exclusion principle, Gause (Gause, 1934)). The delay of the diversity indices in their response to warming until the eighth week of the experiment (Fig. 2.2) indicates a certain resistance from the community to the simulated environmental conditions. This is possibly because of redundancy in the turves, followed by the disappearance of more conservative species. Opportunistic species are able to rapidly benefit from enhanced mineralization and exhibit higher growth rates and productivity relative to conservative species. Hence opportunistic species will cause the exclusion of weaker competitors. Indeed, we saw that opportunistic species, although not abundant before the transplant, became significantly dominant at the cost of more conservative species (Sebastià *et al.*, 2008). We underline that mountain subalpine grasslands consist of a wide range of opportunistic and conservative species with a high variety of survival-strategies. Our results suggest that grassland ecosystems may have the potential to cope well with new climate stress in the short term, thus being resistant to species invasion.



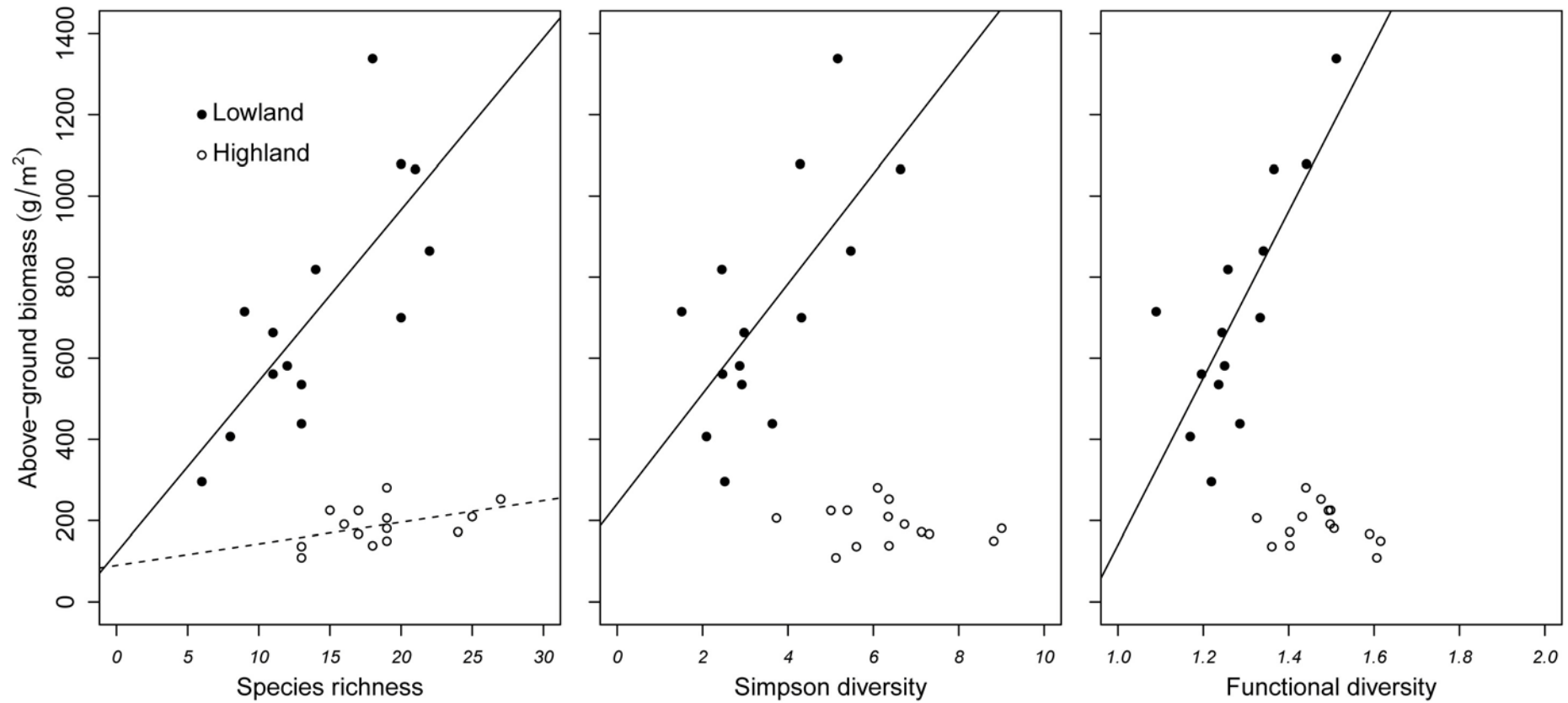
**Figure 2.3** | Relationship between the initial CWM SLA and the change in vegetation composition (Bray-Curtis dissimilarity) between beginning and end of experiment, in both the highland and the lowland. The black points correspond to the lowland, and the white points correspond to the highland. Solid linear regression line reflects significant relationship ( $p < 0.05$ ) in the lowland; and no line represents no significance.

## 2.5.2 Community stability

Our transplant experiment caused a shift in vegetation composition in the lowland favoring species with higher SLA under warming conditions. The biggest change in composition was observed where the turves had originally more species with lower SLA, and therefore species in these turves were replaced by others with greater SLA which acquired the resources more favorably under warming conditions (Fig. 2.3; Tables S2.3 and S2.5). On the contrary no diversity components (species and functional diversity) affected the stability of the grassland community under warming conditions. This shows that functional traits of the species (in our case SLA) exerted the major effect on the vegetation response, but not diversity. These results comply with previous findings highlighting the importance of plant functional traits and interspecific variability on population stability (Polley *et al.*, 2013; Jung *et al.*, 2014; Májeková *et al.*, 2014). We thus conclude that the disappearance of species was greater in turves with originally lower SLA.

### 2.5.3 Community stability-productivity

Sebastià (2007) showed that above-ground biomass increased with warming in the lowland. Similar results were obtained by Lin *et al.* (Lin *et al.*, 2010) and Baldwin *et al.* (Baldwin *et al.*, 2014). Cantarel *et al.* (Cantarel *et al.*, 2013) confirmed this pattern in the short term but reported a progressive decline in the long term of the experiment. In our study, the above-ground biomass had a positive relationship with species richness within both the highland and the lowland (Table 3). This positive relationship between diversity and biomass production is based on Darwin's theory (Darwin, 1859) and has been demonstrated in many studies (Tilman *et al.*, 1996; Hector *et al.*, 1999; Pfisterer & Schmid, 2002; Cardinale *et al.*, 2007; Kirwan *et al.*, 2007). These studies assume that nutrients are captured more efficiently in diverse plant communities because of species complementarity in their resource acquisition, and thus, as they suggest, a greater productivity is reached.



**Figure 2.4** | Relation between above-ground biomass and diversity indices: Species richness (left), Simpson's diversity index with equivalent numbers (centre), and Functional diversity's index Rao with equivalent numbers (right) in both the highland and the lowland. The black points correspond to the lowland, and the white points correspond to the highland. Solid linear regression line reflects significant relationship ( $p < 0.05$ ) between the variables in the lowland; dashed line refers to a marginal significant correlation ( $p < 0.1$ ) in the highland; and no line represents no significance.



In our warming experiment, however, productivity increased and diversity decreased as a response to warming. Interestingly, plant traits and not diversity were the main factor to explain the increased productivity in the lowland. A greater biomass production was the outcome of faster mineralization rate and nutrient acquisition by opportunistic species that are taller, have greater SLA and erect form. Taller plants are considered more competitive since investment in height improves the access to light (Falster & Westoby, 2003), allowing them to acquire nutrients faster thus leading to a faster growth and a greater productivity. The positive correlation between biomass and SLA was already described in Cornelissen et al. (Cornelissen *et al.*, 2003). The positive relationship between biomass and prostrate plant growth may be caused by certain species such as *Koeleria macrantha* (33% prostrate; LEDA traitbase) which contributed greatly to the total biomass in each monolith (Spehn *et al.*, 2000), thus influencing significantly our results. This bias highlights the risk of using data from traitbases which may not be “optimal”. We conclude that the proportion of prostrate species in a community was tightly correlated in the biomass data with the presence of *K. macrantha*. We assume that on a long-term basis biomass production will decline, as previously seen in other warming experiments (Cantarel *et al.*, 2013). This decrease will occur when water and temporary flux of nutrients will get scarce with ongoing warming. Our short-term warming experiment favored the growth of opportunistic fast-growing species in the lowland. Communities dominated by fast-growing species have a higher resilience and a lower resistance to extreme events in comparison to slow-growing conservative species (Lepš *et al.*, 1982; MacGillivray *et al.*, 1995). Therefore we expect that in the long-term, changes in resource availability and climate will lead to a new community at the expense of the old one (Körner & Paulsen, 2004). We speculate that some species will acclimate through their functional traits to the long-term warming and drought and others will simply disappear if warm and dry years occur persistently as projected by climatic models for the region (Llebot, 2005). We also expect the invasion of the lowland by new species (annuals and woody species) more adapted to such extreme conditions, as suggested by the work of de Bello *et al.* (2005, 2006, 2007).

## 2.6 Conclusions

Overall, we found that a short-term warming enhanced productivity and reduced diversity significantly. A change in vegetation composition, manifested by a shift in dominance towards acquisitive fast-growing species, was also observed. Our results also advocate that a higher diversity did not contribute to a greater stability of the community under stress; instead plant functional traits, particularly high SLA, were responsible for

the stability of the vegetation to increased temperature. Also, the changes in CWM of certain traits (% rhizomes, growth form, and start of first flowering) seem to be the main drivers of increased biomass production under climate warming. The relationship between productivity and species richness remained positive within both the highland and the lowland. We postulate that seasonal climate change strongly affects functional traits and diversity. On the long term, however, knowledge of sensitivity of grasslands to climate change is scant and thus more experiments over longer periods are needed. Particularly, complementary observational studies and reciprocal transplanting (from the lowland back to the highland) could be useful tools to better understand the observed patterns. Also, we suggest that further studies should address the recovery of the vegetation after a short period of climate warming, in terms of functional diversity and plant functional traits.

## Acknowledgments

We would like to thank all the people who helped in the experimental set up and sampling. We would also like to thank Dr. Jan Lepš for suggestions on data analyses. This work was carried out in the framework of the projects CAPAS (CGL2010-22378-C03-01), and BIOGEI (CGL2013-49142-C2-1-R), funded by the Spanish Science Foundation. HD was also awarded a FPI short-term visit to the Czech Academy of Sciences (BES-2011-047009) by the Spanish Ministry of Economy and Competitiveness.

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## 2.8 Supporting information

### Supplementary tables

**Table S2.1** | Correlation coefficients between the CWM of traits: SLA, LDMC, height, start of first flowering, % prostrate, % rhizomes.

	SLA	LDMC	Start of first flowering	Height	% prostrate	% rhizomes
SLA	1.000	0.492	-0.627	0.007	-0.043	0.446
LDMC	0.492	1.000	-0.015	0.192	0.097	0.099
Start of first flowering	-0.627	-0.015	1.000	0.018	0.124	-0.365
Height	0.007	0.192	0.018	1.000	0.008	0.062
% prostrate	-0.043	0.097	0.124	0.008	1.000	-0.463
% rhizomes	0.446	0.099	-0.365	0.062	-0.463	1.000

**Table S2.2** | Correlation coefficients between the diversity indices: species richness SR, Simpson's diversity index, and functional diversity FD.

	SR	Simpson	FD
SR	1.000	0.864	0.298
Simpson	0.864	1.000	0.322
FD	0.298	0.322	1.000

**Table S2.3** | Results of multiple regression models to assess the effect of the initial plant functional traits (SLA, LDMC, height, start of first flowering, % prostrate, % rhizomes) at the beginning of the experiment on vegetation composition (Bray-Curtis dissimilarity), with transplant (highland and lowland) and site (p1 and p2 sites). See Fig. 3 for a graphical representation.

	Bray-Curtis					
	Model	R <sup>2</sup> <sub>adj.</sub>	Estimate	Std. Error	t value	P
Site	***a	0.625	-0.01	0.02	-0.74	0.464
Transplant			-0.54	0.21	-2.52	0.015
Initial start of first flowering			0.13	0.09	1.46	0.150
Initial height			-0.007	0.004	-1.67	0.101
Initial SLA			-0.02	0.02	-1.56	0.124
Transplant: initial SLA			0.02	0.01	1.81	<b>0.076<sup>b</sup></b>

<sup>a</sup> \*\*\*P < 0.001

<sup>b</sup> P-values in bold indicate a tendency to a significant relationship.

**Table S2.4** | Results of stepwise regression model to assess the effect of the most significant CWM traits and the most significant diversity indices (according to the previous multiple regression models) on biomass, in the lowland and the highland.

Above-ground biomass						
	Model	R <sup>2</sup> adj.	Estimate	Std. Error	t value	P
<b>Lowland</b>						
Intercept	***a	0.87	-1256.96	796.88	-1.58	0.146
Height			44.85	8.37	5.36	<b>&lt;0.001<sup>b</sup></b>
% rhizomes			-1091.43	297.06	-3.67	<b>&lt;0.01</b>
FD			972.13	540.35	1.80	0.102
<b>Highland</b>						
Intercept	(*)	0.19	119.10	36.41	3.27	<b>&lt;0.01</b>
% rhizomes			136.38	67.66	2.02	0.067

a \*\*\*P < 0.001, (\*) < 0.1

b P-values in bold indicate significant relationships.

**Table S2.5** | Results of stepwise regression model to assess the effect of the CWM traits and diversity indices on the change in vegetation composition (Bray-Curtis dissimilarity) in the lowland.

Bray-Curtis						
	Model	R <sup>2</sup> adj.	Estimate	Std. Error	t value	P
Intercept	*a	0.18	1.95	0.51	3.84	<b>&lt;0.001<sup>b</sup></b>
Initial SLA			-0.06	0.02	-2.78	<b>&lt;0.01</b>
Initial height			-0.01	0.01	-1.56	0.130

a \*P < 0.05

b P-values in bold indicate significant relationships.





# Chapter 3



# Plant guild effects on soil function change along a climatic gradient



An edited version of this chapter is in preparation for submission to *Plant and Soil*.

Debouk H., San Emeterio L., Mari, T., Canals, R.M., Sebastià, M-T. 2017.

## 3.1 Abstract

**G**lobal change modifies plant community composition in mountain grasslands through shifts in the balance of plant functional types (PFTs). Above- and below-ground systems are tightly linked, and changes in plant community composition and structure can be accompanied by changes in soil function and structure. For a thorough understanding of this link, we carried out a study to evaluate the relative importance of environmental variables (regional climate and management, and local soil conditions) and plant diversity (PFT composition and interactions) on soil activity. We sampled soil and vegetation of six sites along a climatic gradient in the Northern Iberian Peninsula. In each site, we sampled aboveground biomass from patches of the dominant plant functional types (PFT) in the site, -grasses, legumes and non-legume forbs-, with three replicates per PFT. We also measured soil chemical variables and the size and activity of soil microbial populations. We performed Variation Partitioning (VP) analysis on soil activity and fertility variables (urease, phosphatase, glucosidase, microbial biomass, dissolved organic carbon (C) and nitrogen (N), nitrate, ammonium, total N, and potential nitrification); and tested simple effects between two groups of variables: a) environmental variables including regional climatic and management, and local soil conditions, and c) local plant diversity variables. Our results show that total variability of soil activity and fertility were best explained by environmental variables (73%), followed by the combined effects of environmental and diversity variables (23.5%). The unique effects of diversity were, however, small (3.4%) compared to the interaction effects, thus suggesting that diversity effects cannot be separated from climatic effects on soil activity and fertility. Among the environmental variables, soil moisture and sheep grazing were the best predictors. PFT evenness and pairwise interactions between PFTs enhanced microbial biomass nitrogen (MBN), dissolved organic nitrogen (DON), total N, urease and phosphatase, and nitrification potential. Overall, our findings suggest that PFT composition affects soil activity and that these effects are mediated by regional and local environmental variables.

## 3.2 Introduction

**G**rasslands and savannas occupy more than 40% of the Earth's surface (Chapin *et al.*, 2001), and provide a wide range of ecosystem services: soil carbon sequestration, source of livestock feed, preservation and enhancement of water quality, soil protection, species conservation, etc. Grasslands are also among the most endangered ecosystems in the world (Noss, 2013), considering that they are vulnerable to global change manifested by land use changes (intensification or abandonment) and climate change (warming and/or drought). Global change alters plant community composition of grasslands (Aguiar, 2005; Fry *et al.*, 2013; Isselin-Nondedeu & Spiegelberger, 2014). Particularly, as a response to climate warming, the balance of forbs and sedges in mountain grassland ecosystems shift (Sebastià, 2007), and an increasing shrub encroachment is observed as a result of both climate change (Sanz-Elorza *et al.*, 2003) and grazing abandonment (Casasús *et al.*, 2007). Plants exert a strong influence on below-ground system because they determine the quantity and quality of the residues returned to the soil (Wardle *et al.*, 2004). Thus, changes in plant community structure and composition imply changes in soil function and structure (Kardol *et al.*, 2010; Cregger *et al.*, 2012; Thakur & Eisenhauer, 2015).

**P**lant functional types (PFTs) have proved to be a useful tool for predicting soil processes related to carbon (C), nitrogen (N), and phosphorus (P) cycles (Ostle *et al.*, 2009; Wang & Moore, 2014; Wullschleger *et al.*, 2014). Also, a number of diversity models have been used to investigate the diversity effects on ecosystem processes (Tilman *et al.*, 1997; Loreau M. *et al.*, 2001; Kirwan *et al.*, 2007, 2009; Connolly *et al.*, 2013). These models provide us with a deeper understanding of the mechanisms behind the effects of plant diversity on soil processes and the feedbacks of plant-soil interactions. While several studies suggest that diversity effects on soil processes are driven by climate (Neilson *et al.*, 2005; Pold & DeAngelis, 2013; Classen *et al.*, 2015), the relative contribution of the regional and local environmental conditions, and the local PFT composition on soil activity remain unclear.

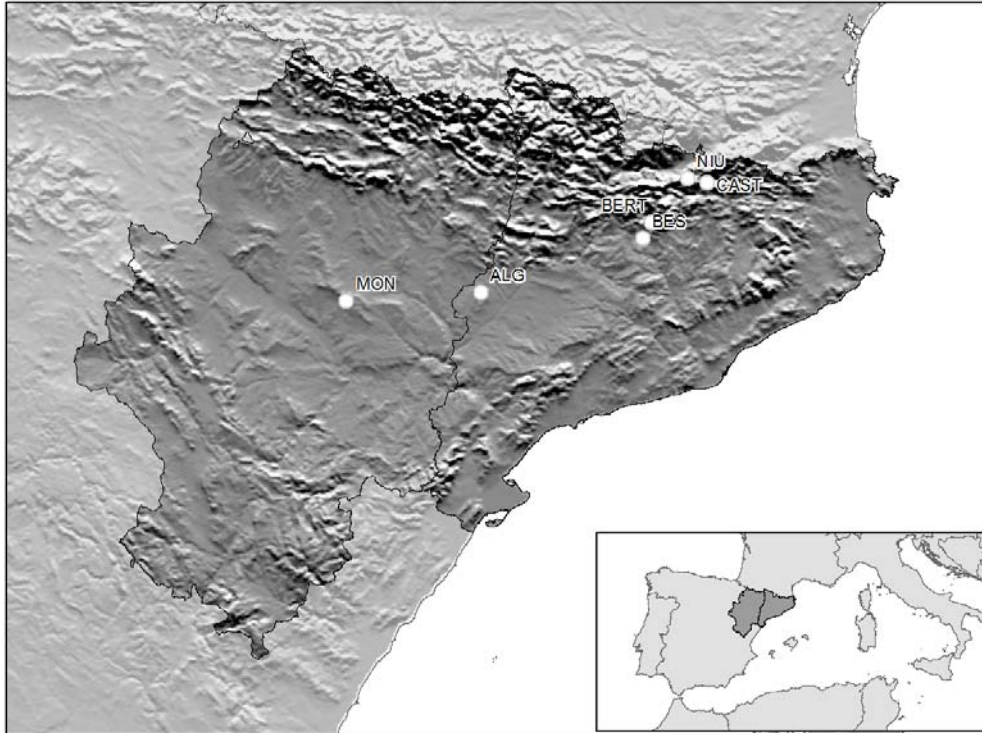
**F**or a thorough understanding of the effects of climate change on plant-soil interactions, we investigated: 1) The effects of PFT composition, including identity effects, pairwise interactions, and evenness, on soil activity and fertility in grasslands; 2) if environmental variables (regional and local) modify the relationship between PFTs and soil activity and fertility. The ideal place to study the diversity-climate effects on ecosystem processes are mountain ecosystems because they can offer a climatic gradient within a relatively small

space. Therefore, we sampled soil and vegetation from six grassland sites along a climatic and altitudinal gradient (334 to 2479 m a.s.l.) in the Northern Iberian Peninsula; the sampled patches representing the dominant plant functional types (PFTs) in the site.

## 3.3 Material and Methods

### 3.3.1 Site description

The six study sites are located in the Northern Iberian Peninsula along a climatic gradient (Fig. 3.1), ranging from arid up to semi-natural subalpine grasslands with a low-intensity management of extensive seasonal grazing. The six locations have different climatic conditions, varying from warm continental in the low-altitude sites, to cold temperate conditions in the high-altitude sites. The main climatic and management characteristics of the sites Monegrillo (MON), Alguaire (ALG), Besora (BES), La Bertolina (BERT), Castell de n'Hug (CAST), and Niu de l'Àliga (NIU) are summarized in Table 3.1. The climatic data were obtained from local climatic atlases (Pons, 1996; Ninyerola *et al.*, 2000, 2003, 2005). The vegetation in all the sites is dominated by grasses and forbs including legumes and non-legumes. In MON and ALG, however, legumes were not dominant. ALG and MON are dominated by *Plantago lanceolata* L. and *Papaver rhoeas* L. BES is dominated by *Plantago lanceolata* L. and *Bromus hordeaceus* L. BERT is dominated by *Festuca arundinacea* Schreb. and *Trifolium repens* L. CAST is dominated by *Festuca rubra* L. and *Carex caryophylla* Latourr. NIU is dominated by *Festuca nigrescens* L. and *Carex caryophylla* Latourr.



**Fig. 3.1** | Map showing the location of the six study sites: MON (Monegrillo), ALG (Alguaire), BES (Besora), BERT (La Bertolina), CAST (Castellar de n'Hug), and NIU (Niu de l'Àliga).

### 3.3.2 Experimental design

The sampling was carried out at the peak of the vegetation biomass – maximum live biomass – in 2014 for the six study sites. The sampling points were spread over a grid on the grassland, covering an area of about 50-70 m radius. We chose the sampling points to represent the typically dominant plant functional types (PFT) in these grasslands, -grasses, legumes, and non-legume forbs-, with three replicates per PFT. In total, we sampled nine points in each study site by placing collars (height = 8 cm; diameter = 25 cm) 3 cm deep into the ground. In ALG and MON, we placed a total of six collars (grasses and forbs) because legumes were not dominant at the peak of the vegetation. To harvest aboveground biomass, we cut the vegetation inside each collar at ground level. For soil analyses, we extracted soil cores of approximately 250 g from the upper soil layer (0-10 cm), without the litter, in each collar. We placed all collected samples in a dark cooling portable refrigerator to preserve them until arrival to the laboratory.

**Table 3.1** | General climatic and management characterization of the six study sites.

Site	Latitude	Longitude	Altitude (m)	MAP (mm)	MAT (°C)	Lang's index	MST (°C)	MSP (mm)	MTmin (°C)	MTmax (°C)	Stocking rate (LSU ha <sup>-1</sup> )	Livestock species
ALG	41°43'46,60"	0°31'30"	334	421.4	13.9	30.32	22.8	95.7	0	28	15.4	Sheep
MON	41°39'45,62"	-1°37'22,15"	657	225.2	12.6	17.87	23	93.8	1	28	0.35	Sheep
BES	42°1'13,07"	1°36'23,48"	712	720.5	11.7	61.58	20	237.5	0	25	0.81	Cattle
BERT	42°5'57,33"	1°39'48,63"	1276	954.8	8.7	109.75	18.2	465.2	-1	24	0.44	Cattle
CAST	42°18'18,84"	2°1'57,58"	1850	1199	5.4	222.04	15.3	319.1	-5	23	0.74	Cattle, sheep
NIU	42°19'18,91"	1°54'4,47"	2479	1302	2.4	542.5	13.8	278.8	-7	22	0.31	Cattle, horses

MAP = mean annual precipitation; MAT = mean annual temperature; Lang's rain factor index (1920); MST = mean summer temperature, MSP = mean summer precipitation; MTmin = mean annual minimum temperature; MTmax = mean annual maximum temperature.

### 3.3.3 Vegetation and soil analyses

Once in the laboratory, we separated the vegetation samples into plant functional types, and into live/standing dead/litter biomass. We placed the samples in the oven at 60°C for 48 hours to obtain the dry weight of each PFT, and we calculated afterwards the proportion of each PFT per site.

Ammonium and nitrate pools were determined in 2M KCl extractions by a segmented flow analyzer AA3 (Braun+Luebbe, Norderstedt, Germany) and microbial biomass C and N (MBC and MBN), dissolved organic nitrogen (DON) and nitrification potential as described in San Emeterio *et al.* (2014). Soil enzyme activities were determined in homogenized and sieved (2 mm) soils. We measured  $\beta$ -glucosidase and acid phosphatase activities using a 96-well microplate approach (Popova & Deng, 2010; Tian *et al.*, 2010), based on p-nitrophenol release after breaking up a synthetic substrate (p-nitrophenyl glucoside and p-nitrophenyl phosphate, respectively) and following San Emeterio *et al.* (2016). We measured urease activity following the method by Kandeler & Gerber (1988), and modified by Rodríguez-Loinaz *et al.* (2008).

### 3.3.4 Data analysis

#### 3.3.4.1 Gradient analysis and variation partitioning

In order to investigate the effect of environmental and diversity variables, we performed direct gradient analysis using Redundancy Analysis (RDA) on soil activity and fertility variables: total nitrogen, nitrate, ammonium, nitrification potential, urease, phosphatase, glucosidase, microbial biomass nitrogen (N), microbial biomass carbon (C), dissolved organic N, and dissolved organic C. We also applied Variation Partitioning (VP) analysis, and calculated the proportion of variability in soil fertility and activity explained by each set of variables separately and their shared explained variation. We tested conditional and simple term effects on two groups of environmental and diversity variables with forward selection of the members of each group and associated Monte Carlo tests of significance. We also generated adjusted P-values estimates calculated by using the false discovery rate (FDR) method for protection against Type I error inflation. We used CANOCO 5 for all the analyses (ter Braak & Šmilauer, 2012). The explanatory sets initially included all the variables recorded in the study: a) environmental (regional climatic and management, and local soil conditions) variables: mean annual temperature (MAT), mean annual precipitation (MAP), mean annual minimum temperature (MTmin), mean annual maximum temperature (MTmax), mean summer temperature (MST), mean summer precipitation (MSP), continentality index of Sebastià (CIS = MST – MAT), sheep grazing, and cattle grazing (the latter including also horses in the alpine site Niu de l'Àliga), and pH and moisture determined for each

sampled soil; b) plant diversity variables: PFT evenness, biomass proportion of grass, legume and non-legume forb, and the pairwise interactions between the three plant guilds. We also tested the interaction between sheep and cattle grazing, but it was not significant and thus was not included in later analyses. Forward selection of the explanatory variables and significance values calculated using Monte-Carlo permutation tests in a preliminary RDA also advised the reduction of environmental variables to a smaller set of six variables: MTmin, CIS, cattle grazing and sheep grazing, and sample pH and moisture. The same procedure suggested the removal of evenness from the plant diversity variables, as species interactions had higher explanatory power. This final RDA model including six environmental and six plant diversity variables explained 81.4% of the adjusted variation in soil activity and fertility.

#### 3.3.4.2 Diversity-interaction (DI) models

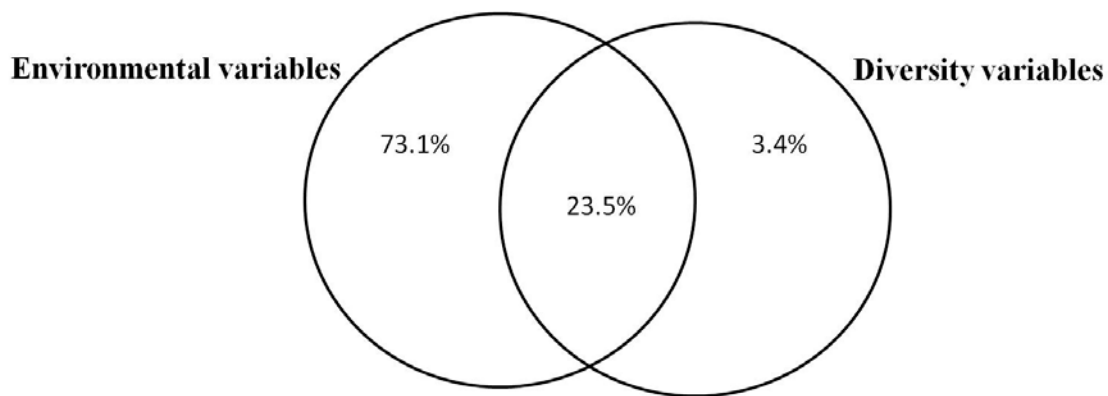
In order to disentangle the effects of environmental and plant diversity variables on soil activity and fertility, we used linear models based on the diversity-interaction approach (Kirwan *et al.*, 2007, 2009). Our modeling framework included four models in total. The first one, the null model, included only environmental variables, and no diversity variables. The second model included environmental variables and the proportions of each plant functional type representing the identity effects. The third one included the environmental variables, the identity effects and all the pairwise interactions between plant functional types. The fourth and last model included the environmental variables, the identity effects and a diversity term, corresponding to evenness calculated as in Kirwan *et al.* (2007). We chose the best fitted model using F tests and the AIC criterion information. Once the diversity model was selected, we performed a backwards stepwise regression to select the regional variables. The interactions between MTmin and diversity variables were also included in the models. DI models were carried out using R software (R Core team, 2013). We also built contour plots to represent with a triangle the soil activity and fertility functions in relation to the relative proportions of PFTs (forbs, grasses, legumes) which sum to a total value of 1. The contour plots were built using the packages: “rsm” (Lenth, 2009), “lattice” (Sarkar, 2008), “lme4” (Bates *et al.*, 2012), “arm” (Gelman & Su, 2013), and “RcolorBrewer” (Neuwirth, 2011) of R software (R Core team, 2013).



## 3.4 Results

### 3.4.1 Relative importance of environmental variables and diversity on soil activity and fertility

**V**ariation partitioning showed that the highest variability was explained by the environmental variables (73.1% of all variation), followed by the overlap among the environmental and diversity sets (23.5%). Unique effects of diversity (3.4%) were small compared with the shared variation (Fig. 3.2).



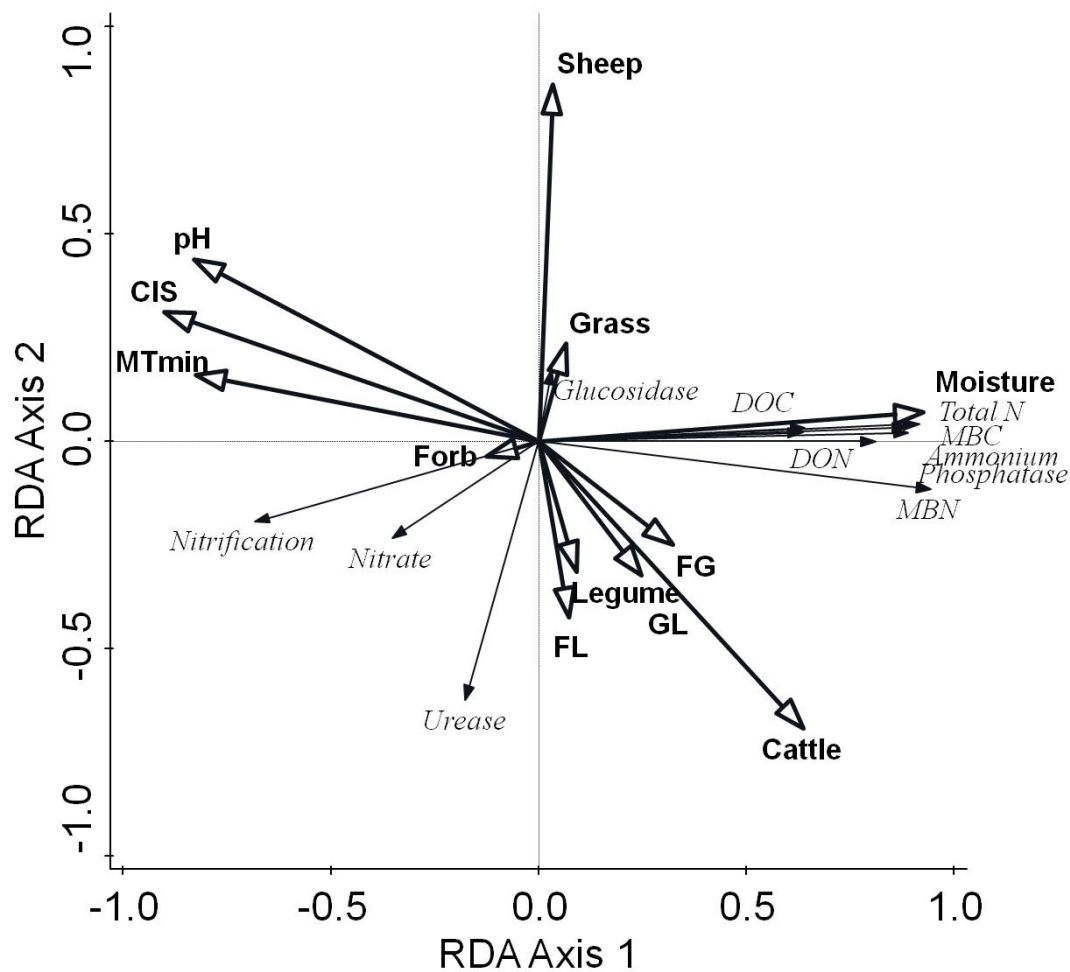
**Fig. 3.2** | Distribution of the explained variation of the two sets of variables. Environmental variables (regional and local climate and management variables): mean minimum temperature (MTmin), continentality index of Sebastià (CIS = mean summer temperature – mean annual temperature), pH, moisture, sheep, cattle. Plant diversity variables: Forb, Grass, Legume, FG, FL, GL.

**R**edundancy analysis (RDA) on soil fertility and activity variables including the two groups of predictors (environmental and plant guild diversity variables) showed that environmental variables accounted for 84.75% of the total variability of soil activity, of which soil moisture was the best predictor (added 73% to explanatory power), followed by pH and sheep grazing (Table 3.2). Diversity variables accounted for only 0.5% of the variability of soil activity and fertility, of which the pairwise interaction between forbs and grasses was the first variable selected by the forward procedure, and added 4.9% to the overall explanatory power. Guild pairwise interactions were more explanatory than the main effects (Table 3.2). From those, the effects of grasses were the most relevant, followed by the interaction between legumes and non-legume forbs (Table 3.2).

**Table 3.2** | Conditional and simple term effects of the RDA analyses. The % explained shows the percentage of variability explained by each variable. The explanatory variables include environmental and diversity variables: FG = pairwise interactions of forbs and grasses; FL = pairwise interactions of forbs and legumes; GL = pairwise interactions of grasses and legumes; MTmin = minimum air temperature.

Variable	Conditional term effects			Simple term effects		
	% explained	F	$P_{adj}$	% explained	F	$P_{adj}$
Moisture	73	124	0.001	73	124	<0.001
FG	4.9	10	<0.01	8.9	4.5	0.08
pH	2.8	6.4	<0.05	58.5	65	<0.001
Sheep	2	4.9	0.06	0.5	0.2	n.s.
FL	0.6	1.5	n.s.	0.6	0.3	n.s.
Forb	0.7	1.7	n.s.	1.4	0.6	n.s.
CIS	0.4	1	n.s.	69.3	104	<0.001
GL	0.2	0.6	n.s.	5.2	2.5	n.s.
Grass	0.8	2.1	n.s.	0.4	0.2	n.s.
MTmin	0.3	0.7	n.s.	57.9	63.2	<0.001
Legume	0.2	0.4	n.s.	0.8	0.4	n.s.
Cattle	0.1	0.3	n.s.	34.6	24.3	<0.001

The first axis of the RDA showed that on one hand total N, ammonium, microbial biomass, dissolved organic C and N, and phosphatase activity increased with soil moisture, but decreased with temperature (MTmin and CIS) and pH (Fig. 3.3). On the other hand, nitrate and nitrification potential decreased with soil moisture. Overall, the RDA axis 1 roughly corresponds to a climatic gradient, where pH increases in the same direction as temperature and temperature seasonal difference (negative side of RDA axis 1), and moisture increases in the opposite direction, towards the cold and moist areas (positive side of RDA axis 1; Fig. 3.3). The second RDA axis showed that glucosidase activity increased with sheep grazing and grass proportion (positive side of RDA axis 2), whereas urease activity increased with legume proportion and the pairwise interaction between forbs and legumes (Fig. 3.3).



**Fig. 3.3** | Biplot axis 1 and axis 2 of the RDA. In bold, the explanatory variables including: environmental variables (regional climate and management, and local soil condition), and diversity variables. In italics are the soil fertility and activity response variables. See Fig. S3.1 in Supporting information for the mean values of soil activity and fertility parameters along the climatic gradient.

### 3.4.2 Diversity effects on soil activity and fertility

**A**mmonium, nitrate, pH, dissolve organic carbon (DOC) and glucosidase were not related to any plant diversity parameters. Microbial biomass nitrogen (MBN), dissolved organic nitrogen (DON) and phosphatase were best described by including all the pairwise interactions between PFT to describe the diversity effects (Table 3.3). For soil activity variables which are particularly related to the nitrogen cycle (total N, urease, and nitrification potential), the diversity effects were explained by the average interaction coefficient, known as evenness ( $E_k$ ). For instance, urease activity and soil total N significantly increased with plant function type evenness ( $P = 0.0095$  for urease activity;  $P = 0.025$  for soil total N). Nitrification potential showed a tendency to increase with increased evenness ( $P = 0.06$ ). Total nitrogen in relatively even legume-forb mixtures was higher than in other PFT combinations, and decreased when grass pro-

portion increased (Fig. 3.4). In fact, total N showed generally higher values near the centroids of the ternary plots compared to main PFT identity effects (Fig. 3.4).

**Table 3.3** | Diversity-interaction models for soil activity and fertility parameters

Soil parameter	Model	Adjusted $r^2$
<i>Model 1. Only environmental variables included</i>		
Nitrate	MTmax <sup>+</sup> + Sheep <sup>***</sup> + Cattle <sup>**</sup>	0.38
Ammonium	MTmax <sup>***</sup> + MAP <sup>+</sup> + Sheep <sup>***</sup> + Cattle	0.84
DOC	MTmax <sup>**</sup> + MTmin <sup>***</sup> + MAP <sup>**</sup> + Sheep <sup>***</sup> + Cattle <sup>***</sup>	0.79
MBC	MTmax <sup>***</sup> + MTmin <sup>*</sup> + MAP <sup>+</sup> + Sheep <sup>***</sup>	0.75
Glucosidase	MTmax <sup>+</sup> + MTmin <sup>+</sup> + MAP <sup>+</sup> + Cattle <sup>-</sup>	0.17
<i>Model 3. Environmental variables + PFT identity effects + PFT pairwise interactions</i>		
MBN	MTmin <sup>***</sup> + MTmax <sup>***</sup> + MAP <sup>**</sup> + Sheep <sup>***</sup> + G + F + L + GF <sup>+</sup> + GL + FL + MTmin:G <sup>+</sup> + MTmin:F <sup>+</sup> + MTmin:L	0.88
DON	MTmin <sup>***</sup> + MST <sup>**</sup> + Sheep <sup>***</sup> + G + F <sup>+</sup> + L + GF + GL <sup>**</sup> + FL	0.60
Phosphatase	MTmin <sup>***</sup> + Sheep <sup>**</sup> + G + F <sup>+</sup> + L + GF <sup>***</sup> + GL + FL	0.63
<i>Model 4. Environmental variables + PFT identity effects + evenness</i>		
Total N	MTmin <sup>***</sup> + MST <sup>**</sup> + Sheep <sup>***</sup> + G + F + L + E <sub>k</sub> <sup>*</sup>	0.84
Urease	MTmin <sup>**</sup> + MTmax <sup>***</sup> + MST <sup>+</sup> + Sheep <sup>***</sup> + Cattle <sup>***</sup> + G + F <sup>**</sup> + L + E <sub>k</sub> <sup>**</sup>	0.79
Nitrification Potential	MTmin <sup>***</sup> + MTmax <sup>***</sup> + Sheep <sup>***</sup> + G <sup>+</sup> + F + L + E <sub>k</sub> <sup>+</sup>	0.78

\*\*\* P<0.001; \*\* P<0.01; \* P<0.05; + 0.05<P<0.08.

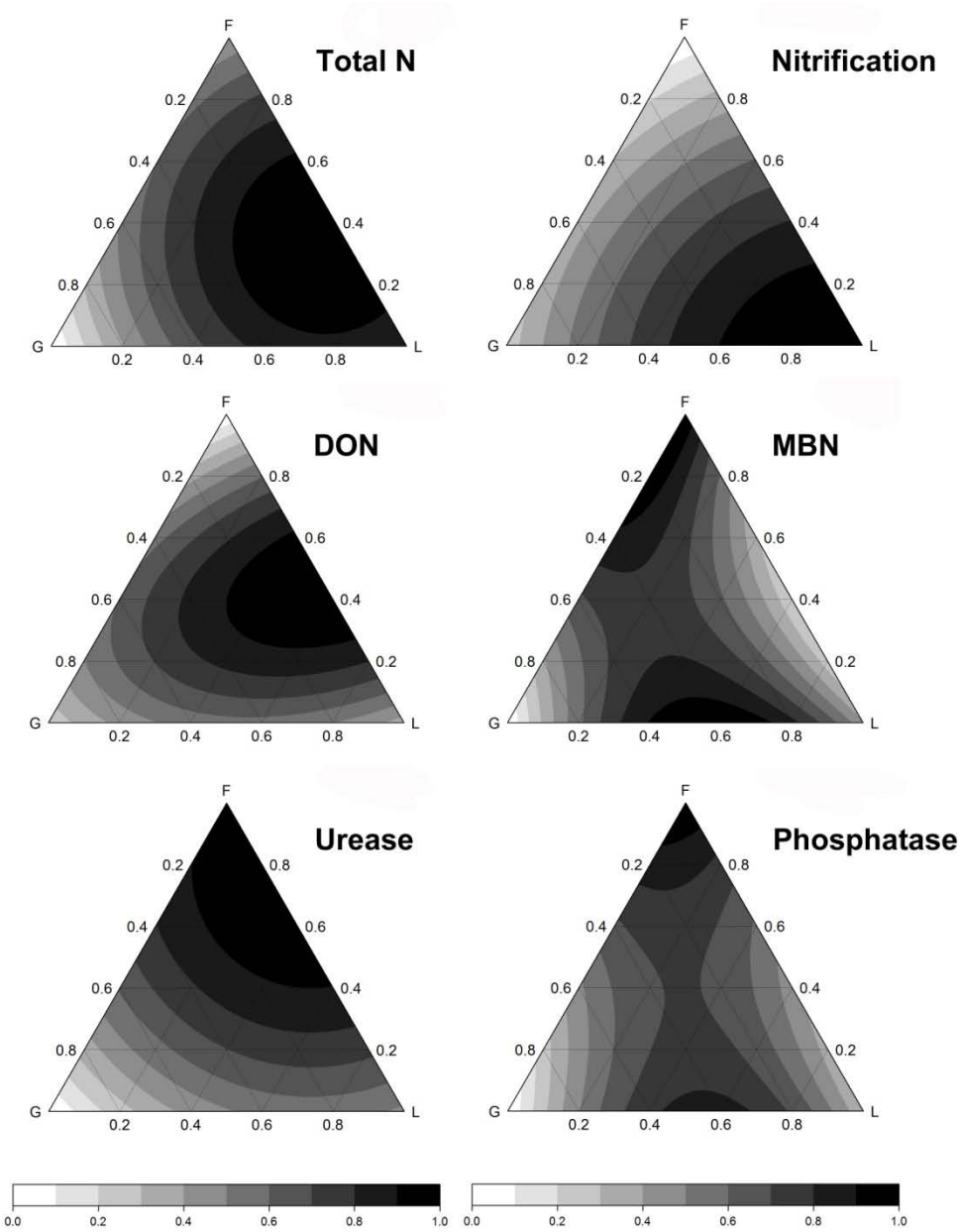
**M**icrobial biomass nitrogen was the only soil parameter showing changes in diversity effects in interaction with climatic variables (Table 3.3). Diversity effects on MBN changed with mean minimum temperature (MTmin). At lowest minimum temperatures, microbial biomass N was higher under forb-dominated patches than under grass- or legume-dominated patches (Figure 3.5).

## 3.5 Discussion

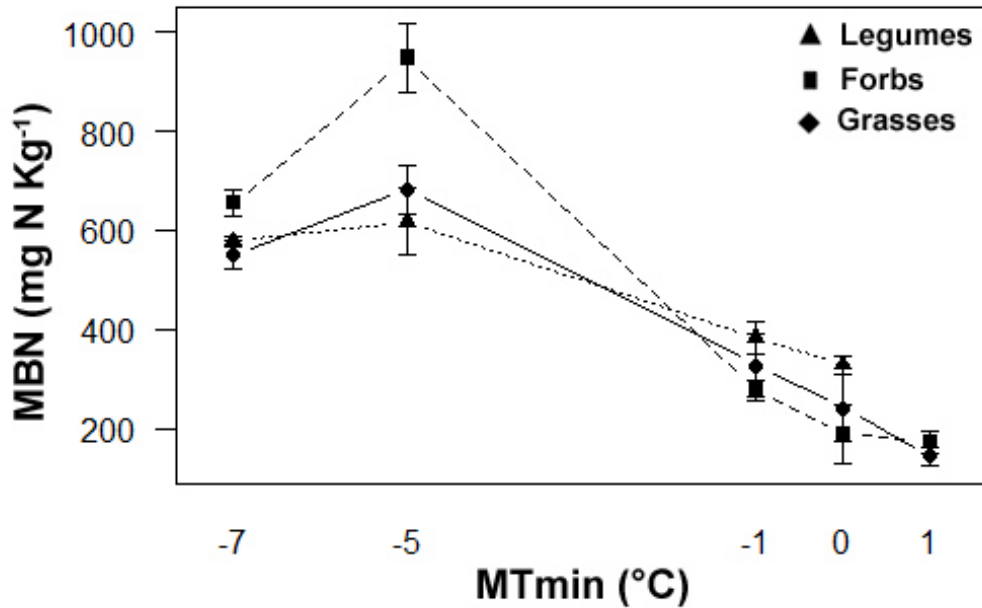
### 3.4.1 Best predictors of soil activity and fertility

**C**limate change affects ecosystem processes directly or indirectly (Classen *et al.*, 2015); indirect effects being defined as those mediated by shifts in diversity, community composition and functional traits. Classen *et al.* (2015) hypothesized that indirect effects of climate change on microbes mediated through plants may be stronger than direct effects of climate on shaping microbial community composition and function. Our results however show that soil activity and fertility were best explained by environmental variables, including regional climatic and management, and local soil conditions. The combined effects of environmental and diversity

variables seemed to have also a strong regulating power on soil functions, and the unique effects of diversity were small compared to the shared variation (Fig. 3.2). Our results also highlight the relative importance of management (grazer type) as a driver of soil activity, and consequently its relevant role to attenuate global change effects on soil activity. In the following sections, we will discuss in detail the effects of regional and local environmental conditions, and diversity on a number of soil activity and fertility indices in grasslands.



**Fig. 3.4 |** Contour plots representing the variations of soil activity and fertility functions in relation to the relative proportions of the plant functional types (F = forb; L = legume; G = grass) on soil parameters: total nitrogen (total N), nitrification potential, dissolved organic nitrogen (DON), microbial biomass nitrogen (MBN), urease and phosphatase activities. Contour plots are normalized and scaled from 0, representing the minimum value plotted, to a maximum of 1. Color intensity shows a gradient from the minimum (white) to the maximum (black) soil activity/fertility.



**Fig. 3.5** | Interaction plot between MTmin and plant functional types (forbs, grasses, legumes) on microbial biomass N. Means  $\pm$  standard error represented. Dashed lines, forb-dominated patches; continuous lines, grass-dominated patches; dotted lines, legume-dominated patches.

**S**oil moisture, temperature and pH are known to be main drivers of soil processes (Fierer & Jackson, 2006; Castro *et al.*, 2010; Bru *et al.*, 2011; Husson, 2013; Jiao *et al.*, 2016). Indeed, our results showed that soil moisture enhanced total N, ammonium, microbial biomass, dissolved organic C and N, and phosphatase activity, while temperature and pH reduced them (Fig. 3.3). The positive effect of soil moisture on microbial biomass and total N was also observed by Yang *et al.* (2016) in shrublands and meadows, and is attributed to enhanced microbial activity and nitrogen mineralization by increased soil water content (Paul *et al.*, 2003; Garten *et al.*, 2009), which consequently increases soil total nitrogen. As for ammonium, its positive correlation with soil moisture, as observed by Mueller *et al.* (2013), can be attributed to the positive impact of soil moisture on organic matter mineralization (Zhalnina *et al.*, 2012), which is converted to inorganic forms of nitrogen such as ammonium (Troy & Tang, 2011). Our findings on phosphatase activity also agree with previous studies advocating that soil moisture is a key factor regulating P in the soil (Grierson & Adams, 2000; Sardans *et al.*, 2007; Shen *et al.*, 2011), and controlling microbial activity (Leirós *et al.*, 1999; Sardans *et al.*, 2007). Higher temperatures generally increase microbial and enzyme activity, as well as mineralization in an ecosystem; however our results showed the opposite. This is most likely attributed to the fact that the warmest sites in our study (mainly the semi-arid) are characterized by dry soil conditions – limiting conditions that can inhibit the positive effect of temperature on mineralization, microbial biomass, and soil phosphatase activity (Sardans & Peñuelas, 2005; Castro *et al.*, 2010). Additionally, the temperature gradient of the study sites is associated with a pH gradient, where the warm semi-arid sites are alkaline compared to the acidic cold subalpine and alpine sites. This

may explain the increase of pH in the same direction as temperature in our results (Fig. 3.3). Similarly to other soil activity and fertility indices, dissolved organic carbon (DOC) and nitrogen (DON) production are also suggested to be enhanced by high temperatures when soil moisture is not limiting (Andersson *et al.*, 2000; Fröberg *et al.*, 2006). High temperatures favor plant growth and litter production while soil moisture enhances and supports DOC and DON transport in the soil horizons (Solinger *et al.*, 2001; Kaiser *et al.*, 2002; Kalbitz *et al.*, 2003). Therefore, the combination of high temperature with limiting soil moisture conditions in the semi-arid grassland sites may reduce or even inhibit DOC and DON production.

**N**itrate and nitrification potential were the only soil activity and fertility indices which decreased with soil moisture (Fig. 3.3). Among environmental drivers, soil moisture and temperature are regarded as the most important factors for nitrification processes, and nitrate uptake and concentrations (Haynes, 1986; Emmett *et al.*, 2004; Butterbach-Bahl *et al.*, 2013). Therefore, this decreasing trend of nitrification potential and nitrate with higher soil moisture levels cannot be separated from temperature, especially that sites with high soil moisture (sub-alpine and alpine) are characterized by low temperatures which would limit these soil processes. Overall, our results highlight the importance of the interplay between temperature and soil moisture, and stress that the effect of each factor cannot be separated from the other when investigating soil activity processes.

**S**oil enzyme activities, such as glucosidase and urease, are indicators of soil organic matter quality (Caldwell, 2005; Burns *et al.*, 2013) which control the availability of nutrients such as carbon, nitrogen and phosphorus, and the rate at which they are produced in the soil for microbial assimilation (Chróst, 1991; Burns & Dick, 2002; Gianfreda, 2015). Plants have a strong influence on enzyme activity because they regulate the quantity and quality of microbial biomass (Sardans *et al.*, 2008; Steinweg *et al.*, 2013). Grazers, on the other hand, exert strong effects on the play-soil system through their impacts on vegetation composition, soil microbial communities and enzyme activities (Prieto *et al.*, 2011; Olivera *et al.*, 2014). Indeed, our results showed the importance of grazers and plant functional types as main drivers of glucosidase and urease activities. In fact, glucosidase activity increased in grasslands grazed by sheep and dominated by grasses. Grass communities and sheep grazing seem to enhance plant litter quantity and quality, a main component of glucosidase activity to produce glucose (Tabatabai, 1994).

### 3.4.2 Diversity effects on soil activity and fertility

**B**eside climatic factors, soil activity and fertility are also affected by plant diversity (Niklaus *et al.*, 2006; Castro *et al.*, 2010), because plants determine the quantity and the quality of residues, soil organic matter, as well as soil structure (Wardle *et al.*, 2004). Indeed, diversity

effects, in addition to environmental effects, were observed on microbial biomass nitrogen (MBN), dissolved organic nitrogen (DON), total N, urease and phosphatase, and nitrification potential (Table 3.2 and Fig. 3.4). Several studies investigated the role of vegetation composition in soil microbial N and C. Some detected no change in MBN and MBC with functional group diversity (Wardle *et al.*, 2003; Niklaus *et al.*, 2006), while others suggest that PFT and particularly legumes with their important effect on total nitrogen play a great role in shaping soil microbial communities (Cui *et al.*, 2016). We found that the interaction between grasses and forbs enhanced MBN and phosphatase activity, but diversity had no effect on MBC (Table 3.3). However in an alternative model, the interaction between forbs and grasses increased MBC ( $P = 0.04$ ), and legumes in sites with lower MT<sub>min</sub> (alpine and subalpine sites) showed a tendency to increase MBC ( $P = 0.08$ ) compared to warmer sites. The increased MBN and MBC related to the interaction between forbs and grasses is most likely the result of the higher quantity and variety of plant-derived compounds available to microbial communities at higher diversity (Strecker *et al.*, 2015). Also among all the soil functions that we assessed, MBN was the only one affected by the interaction between climate and PFT. Particularly, mean minimum temperature with grasses and forbs favored MBN. This may be attributed to the high sensitivity of microbial biomass contents to changes in the climate and environmental conditions compared to soil enzyme activities (Fang *et al.*, 2013). Also, the increased plant productivity as a result of higher diversity can explain the observed increase in DON (Table 3.3), because diversity leads to higher soil organic matter and consequently increased organic N pools (Dijkstra *et al.*, 2007) and thus greater DON production. This pattern was particularly observed in grass-legume interactions. Grasses are suggested to cause higher microbial biomass compared to other PFTs, because of their dense root system and high root exudation rates (Eisenhauer *et al.*, 2010; Roscher *et al.*, 2012). Legumes, in turn, favor organic N sources compared to other PFTs, due to their effectiveness in transferring amino acids between nodules and roots (Moran-Zuloaga *et al.*, 2015), and to their facilitation of N to grasses (Nyfeler *et al.*, 2011; Suter *et al.*, 2015). This may explain why the interaction between legumes and grasses enhanced DON.

**D**iversity also enhanced total nitrogen, nitrification potential, and urease (Table 3.3) which are soil activity variables related mainly to the nitrogen cycle. Our findings are in line with previous studies suggesting that more even plant communities increase bacterial abundance, which consequently enhances the potential for bacterial nitrification (Lamb *et al.*, 2011). Also, this positive effect of diversity on total N and urease activity can be explained by the positive effect of diversity on productivity and nitrogen availability (Cardinale *et al.*, 2007; Kirwan *et al.*, 2007; Oelmann *et al.*, 2011; Niklaus *et al.*, 2016). In fact, increased community aboveground biomass as a result of increased diversity leads to higher litter and soil nutrient content which are closely associated with urease activity (Niklaus *et al.*, 2016; Shen *et al.*, 2016). The latter is a key



enzyme strongly linked to the processes of nitrogen transformation and availability in the soil (Liang *et al.*, 2003; Zhang *et al.*, 2014).

**T**he strong influence of PFT composition and interactions on soil activity and fertility suggest that any shifts in vegetation composition in grasslands, as a result of climate change, can have serious effects on the feedback of the soil to the atmosphere. This is due to the fact that greenhouse gas exchange processes mainly occur in the soil, and thus strongly depend on soil microbial biomass, carbon and nitrogen content and availability, and enzyme activities.

## 3.6 Conclusions

**O**verall, we found that total variability of soil activity and fertility were best explained by environmental variables, followed by the combined effects of environmental and diversity variables. The unique effects of diversity were, however, small compared to the interaction effects, thus suggesting that diversity effects cannot be separated from climatic effects on soil activity and fertility. Among the environmental variables, soil moisture and sheep grazing were the best predictors. This highlights the relative importance of management as a driver of soil activity, suggesting a relevant role of management to attenuate global change effects on soil activity.

**W**e also found that diversity (interactions between PFTs and evenness) strongly influence soil activity and fertility, and that these effects were mediated by environmental (regional and local) variables. In particular, diversity enhanced microbial biomass nitrogen (MBN), dissolved organic nitrogen (DON), total N, urease and phosphatase, and nitrification potential. These findings stress the importance of maintaining community stability in grasslands because any shifts in vegetation composition resulting from climate change can modify soil activity and fertility, and consequently the soil and belowground community feedback to the atmosphere.

## Acknowledgments

**T**his work was funded by the Spanish Science Foundation (FECYT) through the projects CAPAS (CGL2010-22378-C03-01) and BIOGEI (CGL2013-49142-C2-1-R). HD was supported by a FPI fellowship from the Spanish Ministry of Economy and Competitiveness (BES-2011-047009). We would like to thank all the people who helped in the experimental setup and sampling, particularly Helena Sarri.

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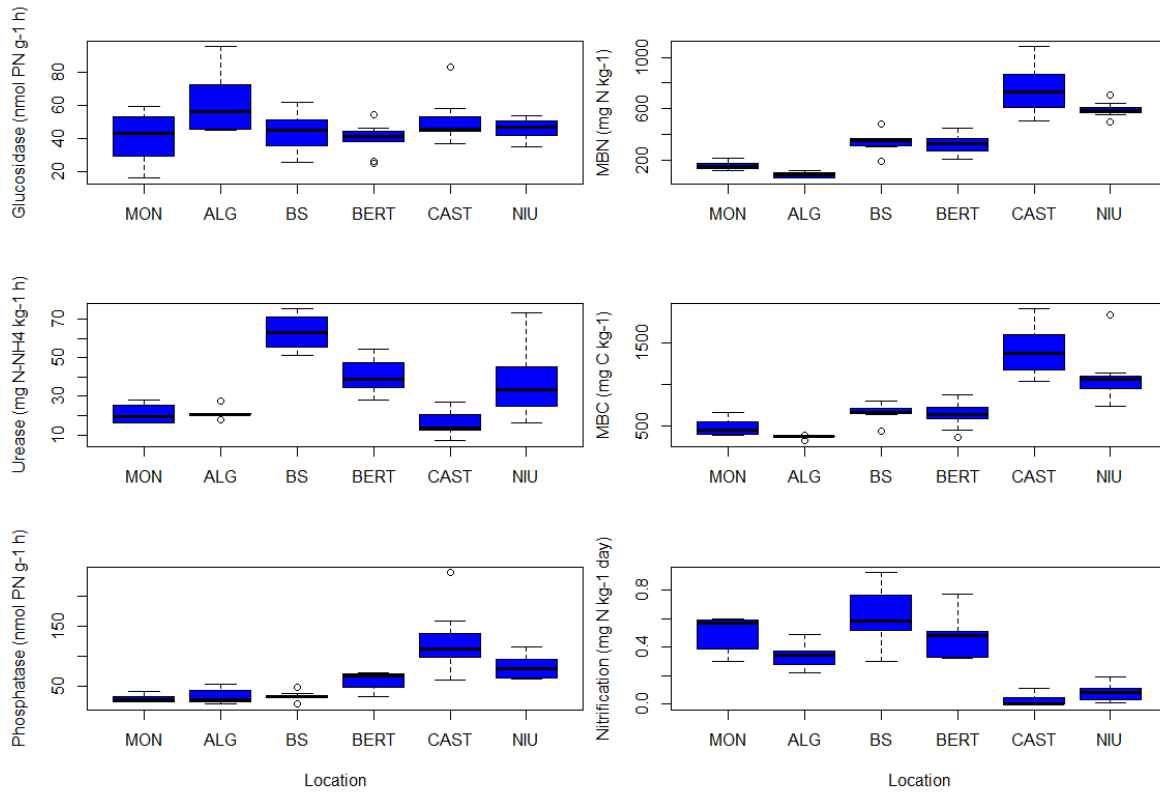
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## 3.8 Supporting information

### Supplementary figures



**Fig. S3.1** | Boxplots representing the variation in mean soil activity and fertility parameters along the climatic gradient, ordered from semi-arid to alpine grasslands: Monegrillo (MON), Alguaire (ALG), Besora (BS), La Bertolina (BERT), Castellar de n'Hug (CAST), Niu de l'Àliga (NIU).





# Chapter 4



# Greenhouse gas fluxes in semi-natural grasslands along an altitudinal gradient in the Eastern Pyrenees



An edited version of this chapter was submitted to *Agricultural and Forest Meteorology* in March 2017.

Debouk H., Altimir N., Ribas A., Sebastià, M-T. 2017.

## 4.1 Abstract

**M**ountain grasslands can be either sources or sinks of carbon dioxide (CO<sub>2</sub>) and other greenhouse gases (GHGs), depending on management and environmental conditions.

Despite the importance of extensively managed semi-natural grasslands as a traditional land-use form in the Pyrenees, data on their GHG emissions are scarce. This creates much uncertainty concerning their contribution to climate change-induced GHG emissions and climate change mitigation. Particularly the responses of methane (CH<sub>4</sub>) and nitrous oxide (N<sub>2</sub>O) are highly complex, hard to predict, and reports on their emission behavior remain controversial. We measured CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O fluxes with a photoacoustic field gas-monitor (INNOVA 1412, LumaSense Technologies) in four semi-natural pastures in the Pyrenees across an altitudinal gradient (1026 to 2436 m a.s.l.) to investigate their magnitude and range of variability, during two growth periods under light and dark conditions. To determine the role of the vegetation in CH<sub>4</sub> and N<sub>2</sub>O emissions, we measured the fluxes on intact vegetation and on bare soil after the vegetation had been harvested. Additionally, we investigated the main drivers of CH<sub>4</sub> and N<sub>2</sub>O fluxes. The four extensively managed semi-natural grasslands showed negative net ecosystem exchange (NEE) values mostly, suggesting small sinks of CO<sub>2</sub>, and were mainly sources of CH<sub>4</sub> and N<sub>2</sub>O. Only the subalpine and alpine grasslands were small sinks of N<sub>2</sub>O. We found that the GHG fluxes decrease with the altitude; the higher the grassland altitude the lower the fluxes. Additionally, the GHG fluxes changed along different seasons, with the highest GHG emissions occurring in the summer period. Aboveground biomass and temperature were the main drivers for all GHG fluxes in the Pyrenean grasslands. Additionally, CH<sub>4</sub> and N<sub>2</sub>O fluxes were highly influenced by grazing. Seasonality and soil water content had also a strong impact on N<sub>2</sub>O, varying however between sites according to their local conditions. Vegetation removal modified only fluxes of CH<sub>4</sub>, particularly in the subalpine and alpine sites; which raises the question about the role of plants as possible methane conduits. As for N<sub>2</sub>O, fluxes were higher under light conditions than under dark conditions in all sites. Therefore, we recommend further studies to unravel the exact role of vegetation and light on CH<sub>4</sub> and N<sub>2</sub>O fluxes, respectively.

## 4.2 Introduction

**C**limate change policy discussions generally underestimate the importance of mountain grasslands (European Commission, 2014), however these ecosystems - together with shrublands - store 60.5 to 82.8 billion metric tons of carbon in their biomass and soils (Ward *et al.*, 2014). Mountain ecosystems are also particularly sensitive to climate change because the seasonality of runoff in snow-melt dominated regions is directly, and at a faster rate, affected by warmer climate compared to other areas (IPCC, 2013). In fact, these ecosystems are witnessing an increase in the atmospheric carbon dioxide (CO<sub>2</sub>) concentration and other greenhouse gases, and a parallel increase in global mean surface temperatures. In the Pyrenees, these effects of warming have been shown to be particularly pronounced (López-Moreno, 2008; López-Moreno and Beniston, 2009). Assessing greenhouse gases (GHG) exchange in mountain grasslands, particularly in the Pyrenees, is crucial since these ecosystems can act as both sinks and sources of carbon dioxide (Galvagno *et al.*, 2013) depending on resource availability and land use practices. Increased photosynthetic capacity, resource use efficiency, and decreased decomposition rates favor CO<sub>2</sub> fixation whereas enteric fermentation and manure deposition by cattle enhance off-site CO<sub>2</sub> emissions in grasslands (Soussana *et al.*, 2007). For example in grazed grasslands in the Eastern Pyrenees, Gilmanov *et al.* (2007) found lower levels of photosynthetic CO<sub>2</sub> uptake than those usually found in temperate Central European mountain grasslands; referring these findings to lower water supply and resource availability. Berninger *et al.* (2015) also found net ecosystem exchange (NEE) values close to zero in Eastern Pyrenean grasslands.

**W**hile most studies focus on CO<sub>2</sub> fluxes (e.g. Byrne *et al.*, 2005; Fu *et al.*, 2009; Leiber-Sauheitl *et al.*, 2014; Merbold *et al.*, 2012; Wohlfahrt *et al.*, 2009), the knowledge of the temporal and spatial variability of CH<sub>4</sub> and N<sub>2</sub>O fluxes in mountain grasslands is scarce (Allard *et al.*, 2007; Soussana *et al.*, 2007; Skiba *et al.*, 2013), and even more so in semi-natural grasslands; traditional land-use form in the Pyrenees (López-i-Gelats *et al.*, 2015). Although atmospheric concentrations and fluxes of methane (CH<sub>4</sub>) and nitrous oxide (N<sub>2</sub>O) are small, their increased emissions can offset potential CO<sub>2</sub> sequestration (Leahy *et al.*, 2004; Schulze *et al.*, 2009; Ciais *et al.*, 2010), and enhance global warming due to their high global warming potential (GWP), notably in extensively managed grasslands in the Eastern Pyrenees, where NEE values were found to be neutral (Berninger *et al.*, 2015). Thus, a major concern addresses the question of how management in mountain grasslands can contribute to enhance soil carbon sequestration and prevent its release to the atmosphere. Understanding better the main drivers and the mechanisms of GHG emissions – especially for CH<sub>4</sub> and N<sub>2</sub>O – is hereby a prerequisite for determining adaptive management strategies.

The main drivers of CO<sub>2</sub> fluxes such as temperature (T<sub>a</sub>), photosynthetically active radiation (PAR), soil water content (SWC), and vegetation biomass (AGB) are already well established (e.g. Raich & Schlesinger, 1992; Ruimy *et al.*, 1995; Reverter *et al.*, 2010; Leiber-Sauheitl *et al.*, 2014). However in the case of CH<sub>4</sub> and N<sub>2</sub>O, and despite the great number of experiments investigating these gases, establishing strong predictive relationships between their fluxes and environmental parameters remains complex due to their non-uniform spatial patterns (Luo *et al.*, 2013). In grasslands, this spatial irregularity of CH<sub>4</sub> and N<sub>2</sub>O fluxes is often controlled by soil properties and/or site management (Dalal & Allen, 2008), making grasslands both potential sources and sinks of methane and nitrous oxide. Several studies report SWC, temperature, soil redox potential, and substrate availability as determining factors for the balance between methanogenesis and CH<sub>4</sub> oxidation of methane (e.g. Bowden *et al.*, 1998; He *et al.*, 2014; Imer *et al.*, 2013; Luo *et al.*, 2013). Other studies emphasize additionally the role of vegetation as emitter of methane (Keppler *et al.*, 2006; Fraser *et al.*, 2015), or simply as a conduit for methane (Whiting *et al.*, 1991; Nisbet *et al.*, 2009) and a possible methane exchange regulator (Ribas *et al.*, 2015). As for N<sub>2</sub>O, soil temperature, pH, moisture, and mineral N content are considered to be the main drivers of its fluxes in grasslands (e.g. Rudaz *et al.*, 1999; Wang *et al.*, 2005; Jones *et al.*, 2007; Imer *et al.*, 2013).

The role of grazing on CH<sub>4</sub> and N<sub>2</sub>O fluxes remains ambiguous. While some studies found no clear effect of grazing intensity on CH<sub>4</sub> emissions from grasslands (Van Den Pol-Van Dasselaar *et al.*, 1999; Zhou *et al.*, 2008), others advocate that increased grazing intensity enhance CH<sub>4</sub> emissions in grasslands because excessive ruminant excretions are able to shift the exchange of CH<sub>4</sub> from consumption to emission (Wang *et al.*, 2009b). As for N<sub>2</sub>O, while some studies claim that grazing enhances emissions (Saggar *et al.*, 2004; Bhandral, 2005; Clark *et al.*, 2005; Rafique *et al.*, 2012), others report the opposite trend (Xu *et al.*, 2008; Wolf *et al.*, 2010). Factors like precipitation or soil texture, due to their effect on plant growth and vegetation composition, seem to be drivers for changes in the grazing effect (Kinraide, 1984; Britz & Ward, 2007; Chimner & Welker, 2011). The excretion patches of different grazing animals can act as GHG “hot-spots” because they lead to high C and N deposition (Ma *et al.*, 2006). However the excrement effect on GHG fluxes varies according to the animal type, because of the difference in height of fall of excreta between sheep and cattle for example, and the different nutrient transformation characteristics (Williams & Haynes, 1994). For instance, cattle excretions are believed to be a great source of N<sub>2</sub>O and CH<sub>4</sub> compared to sheep and horses (IPCC, 2006) because of the highly GHG-emitting rumen of the cattle and the direct enteric emissions.

The effect of vegetation presence and light on CH<sub>4</sub> and N<sub>2</sub>O fluxes remains uncertain and highly controversial. For example, Günther *et al.* (2014) suggested that the effect of light in com-

parison to dark conditions on methane depends on the vegetation type. In a study in an alpine meadow comparing chamber fluxes with intact vegetation and those with only bare soil, Wang *et al.* (2009a) suggest that the difference in fluxes observed arise in fact from differences in soil temperature and water-filled pore space caused by the removal of plants, and that the vegetation itself is not the source. While several studies have reported that vegetation can contribute to N<sub>2</sub>O emissions (Chang *et al.*, 1998; Müller, 2003), it is argued that plants in fact serve only as a conduit of N<sub>2</sub>O produced by soil microorganisms (Du *et al.*, 2010), and that the observed increase in N<sub>2</sub>O fluxes in the presence of plants is connected mainly to higher soil moisture and temperature (Uchida, 2010; Cheng *et al.*, 2014). Further, Müller (2003) advocates that the difference between N<sub>2</sub>O emissions before and after plant removal depends highly on the type of closed chamber; with fluxes measured with chambers transparent to light being twice as high as those measured with dark chambers. Unlike most customary measurements for N<sub>2</sub>O and CH<sub>4</sub> fluxes using dark chambers on bare soil, we endeavoured to unravel the controversial role of vegetation and the effect of light on CH<sub>4</sub> and N<sub>2</sub>O fluxes by carrying measurements on intact vegetation under both light and dark conditions, and on bare soil under dark conditions.

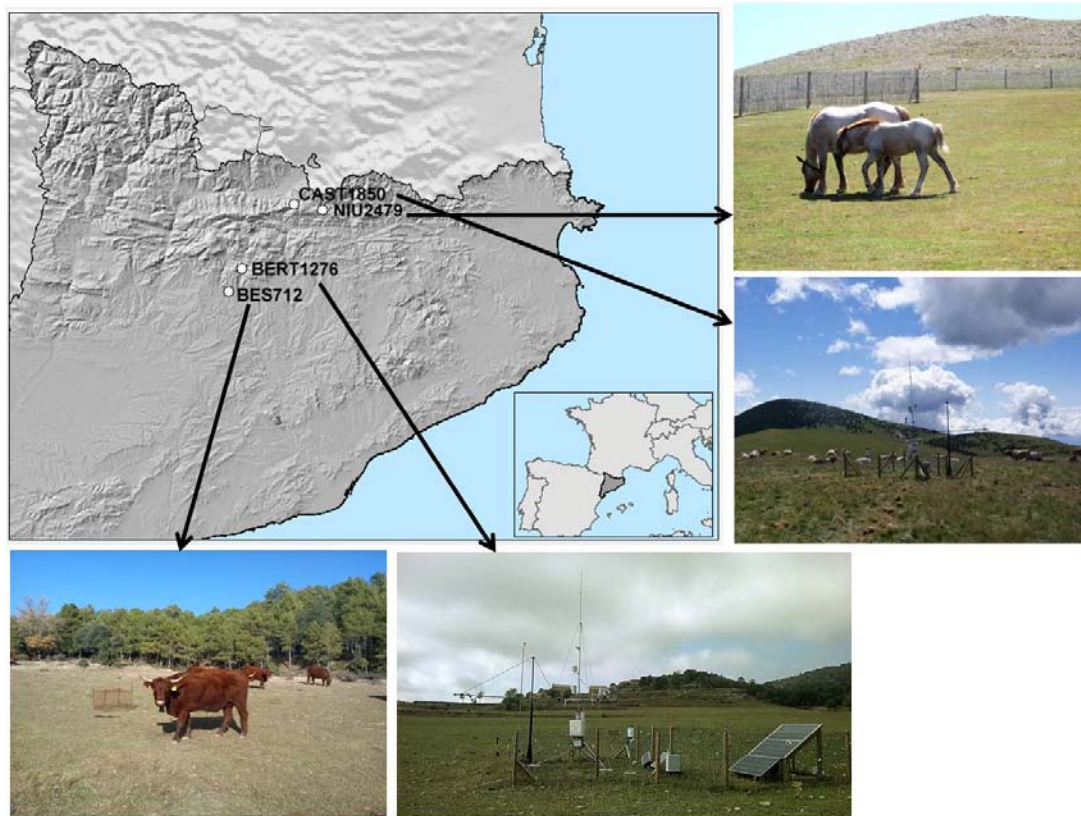
**T**his study presents chamber measurements of CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O fluxes in four semi-natural grasslands in the Pyrenees across an altitudinal gradient and along different seasons covering the grazing and the non-grazing periods. Extensive grazing with few external inputs has been traditionally used in the Pyrenees since prehistoric times, mainly during the summer period where forage production in lowland areas is poorer (Sebastià, 2004; Sebastià *et al.*, 2008). Nowadays, there is a trend towards abandonment of those marginal systems (Komac *et al.*, 2014), contrary to the intensification trend found in the lowland areas (Reiné *et al.*, 2004). We carried out the measurements under both light and dark conditions, as well as on vegetation and on bare soil, using static chambers connected to a field gas photoacoustic monitor (PAS). Information on climatic variables, aboveground biomass, and grazing per site were also obtained. Our study aimed to: 1) describe the magnitude and range of variability of the fluxes of CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O from semi-natural grasslands in the Pyrenees at different altitudes and along different seasons, 2) identify the main biotic (e.g. grazing animals, aboveground biomass) and abiotic drivers (e.g. SWC, PAR, T<sub>a</sub>) of the recorded CH<sub>4</sub> and N<sub>2</sub>O fluxes in those grasslands, and 3) determine the effect of vegetation and light presence on the measured CH<sub>4</sub> and N<sub>2</sub>O fluxes. Overall, we aimed to shed light on the patterns of GHG in extensively managed semi-natural grasslands in the Eastern Pyrenees and their main drivers.

## 4.3 Material and Methods

**W**e measured vegetation and soil fluxes of CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O from four grassland locations along an altitudinal gradient in the Eastern Pyrenees (Fig. 4.1). The fluxes were measured intermittently during 2012 and 2013 with a portable gas-exchange system. The data presented in this study is pooled from several field campaigns providing a fundamental set of data from which information on the range and variability of flux values is obtained.

### 4.3.1 Site description

**T**he four locations are distributed along an altitudinal and climatic gradient in the South-Eastern Pyrenees and represent typical extensively grazed semi-natural mountain grasslands. The four locations are: Besora (712 m a.s.l.; BES712; 42°1'6.40"N, 1°36'19.54"E), La Bertolina (1276 m a.s.l.; BERT1276; 42°5'50.66"N, 1°39'44.68"E), Castellar de n'Hug (1850 m a.s.l.; CAST1850; 42°18'12.19"N, 2°1'53.60"E), and Niu de l'Àliga (2479 m a.s.l.; NIU2479; 42°19'12.24"N, 1°54'0.49"E).



**Figure 4.1** | Locations of the four study sites: Besora (BES712), La Bertolina (BERT1276), Castellar de n’Hug (CAST1850), and Niu de l’Àliga (NIU2479).

**T**he BES712 and BERT1276 sites are sub-Mediterranean montane meso-xerophytic grasslands located in Pla de Busa. The CAST1850 and NIU2479 sites are mesic subalpine and alpine grasslands respectively. The CAST1850 site is located in Plans del Ginebrar, and the alpine site NIU2479 is situated close to La Molina ski resort (see Table 4.1 for a detailed description of the sites’ characteristics). Bedrock in all sites is calcareous, but soils of high-altitude grasslands in the Pyrenees are acidified (Sebastià, 2004). The BES712 and BERT1276 sites consist of polygenic conglomerates with a high stoniness. Meanwhile, the CAST1850 and NIU2479 sites are over lutites and microconglomerates, with less stony soil (ICGC, 2007). The BES712 and BERT1276 sites are characterized by a sandy loam texture. The soil texture in the CAST1850 site is loamy, and in the NIU2479 site clayey. Both BERT1276 and CAST1850 sites are equipped with eddy covariance flux towers, from which meteorological measurements were acquired.

**Table 4.1** | General characterization of the four study sites; MAT = mean annual temperature; MAP = mean annual precipitation.

Site	MAP (mm)	MAT (°C)	Stocking rate (LSU ha <sup>-1</sup> )	Soil pH	Soil texture	Livestock species	Grazing period
BES712	720.5	11.7	0.81	8.19	Sandy loam	Cattle	November-May
BERT1276	954.8	8.7	0.44	8	Sandy loam	Cattle	May-November
CAST1850	1199	5.4	0.74	5.42	Loam	Cattle, sheep	June-November
NIU2479	1302	2.4	0.31	6.09	Clay loam	Cattle, horses	July-November

\*MAP and MAT are according to the Atlas climatic digital de Catalunya (Ninyerola et al., 2000).

Accordingly, the vegetation in the four locations consists of montane, subalpine and alpine grasslands respectively. BES712 and BERT1276 are dominated by grasses, where forbs (legume and non-legume forbs) are abundant and sedges are rare. The dominant species in BES712 are *Plantago lanceolata* L. and *Bromus hordeaceus* L. and in BERT1276 they are *Festuca arundinacea* Schreb., and *Trifolium repens* L. CAST1850 is dominated by *Festuca nigrescens* LAM., and *Carex caryophyllea* Latourr., and NIU2479 is dominated by *Festuca airoides* Lam. and *Carex caryophyllea* Latourr. The grazing period in the four grassland sites extends from May until November, except for BES712 where it extends from November until May.

### 4.3.2 Sampling design

The common sampling strategy was based on periodic one-day visits to the sites to collect chamber-based flux data and biomass. The measurements took place during daylight (between 8 am and 4 pm approximately; solar time). The sites were visited at four-to-six-week intervals (depending on the length of the growing season at each site) in order to represent the different stages of development. Sampling points were spread over a grid in the grassland; the diameter of the circular samples represented by metallic collars was 25 cm. At BERT1276 and CAST1850, the chamber sampling points were placed inside the footprint of the environmental-data measuring equipment, a target area of about 50-70 m radius. At BES712 and NIU2479, the sampling points were spread over a grid on the grassland, covering a similar area. The points were chosen anew for every sampling date because of destructive biomass sampling, and chambers were placed over the collars, to obtain flux measurements.

The campaigns differed in 2012 and 2013 in the following ways: In 2012 the points were randomly distributed on the grassland (N=9 per site), and only measures with intact vegetation under light and dark conditions were performed. In 2013, the points were chosen to represent the dominant plant functional types (PFT), -grasses, sedges, legumes, and non-legume forbs-, with three replicates per PFT (thus N= 9 or 12 per site). Therefore, we had a total of nine to twelve sampling points per measurement campaign and site, depending on whether the site was dominated by three or four PFT. Also, during 2013, additionally to the flux measures with

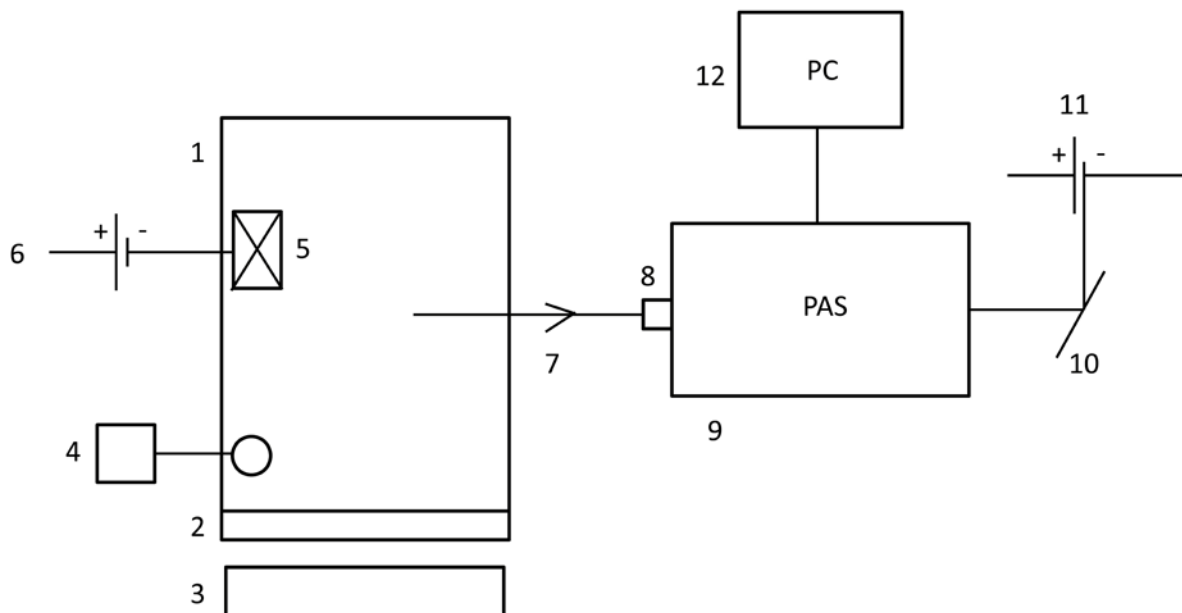


intact vegetation in light and dark, soil dark measurements were taken after the vegetation had been harvested.

### 4.3.3 Flux measurements

#### 4.3.3.1 System setup

We used a self-assembled portable gas-exchange system to perform *in-situ* field surveys. The system consisted of a cylindrical chamber (20L nominal volume), connected to a multi-gas analyzer through Teflon tubing. The chamber was made of uncoated transparent methacrylate that was darkened when needed with a reflective cover manually placed on its top (see details of the set up in Fig. 4.2).



**Figure 4.2** | Scheme of the chamber-scale gas-exchange measurement system set-up. The enclosure consisted of (1) a methacrylate chamber (h =38.5 cm; Ø= 25 cm), (2) a rubber joint at its base to provide sealing at the chamber/collar junction, and (3) a metal collar (h = 8 cm; Ø= 25 cm) installed 3 cm deep in the soil. The chamber was equipped with (4) monitoring of the internal air temperature with thermocouple connected to an AMPROBE multi-logger thermometer TMD-56, (5 and 6) air mixing to homogenize the air in the headspace with a small fan powered by its own battery, and (7) a 20-m long Teflon sampling tube. Air was intermittently drawn at a rate of 1LPM and passed through an external air-filter before entering the (8) PAS analyzer (9) and being eventually exhausted. The flow rate was produced and determined by the analyzer, therefore there was no additional pump or flow controller. The system was powered by 12V batteries + 12V-220V converter (10 and 11). Communication to PAS and data storing was done with a laptop (12). To allow portability, the equipment was housed on a carriage and it was shaded from direct sun-light to avoid overheating.

We measured all gases simultaneously with a photoacoustic spectroscopy (PAS) analyzer (INNOVA 1412, LumaSense Technologies, Denmark). PAS has a measurement cycle that implies intermittent air flow from the chamber. The cycle starts by drawing air from the sampling point in order to flush the old air in the system and thereafter obtain a sample of fresh air. When the required volume of the sample is reached, the flow stops. From this, the concentration of the desired gases will be consecutively determined inside the cell of the analyzer. The

sample is irradiated in a modulated way to produce intermittent expansions, which can be detected photoacoustically. Each gas of interest is determined separately, as the irradiation is delivered through optical filters with selected wavelengths, and the filters are applied in sequence. The response time depends on the sampling integration and the flushing time defined; which in this study was approximately 60 seconds including the three gases and water vapor. This implies that the concentration output rate was of approximately one value per minute. The air removed from the headspace including flushing and sampling represents about 1% of the total chamber headspace. The removal happens during less than 10 seconds, leaving the system more than 50 seconds to replace the air -which will be homogenized by the small ventilator- before the next sampling volume is removed.

**T**he nominal detection limits of the various gases are: 5, 0.03, and 0.24 ppm for CO<sub>2</sub>, N<sub>2</sub>O, and CH<sub>4</sub>, respectively. The PAS was calibrated prior to the field campaigns by the vendor in the customary way (Moody *et al.*, 2008). The analyzer was used in the cross-interference and the water-interference modes, to take into account the cross interference between gases and the interference of water vapor in the measure of gases (for more details on PAS modus-operandi and comparability see Iqbal *et al.*, 2013).

**T**he collars were placed into the ground (3 cm deep) two to three weeks before each measurement period, in order to limit any disturbance in the soil prior to sampling. During measurements, the chambers were moved manually between sampling points. Flux measurements were done by placing the chamber around the collar to enclose the vegetation and soil for about four minutes. The chamber was always left open for four minutes before each flux measurement to ensure ventilation of the chamber headspace, and to obtain the values of ambient gas concentrations. We first measured fluxes of intact vegetation and soil under light, then under dark conditions. Afterwards we cut the aboveground vegetation and measured soil fluxes without vegetation and under dark conditions. Despite studies suggesting an increase in methane emissions (Wang *et al.*, 2009a) after plant removal, we did not observe any remarkably rising peaks of methane fluxes linked to this sampling effect.

#### **4.3.3.2 Flux calculations**

**T**he flux is computed using the change in gas concentration monitored during the closure. When the chamber is placed in the collar, the plant-soil system is forced into a dynamic state where light, temperature, humidity and gas concentrations change due to the activity of the soil-plant system, which in turn responds to the change. The flux of interest is the rate of concentration change since the time of closure. This should preferably be estimated through a non-linear fitting procedure, as reported extensively (Kutzbach *et al.*, 2007 and references therein). In our case, non-linear fitting is challenged by the small amount of measurements per closure,

which makes several-parameter fitting spurious (Parkin & Venterea, 2010). In addition, N<sub>2</sub>O and CH<sub>4</sub> present small noise to signal ratio so that flux values rendered through calculation might not be statistically significant from 0 (Pedersen *et al.*, 2010). Therefore, we used a linear estimation (Fig. S4.1 in Supplementary material) to calculate the flux as the slope of the relationship of gas concentration versus time:

$$F = \frac{V\delta C}{A\delta t}$$

where F is the flux in mol/s, V is the chamber volume in m<sup>3</sup>, A is the chamber surface area in m<sup>2</sup>, δC is the gas concentration in mol/m<sup>3</sup>, and t is the time in s. Positive flux values refer to gas emissions to the atmosphere, and negative values represent uptake of the gas by the vegetation and/or the soil. The obtained total measured flux is the net result of all the mechanisms generating a change in concentration in the headspace, that is, fluxes generated by the soil ( $F_{soil}$ ) and the plants ( $F_{plants}$ ) as well as potential artifacts of the measuring system ( $F_{system}$ ); which refer to the error margin that may occur in the system.

$$F_{measured} = F_{plants} + F_{soil} + F_{system}$$

#### 4.3.3.3 Quality control

All collected raw data were screened for integrity and outliers. As the examination of the concentration data revealed a remaining interference of water vapor on CH<sub>4</sub> concentration, a correction was applied similarly as in Tirol-Padre *et al.* (2014). The slope between the concentrations of these two gases showed an increase of 1.104 per 1 mol/m<sup>3</sup> of water vapor, and this happened at water vapor concentrations higher than 12 mol/m<sup>3</sup> (Fig. S4.2 in Supplementary material). Correspondingly, the CH<sub>4</sub> value was lowered by a factor of 1.104 proportional to the change in water vapor since the previous measurement.

The overall background noise of the system was assessed from the measurements of the ambient concentration as the standard deviation (SD) over the average. This was also used to estimate the flux detection limit (DL) over 4 min as:

$$DL = \frac{V \times SD}{A \times t}$$

where t is total closure time (in our case 240 s). The goodness of fit of the flux calculation was assessed from the r<sup>2</sup> value.

The comparability of the data for different days was compromised by some technical failures that left temporarily unrecorded some variables. Thus in a 13% of the measurements, the chamber temperature was missing and in a 20%, the photosynthetic active radiation (PAR) failed. The missing values were gap-filled in approximately 70% of the missing cases, particularly in the sites BERT1276 and CAST1850, using an empirical relation between PAR and temperature when it was simultaneously available. Temperature and relative humidity increase inside the chamber, especially during CO<sub>2</sub> measurements on intact vegetation, were considered during chamber closure due to their effect on the stomatal behavior of the plants. In general, temperature increased in a range of 2-3 °C during chamber closure, reaching up to 5 °C in certain measurements days of the hot summer period, and the temperature change was considered during flux calculations. As for relative humidity, our inspection of the CO<sub>2</sub> data before and after chamber closure (Fig. S4.1 in Supplementary material) showed no saturation of the system by the relative humidity.

#### 4.3.3.4 Environmental variables

At each location and simultaneously to the gas flux measurements in each collar, the temperature inside the chamber was measured every 10 seconds. The photosynthetic active radiation (PAR) outside the chamber was also measured using an AccuPAR model LP-80 PAR/LAI ceptometer (DECAGON DEVICES INC.). In the BERT1276 and CAST1850 sites, the environmental variables that we acquired from the eddy covariance flux towers at 30 minutes average, were: incoming PAR, (SKP215, Skye Instruments, UK), T<sub>a</sub> (HMP45C, Vaisala, Finland), soil temperature T<sub>s</sub> (TCAV, Campbell, USA) and SWC (CS616, Campbell, USA).

#### 4.3.4 Above-ground biomass and grazing

Once the GHG measurements of the vegetation were completed in each collar, we harvested the plants at ground level. We later separated these samples in the laboratory into live and standing dead above-ground biomass. We placed the samples in the oven at 60°C for 48 hours to obtain the dry weight. We later calculated live aboveground biomass (AGB<sub>L</sub>), and total aboveground biomass (AGB<sub>T</sub>) by adding standing dead to AGB<sub>L</sub>. The grazing calendar and stocking rates were provided by the farmers, and later confirmed in-situ during the sampling visits.

#### 4.3.5 Statistical analyses

To determine the range of the fluxes in all the sites along time, we calculated the mean flux value of each gas per sampling date and site. We then calculated the standard deviation (SD) of the mean flux values. In total, we obtained three mean flux values for CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O

per sampling date per site: mean fluxes from intact vegetation under light conditions, from intact vegetation under dark conditions, and from bare soil under dark conditions.

To test the effect of the biotic and abiotic variables along the altitudinal gradient on GHG fluxes, we carried out multiple regression models. Our dependent variable consisted of each of the mean chamber GHG fluxes ( $\text{CO}_2$ ,  $\text{CH}_4$ , and  $\text{N}_2\text{O}$ ) of each site per sampling date, while our explanatory variables representing the altitudinal gradient were: mean annual precipitation (MAP), mean annual temperature (MAT), mean minimum temperature ( $\text{MT}_{\text{min}}$ ), and mean maximum temperature ( $\text{MT}_{\text{max}}$ ).  $\text{CO}_2$  fluxes are represented by: net ecosystem exchange (NEE; on intact vegetation under light conditions), ecosystem respiration ( $R_{\text{eco}}$ ; on intact vegetation under dark conditions), and soil respiration ( $R_{\text{soil}}$ ; on bare soil under dark conditions). We acquired the climatic variables from the Atlas climatic digital de Catalunya (Ninyerola *et al.*, 2000). We also included seasonal and daily environmental variables:  $T_a$ , PAR and date as they represent seasonality, in addition to grazing (dummy variable where presence of grazing = 1 and absence = 0), and aboveground biomass; both live and total ( $\text{AGB}_L$  and  $\text{AGB}_T$ ). We selected the best models based on the Akaike criterion (Akaike, 1973). No significant multicollinearity between biotic and abiotic predictors was detected.

A more detailed analysis of the role of local environmental factors on  $\text{CH}_4$  and  $\text{N}_2\text{O}$  fluxes was carried out in the two sites equipped with continuous meteorological data, BERT1276 and CAST1850. In addition to  $T_a$ , PAR, AGB, and grazing, the effect of SWC and  $T_s$  was also analyzed in those two sites, using multiple regression models. Since  $\text{CH}_4$  fluxes in BERT1276 and CAST1850 showed exponential relationships with soil water content (SWC), we log-transformed the methane flux for the multiple linear models. The selection of the best multiple regression model was also based on the Akaike criterion (Akaike, 1973). All statistical analyses were carried out using the R software (R Core team, 2013). We used  $\text{CH}_4$  and  $\text{N}_2\text{O}$  fluxes under light conditions as proxies for methane and nitrous oxide behaviors. We did not include in the statistical analyses the flux values below the detection limit, which also had very low  $r^2$  values.

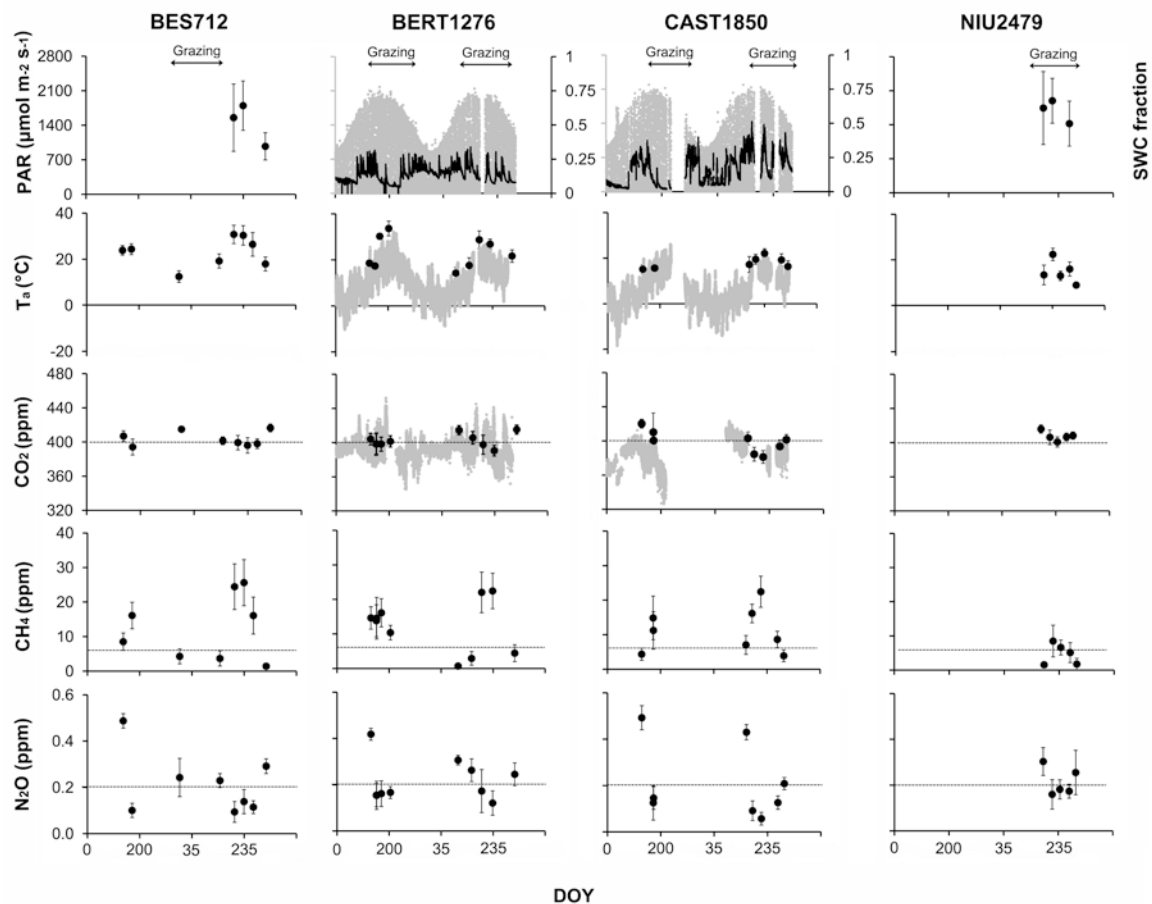
To test for differences between total  $\text{CH}_4$  and  $\text{N}_2\text{O}$  fluxes under light versus dark conditions, as well as the fluxes on intact versus removed vegetation, we conducted paired t-tests grouping all data (considering the four grassland sites). In the case of  $\text{CH}_4$ , and due to its high variability within each site, we also carried out paired t-tests for each site separately.

## 4.4 Results

### 4.4.1 Overview and system performance

A summary of the data can be seen in figure 4.3, which shows the temporal course of ambient measurements to give an overview of the environmental conditions, the sampling frequency and the comparability of the values between sites. The continuous recordings of the PAR,  $T_a$ , and SWC from the eddy-covariance towers provide a description of the seasonal patterns and give a context for the campaign dates, which covered the growing season. The altitudinal gradient is clearly reflected in the air temperature, with sites going from warmer to cooler with altitude. In the sites with SWC data, the lowest altitude site presents the driest soil. Note that in 2012 there was a rather intense drought period that affected also the highest-altitude sites.

It is also interesting to see the level of ambient gas concentrations. In the case of  $CO_2$ , we can see by comparison with the meteorological data that they are about the same level and follow the same temporal patterns. The levels of  $N_2O$  are in the order of magnitude of the background atmospheric concentration, 0.2 ppm, although spanning from 0.1 to 0.5 ppm.  $N_2O$  seems to be higher at the vegetation peak, and decreasing along the grazing season. An opposite pattern is seen for  $CH_4$ , with the lowest concentrations shown around the vegetation peak. The lowest measured  $CH_4$  concentrations are in the range of the background atmospheric concentration, 2 ppm, whereas the highest values are an order of magnitude higher.



**Figure 4.3** | Temporal course (expressed in day of the year - DOY) of ambient measurements throughout the campaigns at the four grassland sites: BES712, BERT1276, CAST1850, and NIU2479. Values are daily averages and bars denote standard deviation (SD). In the case of BERT1276 and CAST1850, the continuous recordings from the meteorological station are also shown: CO<sub>2</sub>, photosynthetically active radiation (PAR), volume fraction of soil water content (SWC), and air temperature (T<sub>a</sub>). The horizontal lines in the CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O concentrations mark the global average ambient concentration for these gases. The horizontal arrows indicate the grazing period.

The overall background noise of the ambient gas concentrations measurements is reflected by the standard deviation (SD) of the mean, and can be seen in figure 4.3. We observed certain variability in SD between dates and between sites. Particularly, we detected temporal patterns of SD for all gases, with higher variability at the vegetation peak in comparison to very low variability during the early season or in autumn. This higher range of variability may be attributed to increasing temperatures and higher biological activities and emissions at the vegetation peak. The observed variability in the SD on the ambient concentration of the gases, on daily or seasonal basis could also be connected to the varying local wind speed (Murayama *et al.*, 2003).

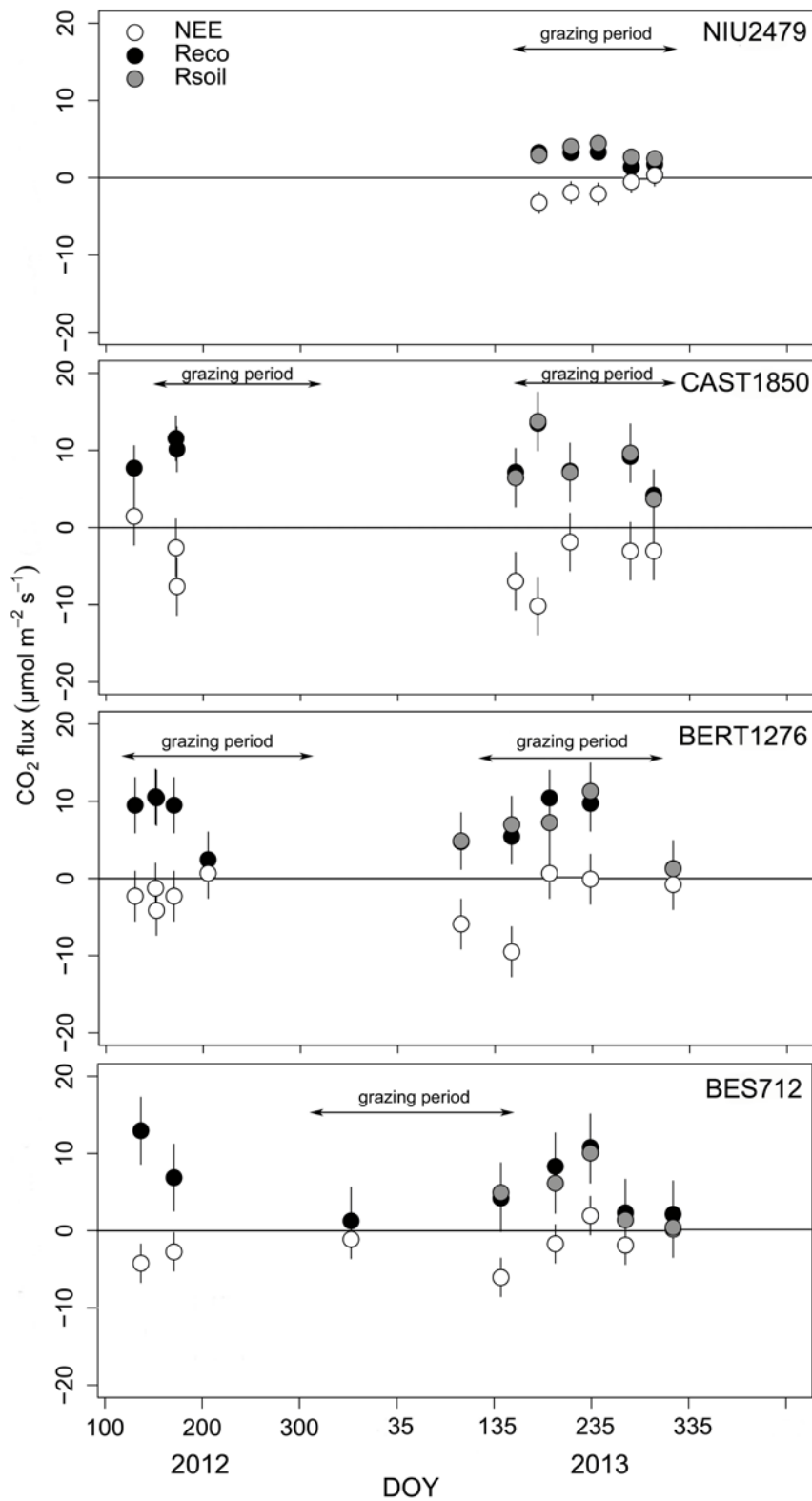
The calculated DL of the fluxes changed according to the variability in the standard deviation. In the case of CH<sub>4</sub>, 53% of all measured fluxes exceeded DL in all sites (Fig. S4.3 in Supplementary material), while 63% of all measured N<sub>2</sub>O fluxes exceeded DL (Fig. S4.4 in Supplementary material).

## 4.4.2 Seasonal flux range and patterns along the altitudinal gradient

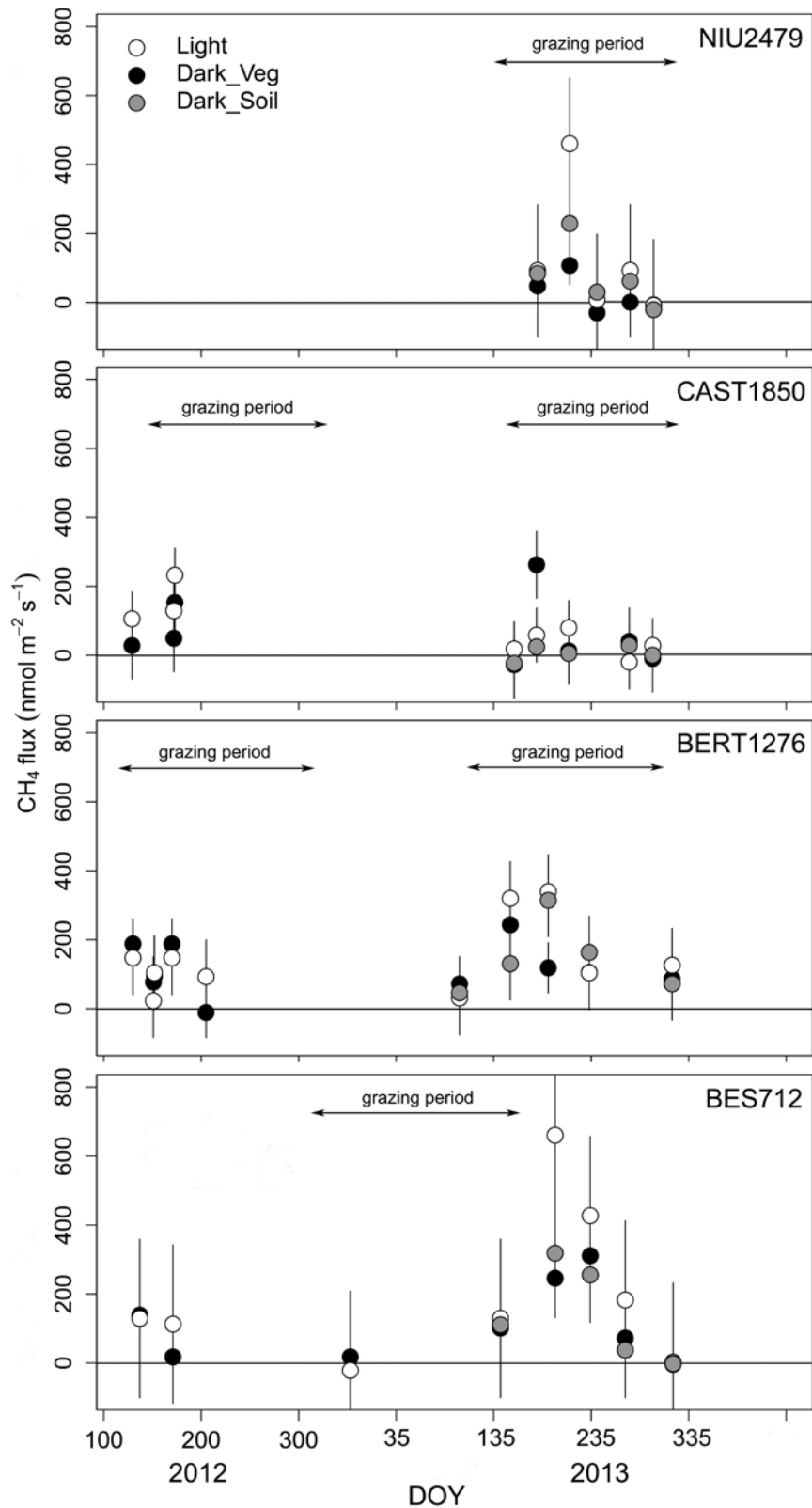
**O**verall, we did not observe differences in GHG fluxes between the years 2012 and 2013. We also observed that the GHG fluxes decreased along the altitudinal gradient (Figs. 4.4 to 4.6; Tables 4.2 and 4.3), with the lowest fluxes found in the high-altitude NIU2479 site (Figs. 4.4 to 4.6). Fluxes of CO<sub>2</sub> in the site at the lowest altitude (BES712; -6.05 to 12.95 μmol m<sup>-2</sup> s<sup>-1</sup>) were approximately twice to three times the values observed in the alpine site (NIU2479; -3.62 to 4.45 μmol m<sup>-2</sup> s<sup>-1</sup>). CH<sub>4</sub> (-41.03 to 746.5 nmol m<sup>-2</sup> s<sup>-1</sup>) and N<sub>2</sub>O (-3.10 to 17.54 nmol m<sup>-2</sup> s<sup>-1</sup>) emissions in BES712 were also higher than in NIU2479; the latter being a smaller emitter of methane (-58.65 to 521.32 nmol m<sup>-2</sup> s<sup>-1</sup>), and a sink of nitrous oxide (-27.09 to 0.28 nmol m<sup>-2</sup> s<sup>-1</sup>). In summary, in the four grassland sites we observed mainly positive values of methane (Fig. 4.5). While the two sites at the lowest altitudes showed generally positive values of N<sub>2</sub>O, we detected mostly negative N<sub>2</sub>O flux values in the subalpine CAST1850 and alpine NIU2479 sites (Fig. 4.6). The most negative peaks of CH<sub>4</sub> and N<sub>2</sub>O fluxes occurred in the alpine grassland site at the end of August 2013 (Figs. 4.5 and 4.6).

**F**luxes of CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O showed the maximum positive rates, thus emissions, during the summer months of July and August 2013 which also corresponds to the grazing period in the four sites, except for BES712. In contrast, we observed the lowest GHG flux values during the autumn period in all the sites (Figs. 4.4 to 4.6). Notably in BERT1276, the low GHG fluxes in autumn also corresponded to the non-grazing period. We also observed greater methane positive flux values during the summer months of July and August 2013, with the highest peaks observed in the site at the lowest altitude (BES712) and in the alpine site (NIU2479) in July at the peak of the vegetation.

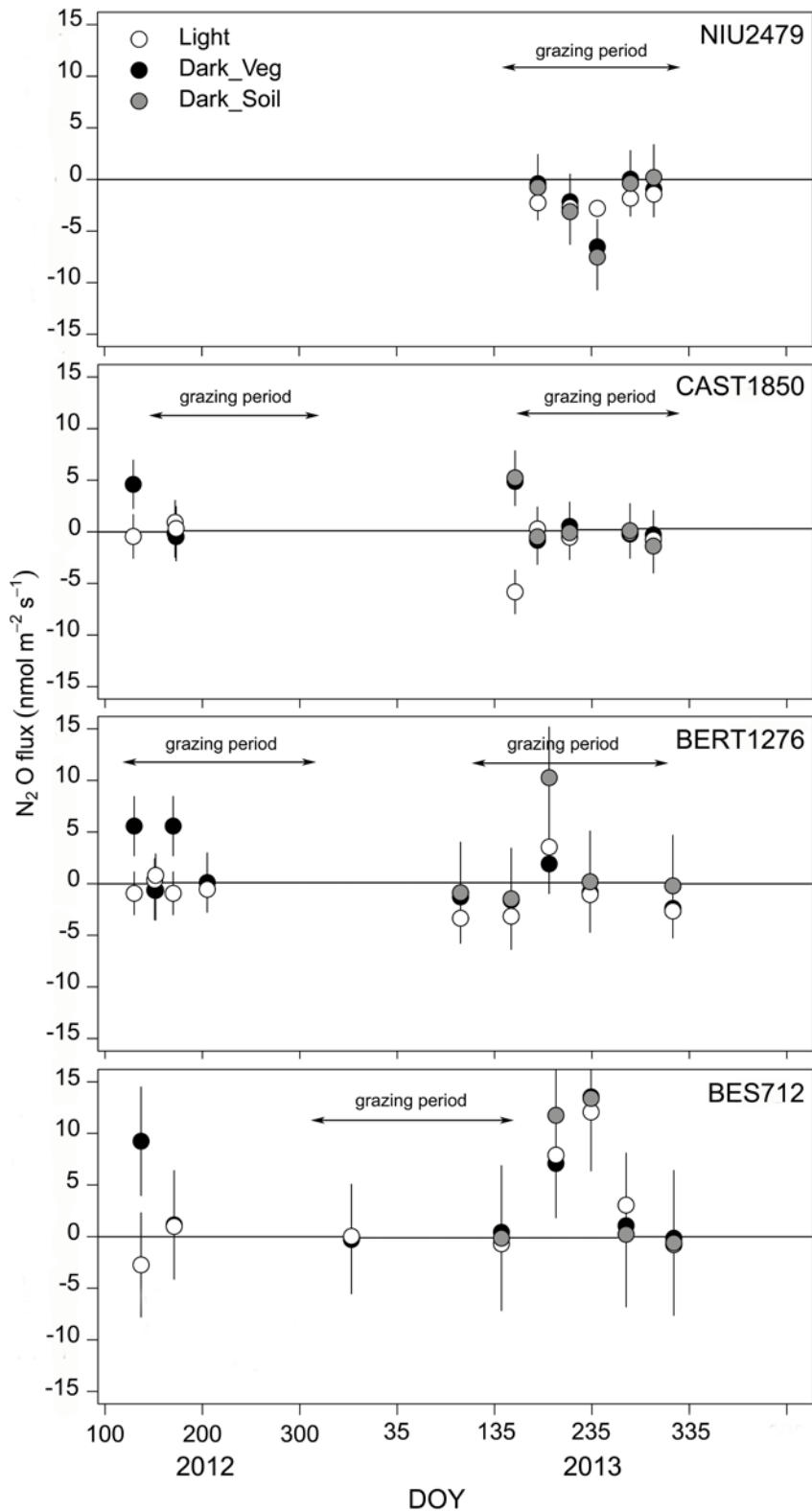




**Figure 4.4** | Daily averaged fluxes of CO<sub>2</sub> in the four study sites along the altitudinal gradient: BES712; BERT1276; CAST1850; and NIU2479. The vertical bars denote standard deviation (SD) of the mean flux values. Negative values represent uptake by the soil, and positive values represent release to the atmosphere. NEE = net ecosystem exchange; R<sub>eco</sub> = ecosystem respiration, R<sub>soil</sub> = soil respiration; DOY = day of the year (between May 2012 and November 2013).



**Figure 4.5** | Daily averaged fluxes of CH<sub>4</sub> in the four study sites along the altitudinal gradient: BES712; BERT1276; CAST1850; and NIU2479. The vertical whiskers denote the standard deviation (SD) of the mean flux values. Negative values represent uptake by the soil, and positive values represent release to the atmosphere. Light = CH<sub>4</sub> fluxes with intact vegetation under light conditions; Dark\_Veg = CH<sub>4</sub> fluxes with intact vegetation under dark conditions; Dark\_Soil = CH<sub>4</sub> fluxes on bare soil (after vegetation removal) under dark conditions. DOY = day of the year (between May 2012 and November 2013).



**Figure 4.6** | Averaged daily fluxes of  $N_2O$  in the four study sites along the altitudinal gradient: BES712; BERT1276; CAST1850; and NIU2479. The vertical whiskers denote the standard deviation (SD) of the mean flux values. Negative values represent uptake by the soil, and positive values represent release to the atmosphere. Light =  $N_2O$  fluxes with intact vegetation under light conditions; Dark\_Veg =  $N_2O$  fluxes with intact vegetation under dark conditions; Dark\_Soil =  $N_2O$  fluxes on bare soil (after vegetation removal) under dark conditions. DOY = day of the year (between May 2012 and November 2013).

### 4.4.2.1 CO<sub>2</sub> fluxes

We detected a clear difference between net ecosystem exchange (NEE), ecosystem respiration ( $R_{\text{eco}}$ ), and soil respiration ( $R_{\text{soil}}$ ) in all the sites (Fig. 4.4). The results of our best multiple linear models showed that the mean NEE and  $R_{\text{soil}}$  fluxes per sampling date varied from one site to another based on the climatic conditions of each: MAP and MTmax for NEE, and MAP and MTmin for  $R_{\text{soil}}$  (Table 4.2). However unlike NEE and  $R_{\text{soil}}$ , the regional climatic variables had no significant effect on  $R_{\text{eco}}$ . Instead, the local environmental factors of each site, particularly  $T_a$  were the most significant variables for  $R_{\text{eco}}$  (Table 4.2). Local  $T_a$  had also a significant effect on  $R_{\text{soil}}$ . For NEE, total (AGB<sub>T</sub>) and live (AGB<sub>L</sub>) above-ground biomass exerted a high influence on the fluxes; the higher the AGB<sub>L</sub> the more negative the NEE fluxes, indicating a higher absorption of CO<sub>2</sub> (Table 4.2).

**Table 4.2** | Results of the best multiple linear regression models testing the effect of the biotic and abiotic variables along the altitudinal gradient on mean CO<sub>2</sub> fluxes per sampling date (NEE = net ecosystem exchange;  $R_{\text{eco}}$  = ecosystem respiration;  $R_{\text{soil}}$  = soil respiration) at the four sites: *p* values are given for the individual explanatory variables (MAP = mean annual precipitation; MTmax = mean maximum temperature; AGB<sub>L</sub> = live aboveground biomass; AGB<sub>T</sub> = total aboveground biomass; DOY = day of the year;  $T_a$  = air temperature; MTmin = mean minimum temperature); N = 27.

Model	R <sup>2</sup> <sub>adj.</sub>	Estimate	Std. Error	<i>P</i>
<b>NEE</b>				
	**	0.33		
MAP		-0.072	0.023	**
MTmax		-14.419	4.616	**
AGB <sub>L</sub>		-0.064	0.016	***
AGB <sub>T</sub>		0.056	0.014	***
<b><math>R_{\text{eco}}</math></b>				
	*	0.20		
DOY		-0.008	0.005	n.s.
$T_a$		0.296	0.126	*
AGB <sub>L</sub>		0.007	0.007	n.s.
<b><math>R_{\text{soil}}</math></b>				
	*	0.35		
DOY		-0.020	0.011	n.s.
MAP		0.034	0.014	*
MTmin		2.328	1.039	*
$T_a$		0.430	0.145	**

n.s. not significant. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001. *P*-values in bold indicate significant relationships.

### 4.4.2.2 CH<sub>4</sub> fluxes

Our best model explaining CH<sub>4</sub> fluxes revealed that the mean fluxes per sampling date varied according to the MAT and the MTmax of the site; the higher the MAT the higher the methane fluxes (Table 4.3). We also observed that  $T_a$  and AGB<sub>T</sub> were the variables with the highest influence on CH<sub>4</sub> in all sites.

**Table 4.3** | Results of the best multiple linear regression models testing the effect of the biotic and abiotic variables along the altitudinal gradient on mean CH<sub>4</sub> and N<sub>2</sub>O fluxes per sampling date, with intact vegetation under light conditions at the four sites: *p* values are given for the individual drivers (MAT = mean annual temperature; altitude; MTmax = mean maximum temperature; T<sub>a</sub> = air temperature; AGB<sub>T</sub> = total aboveground biomass; grazing = 0 or 1; MAP = mean annual precipitation); N = 27.

Model	R <sup>2</sup> adj.	Fluxes		P
		Estimate	Std. Error	
<b>CH<sub>4</sub></b>				
	***	0.64		
MAT		1.607e+03	3.636e+02	***
MTmax		-4.989e+03	1.134e+03	***
T <sub>a</sub>		2.544e+01	5.102e+00	***
AGB <sub>T</sub>		5.035e-01	2.421e-01	*
<b>N<sub>2</sub>O</b>				
	***a	0.65		
Grazing		-3.312	1.110	**
MAP		-0.011	0.004	**
T <sub>a</sub>		0.333	0.110	**
AGB <sub>T</sub>		0.016	0.005	**

n.s. not significant. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001. P-values in bold indicate significant relationships.

In the montane grassland BERT1276, local PAR and AGB were the main variables influencing methane fluxes. Particularly, CH<sub>4</sub> fluxes increased with PAR and live biomass (Table 4.4). In the subalpine grassland site CAST1850, grazing together with aboveground biomass were the most significant drivers of methane fluxes (*P* < 0.001). CH<sub>4</sub> decreased under grazing conditions and increased with increasing live biomass.

**Table 4.4** | Multiple linear model testing the effect of environmental variables, seasonality (DOY), grazing, and vegetation on log(CH<sub>4</sub>) fluxes under light conditions at the two sites BERT1276 and CAST1850 in the year 2013; *p* values are given for the individual drivers (T<sub>a</sub> = air temperature; PAR = photosynthetically active radiation; AGB<sub>L</sub> = live aboveground biomass; AGB<sub>T</sub> = total aboveground biomass; grazing = 0 or 1).

Model	R <sup>2</sup> adj.	Log(CH <sub>4</sub> ) fluxes		P
		Estimate	Std. Error	
BERT (montane grassland); N = 31				
	***	0.46		
T <sub>a</sub>		0.074	0.043	n.s.
PAR		0.001	0.0005	*
AGB <sub>L</sub>		0.014	0.005	**
AGB <sub>T</sub>		-0.012	0.004	**
CAST (subalpine grassland); N = 46				
	***	0.33		
Grazing		-44.982	10.929	***
AGB <sub>L</sub>		0.199	0.042	***
AGB <sub>T</sub>		-0.196	0.039	***

n.s. not significant. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001. P-values in bold indicate significant relationships.

#### 4.4.2.3 N<sub>2</sub>O fluxes

Mean N<sub>2</sub>O fluxes varied according to the MAP of the site; where the site with the highest MAP (NIU2479) had the lowest or most negative N<sub>2</sub>O fluxes (Table 4.3). Additionally, grazing, T<sub>a</sub> and AGB<sub>T</sub> showed high influence on nitrous oxide in all the sites. Higher temperature and aboveground biomass increased N<sub>2</sub>O fluxes, and grazing reduced them.

In both the montane (BERT1276) and the subalpine (CAST1850) grasslands, local SWC and DOY were good predictors of N<sub>2</sub>O (Table 4.5). Soil water content had a significant negative effect on the fluxes; the higher the SWC the more negative the N<sub>2</sub>O fluxes. However DOY, reflecting seasonality, had a different impact on nitrous oxide according to the site; negative in BERT1276 and positive in CAST1850 as the growing season progressed. In addition to those drivers, T<sub>s</sub> in BERT1276 ( $P < 0.01$ ) and grazing in CAST1850 ( $P < 0.05$ ) showed a strong positive influence on N<sub>2</sub>O fluxes.

**Table 4.5** | Multiple linear model testing the effect of environmental variables, seasonality (DOY), grazing, and vegetation on N<sub>2</sub>O fluxes under light conditions at the two sites BERT1276 and CAST1850 in the year 2013; *p* values are given for the individual drivers (DOY = day of the year; T<sub>s</sub> = soil temperature; SWC = soil water content; AGB<sub>T</sub> = total aboveground biomass; grazing = 0 or 1).

N <sub>2</sub> O fluxes				
Model	R <sup>2</sup> adj.	Estimate	Std. Error	<i>P</i>
BERT1276 (montane grassland); N = 31				
	<b>***</b>	0.55		
DOY		-0.047	0.015	<b>**</b>
T <sub>s</sub>		0.395	0.096	<b>***</b>
SWC		-90.018	24.458	<b>**</b>
AGB <sub>T</sub>		-0.008	0.004	n.s.
CAST1850 (subalpine grassland); N = 38				
	<b>***</b>	0.49		
DOY		0.069	0.015	<b>***</b>
Grazing		6.328	2.337	<b>*</b>
SWC		-39.560	18.289	<b>*</b>

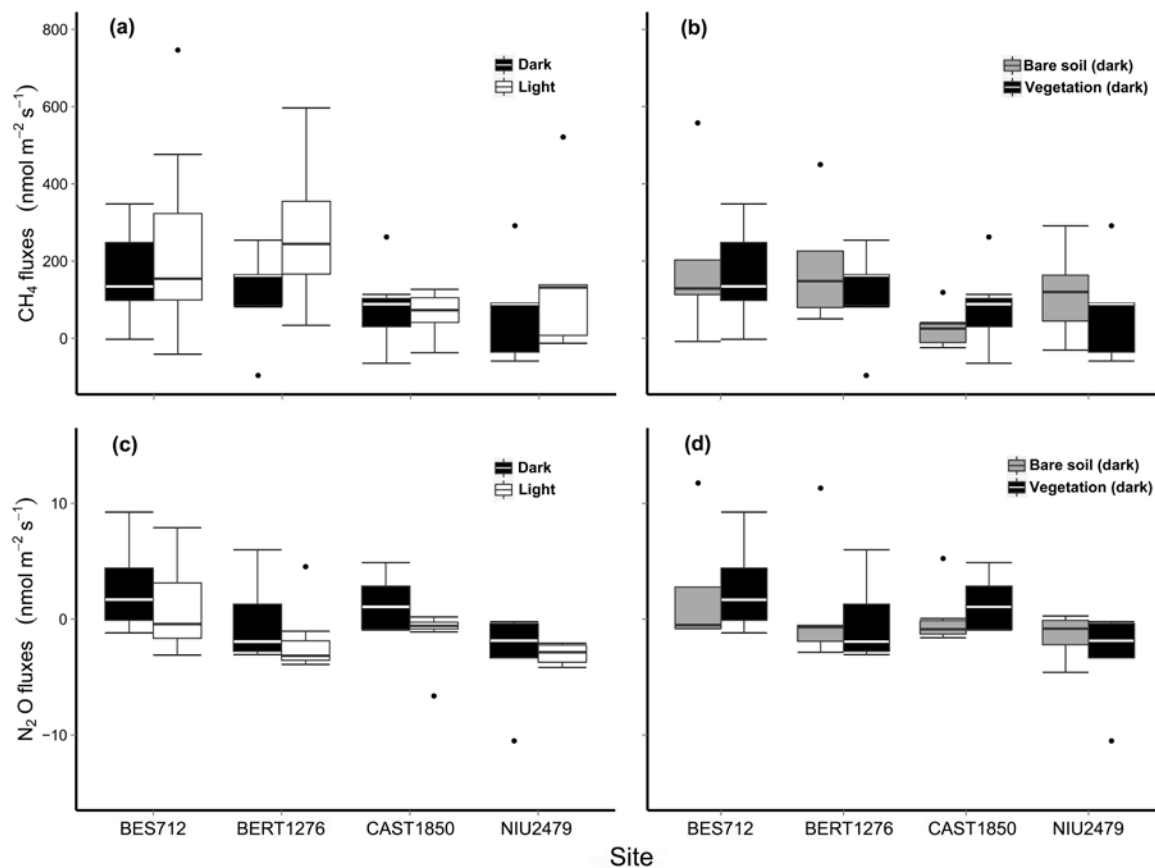
n.s. not significant. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . *P*-values in bold indicate significant relationships.

### 4.4.3 Comparison between GHG fluxes under light and dark conditions, with and without vegetation

Paired *t*-tests on methane fluxes, conducted on data from the four grassland sites, suggested no difference between measurements on intact vegetation and those on bare soil ( $P = 0.93$ ;  $t = -0.089$ ), and no difference between fluxes under light conditions and fluxes under dark conditions ( $P = 0.16$ ;  $t = 1.396$ ). Yet, the high variability in CH<sub>4</sub> fluxes, especially when grouping all the sites together, could be hiding possible differences (Fig. 4.7). In fact, per-site analyses showed that CH<sub>4</sub> fluxes on intact vegetation were significantly higher than those on bare soil in CAST1850 ( $P < 0.05$ ;  $t = 2.623$ ), and lower in NIU2479 ( $P < 0.05$ ;  $t = -2.592$ ). No significant differences were observed in BES712 ( $P = 0.07$ ;  $t = -2.037$ ) and BERT1276 ( $P = 0.90$ ;  $t = 0.120$ ). For light effect, per-site analyses showed similar results as all-sites analyses and no difference was detected between CH<sub>4</sub> fluxes under light conditions and those under dark conditions.

In the case of N<sub>2</sub>O, paired-*t* tests including data of all sites showed that their fluxes were significantly lower under light conditions than under dark conditions ( $P < 0.001$ ;  $t = -4.170$ ). However, only a marginal difference was detected between fluxes with vegetation presence and flux-

es after plant removal ( $P = 0.05$ ;  $t = -1.963$ ).



**Figure 4.7** | Boxplots of: a) CH<sub>4</sub> fluxes under light and dark conditions (top left); 2) CH<sub>4</sub> fluxes on intact vegetation and bare soil, under dark conditions (top right); 3) N<sub>2</sub>O fluxes under light and dark conditions (bottom left); 4) N<sub>2</sub>O fluxes on intact vegetation and bare soil, under dark conditions (bottom right) in the four grassland sites: Besora (BES712), La Bertolina (BERT1276), Castellar de n'Hug (CAST1850), Niu de l'Àliga (NIU2479).

## 4.5 Discussion

### 4.5.1 Flux measurements with manual chamber and PAS

Several methods have been conducted in grasslands to measure GHG emissions; some used the eddy-covariance technique (e.g. Dengel *et al.*, 2011; Merbold *et al.*, 2014) and others used chambers (e.g. Blankinship *et al.*, 2010; Imer *et al.*, 2013). We chose to use a combination of manual chambers and PAS analyzer, due to the great advantage of portability and multi-gas instantaneous measurements. However, the system is limited to detect N<sub>2</sub>O and CH<sub>4</sub> when their fluxes become too small at the sampling site, which was the case for 37% and 47 % of our measurements of N<sub>2</sub>O and CH<sub>4</sub>, respectively. The rest of the fluxes were detectable and could be analyzed further. Several studies aiming to measure soil fluxes of methane and nitrous oxide tend to use non-transparent chambers and close them for as long as 40 minutes (e.g. Pihlatie *et al.*, 2013; Ribas *et al.*, 2015). In our case, the vegetation presence in the chamber headspace made the closure time necessarily short (four minutes) as in other studies using transparent

chambers which chose a closure time of five (Pirk *et al.*, 2015) and three minutes (Luan & Wu, 2014) to avoid condensation. This short accumulation time was obviously limiting the capacity to detect the smaller fluxes, yet it allowed us to investigate and compare fluxes with and without vegetation, under light and dark conditions.

**T**he PAS analyzer has recently received attention (e.g. Nicoloso *et al.*, 2013; Tirol-Padre *et al.*, 2014) concerning a potential cross-interference between gases, which is not sufficiently taken into account by the default settings. The interference of water vapor with CH<sub>4</sub> is particularly obvious. Indeed, the PAS shows stable readings in the laboratory but the environmental variability under field conditions in terms of temperature and humidity seems to compromise the precision of the measurements, in such a way that the baseline of the readings seems to drift in the field. As the interference of water vapor with CH<sub>4</sub> seems to be strong, we minimized a posteriori its effect on the flux calculation, by removing the virtual flux of CH<sub>4</sub> created by the rising water vapor concentration during closure. Yet, it was impossible for us to fully get rid of this deviation in absolute numbers, but since the objective of the measurements was the calculation of fluxes from concentration differences, then the level of the absolute value is not critical. Despite the above discussed technical limitations of the system, the combination of the PAS and the chamber technique allowed us to measure GHGs in remote mountain areas in the Pyrenees, where to our best knowledge data on GHG, particularly CH<sub>4</sub> and N<sub>2</sub>O are scarce or even lacking.

#### 4.5.2 Magnitude and range of GHG fluxes in extensively managed grasslands in the Pyrenees

**N**et ecosystem exchange values of the studied extensively managed grasslands in the Pyrenees indicate mainly uptake of CO<sub>2</sub> on the measurement dates (Fig. 4.4). Our results also show that these grasslands were mainly sources of methane (Fig. 4.5) and with the exception of the subalpine and alpine sites, small emitters of N<sub>2</sub>O on the sampling dates (Fig. 4.6). These findings are in agreement with previous studies in grazed temperate grasslands reporting uptake of CO<sub>2</sub> (e.g. Wohlfahrt *et al.*, 2008), emissions of methane (e.g. Dengel *et al.*, 2011; Merbold *et al.*, 2014), and nitrous oxide (e.g. Tilsner *et al.*, 2003; Imer *et al.*, 2013). Considering the fact that C sequestration in grassland soils can partly counterbalance the effects of non-CO<sub>2</sub> gases (CH<sub>4</sub> and N<sub>2</sub>O) and that under climate change and warming, a reduction of carbon storage in the soil can be expected, increased emissions of CH<sub>4</sub> and N<sub>2</sub>O to the atmosphere, as observed in our study, may have dramatic effects on the GHG balance of these ecosystems.

**G**ilmanov *et al.* (2007) and Berninger *et al.* (2015) found neutral to small negative NEE budget in grasslands in the Pyrenees, with lower levels of photosynthetic CO<sub>2</sub> uptake than those usually found in temperate Central European mountain grasslands. Our NEE



values for the measurement dates showed mostly negative values and hence uptake of CO<sub>2</sub>. The range of NEE in all the sites varied between -10 and 1 μmol m<sup>-2</sup> s<sup>-1</sup> (Fig. 4.4), and compare well with values obtained by Wohlfahrt *et al.* (2008) in the Eastern Pyrenees. As for methane, there is an ongoing debate whether or not CH<sub>4</sub> emissions from mountain grasslands contribute to GHG emissions. While most studies agree that temperate grasslands are sinks of methane (e.g. Imer *et al.*, 2013; Liebig *et al.*, 2010), our findings suggest that extensively managed grasslands in the Pyrenees acted as methane sources. It is also interesting that our CH<sub>4</sub> fluxes, with an average of 150-200 nmol m<sup>-2</sup> s<sup>-1</sup> and reaching maxima > 500 nmol m<sup>-2</sup> s<sup>-1</sup>, are higher than previously reported fluxes measured with chamber techniques. We attribute the observed high CH<sub>4</sub> emissions partly to the animal urine and dung patches during our measurement campaigns. Another possible factor contributing to higher CH<sub>4</sub> emissions may be trampling and soil compaction by grazing animals, which leads to an increased formation of anaerobic microsites in the soil (Laubach *et al.*, 2016). Also, the high CH<sub>4</sub> values may be attributed to our regional climatic conditions with Mediterranean influence; low SWC combined with high temperatures particularly in the summer (Fig. 4.3). In fact, the combination of low SWC with elevated soil temperature inhibits soil respiration (Chang *et al.*, 2014), resulting in the formation of anaerobic conditions in the centre of the soil macropores. Such conditions favor the production of N<sub>2</sub> through denitrification, and eventually favor CH<sub>4</sub> formation instead of oxidation (Butterbach-Bahl *et al.*, 2013). Beside drought stress, clayey soil texture – as found in the alpine grassland NIU2479 – can contribute to favorable conditions for methane production, because clayey soil is characterized by a small amount of macropores which could increase anaerobic microsites (Signor and Pellegrino Cerri, 2013). Our methane flux values are rather close to the order of magnitude of CH<sub>4</sub> emissions measured with eddy-covariance technique on intensively managed grassland in Switzerland with values up to 400 nmol m<sup>-2</sup> s<sup>-1</sup> (Merbold *et al.*, 2014), and a sheep pasture in Scotland with values up to 150 nmol m<sup>-2</sup> s<sup>-1</sup> (Dengel *et al.*, 2011). Beside methane, studying N<sub>2</sub>O emissions in mountain grasslands is important to determine their contribution to the GHG balance in these ecosystems. In general, our N<sub>2</sub>O flux values (-5 to 15 nmol m<sup>-2</sup> s<sup>-1</sup>) were comparable to values obtained by Soussana *et al.* (2007) in European grasslands and Laubach *et al.* (2016) in pastures in New Zealand. Several studies in European grasslands report these ecosystems as net sources of nitrous oxide (e.g. Imer *et al.*, 2013; Soussana *et al.*, 2007). Our findings are in line with these studies, since we observed mainly emissions of N<sub>2</sub>O in the extensively managed grassland sites, except for the alpine site NIU2479. This is most likely attributed to the low nutrient mineralization rates in such cold and moist ecosystem, causing the denitrifying microorganisms to favor N<sub>2</sub>O reduction activity (Sørensen *et al.*, 2008). However our results of the alpine grassland NIU2479 are in contrast to the results of continuous N<sub>2</sub>O measurements presented by Imer *et al.* (2013), where an extensively managed grassland in the Swiss Alps was a small source of N<sub>2</sub>O.

### 4.5.3 Spatial and temporal variability of GHG fluxes

**O**ur results show a large spatial and temporal variability in GHG fluxes: CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O fluxes changed along the altitudinal gradient and along different seasons, but not much between different years (Figs. 4.4 to 4.6, tables 4.2 and 4.3). Our results also show that grasslands at higher altitudes with cold and humid climatic conditions, exhibit lower range of GHG fluxes (Figs. 4.4 to 4.6, tables 4.2 and 4.3). For instance, the highest respiration and uptake rates (NEE and R<sub>soil</sub>) were observed in the low-altitude grassland site BES712 (Fig. 4.4 and Table 4.2). In fact, respiration was higher than uptake in BES712 (12.95 compared to -6.05 μmol m<sup>-2</sup> s<sup>-1</sup>). Higher CO<sub>2</sub> emissions compared to uptake in the low-altitude site may be the result of higher decomposition rates under elevated temperatures. Alpine grasslands, in contrast, are characterized by slow growth and low root turnover corresponding to low carbon flux (Leifeld *et al.*, 2015), due to low temperatures (Gill & Jackson, 2000). Our findings confirm this pattern through the lowest CO<sub>2</sub> emission and uptake rates observed in the alpine site NIU2479 (Fig. 4.4 and Table 4.2). Temperature alone, however, does not explain the observed NEE fluxes. Live above-ground biomass did also contribute significantly to the ecosystem CO<sub>2</sub> fluxes (Table 4.2), by enhancing NEE in all the sites. The alpine grassland emitted less CH<sub>4</sub> and absorbed more N<sub>2</sub>O in comparison to the grasslands at lower altitudes (Figs. 4.5 and 4.6). Imer *et al.* (2013) also reported a similar trend along an altitudinal and management gradient in the Alps, where CH<sub>4</sub> and N<sub>2</sub>O fluxes in the extensively-managed alpine grassland (around 2000 m a.s.l.) were lower than those at low-altitude sites. The lower emissions of CH<sub>4</sub> and N<sub>2</sub>O in the subalpine and alpine grasslands compared to the low-altitude sites can be attributed to the grazer type in these grasslands (mixed grazing in the subalpine and alpine sites compared to cattle grazing in the montane sites), and its effect on vegetation composition. For example, cattle grazing emit more CH<sub>4</sub> than horse or sheep grazing because of the highly GHG-emitting rumen from cattle (IPCC, 2006). Also, cattle grazing favor higher heterogeneity in vegetation composition than sheep grazing (Sebastià *et al.*, 2008). Vegetation composition, in turn, regulates the microbial activity by influencing the composition and the quantity of root exudates (De Deyn *et al.*, 2008) - which are a substrate for microbial respiration (Hütsch *et al.*, 2002) and a key control of microbial communities (Bais *et al.*, 2006). As for N<sub>2</sub>O, the lower emissions in the subalpine and alpine sites (CAST1850 and NIU2479) compared to the montane sites can be attributed to their acidic soils, because acidic soil conditions are believed to reduce soil emissions (Oertel *et al.*, 2016). Although developed on limestone, these high-altitude grasslands show acidic soils (Sebastià, 2004), compared to the montane grassland sites with alkaline soils (pH = 8.19 in BES712; and pH = 8 in BERT1276).

**I**n the studied grassland sites, we observed a pronounced temporal variability in all GHGs, and we believe that it was mainly temperature- and vegetation-driven. In fact, the highest GHG

emissions occurred during the summer period of July and August 2013, with the highest emission peaks detected in the warmest grassland site – the low-altitude site BES712 (Figs. 4.4 to 4.6). Previous studies in grasslands also observed higher CO<sub>2</sub> (Imer *et al.*, 2013; Oertel *et al.*, 2016) and N<sub>2</sub>O (Imer *et al.* 2013; Mori & Hojito 2011) emissions during summer, and underline the importance of temperature as a main driver. For methane, however, our results are in contrast to previous findings in temperate and semi-arid grasslands which reported lower emissions in summer resulting from lower SWC (e.g. Imer *et al.*, 2013; Luo *et al.*, 2013). Nonetheless, Guillet *et al.* (2015) observed a similar trend as in our study, where CH<sub>4</sub> emissions in temperate grasslands increased in late summer. They attribute these CH<sub>4</sub> peaks to the increasing flow of methane to the atmosphere from deep saturated soil layers, favored by soil cracks formed in the dry summer period. In the next section, we will discuss in detail the mechanism and processes behind the effects of temperature and vegetation on the temporal variability of GHG fluxes.

## 4.5.4 Drivers of GHG fluxes

### 4.5.4.1 Carbon dioxide

**O**ur findings indicate that live aboveground biomass enhances NEE in all the sites (Table 4.2). Several studies already suggested the control of aboveground biomass on ecosystem CO<sub>2</sub> fluxes (Ganjurjav *et al.*, 2015; Peng *et al.*, 2015), through positive correlations between live shoot and root biomass quantity and both NEE and R<sub>eco</sub>; higher above- and below-ground biomass implies higher photosynthesis and respiration rates, particularly at the peak of the vegetation growth. In our study, however temperature, and not aboveground biomass, was the main driver of R<sub>eco</sub> and R<sub>soil</sub> (Table 4.2). The important role of temperature on R<sub>eco</sub> and R<sub>soil</sub>, as a regulator of the rate at which CO<sub>2</sub> is produced in the soil, was already extensively established in previous studies (Singh & Gupta, 1977; Raich & Schlesinger, 1992; Flanagan & Johnson, 2005; Hartley *et al.*, 2006).

### 4.5.4.2 Methane

**W**hen studying the processes behind methane fluxes, we found that aboveground biomass and temperature were the main drivers in all sites (Table 4.3), as previously reported in a number of studies in grasslands (e.g. Le Mer & Roger 2001; Luo *et al.*, 2013; Whiting *et al.*, 1991). In fact, temperature not only affects CH<sub>4</sub> emissions through its role in controlling microbial activities (Le Mer & Roger, 2001) but it also regulates the way how plants affect CH<sub>4</sub> fluxes (Praeg *et al.*, 2017), because plants provide substrates for methanogenic bacteria through root exudation or decay (Raimbault *et al.*, 1977).

**I**n the subalpine site CAST1850, in addition to biomass, grazing was an important driver of CH<sub>4</sub> fluxes. In fact, grazing decreased CH<sub>4</sub> fluxes in the subalpine site CAST1850 (Table 4.4). The

negative effect of grazing may be explained by: 1) the reduced vegetation biomass and the lower litter production, resulting in less substrates available for methanogenic bacteria (Zhou *et al.*, 2006), or 2) altered plant species composition by grazing (Sebastià *et al.*, 2008), causing changes in soil N and pH (Semmartin *et al.*, 2010), or 3) possible trampling effect, which alters soil aeration and moisture (Lavado & Taboada, 1988; Lavado *et al.*, 1993); and consequently influences methane oxidation and production (Singh *et al.*, 1997; Benstead & King, 2001). In the montane site BERT1276, PAR was also an important driver of methane emissions (Table 4.4). The effect of PAR on methane is through both its role as a main driver of the photosynthetic production and plant growth, and by the relationship between PAR and  $T_a$ ; the latter being a driver of  $CH_4$  emissions through its role in controlling microbial activities (Le Mer & Roger, 2001).

#### 4.5.4.3 Nitrous oxide

In all the grassland sites,  $N_2O$  fluxes were regulated by grazing,  $T_a$ , and  $ABG_T$  (Table 4.3). As observed in previous studies (e.g. Brentrup *et al.*, 2000; Luo *et al.*, 2013), temperature exerted a strong impact on  $N_2O$  fluxes (Table 4.3). This is attributed to the crucial role of temperature in determining the activity of microorganisms. In particular, the higher the temperatures, the higher the observed  $N_2O$  emissions (Fig. 4.6; Table 4.3). As for grazing, there is a controversy as to whether grazing reduces or enhances  $N_2O$  emissions. We found a negative effect of grazing on  $N_2O$  emissions in all sites (Table 4.3), as observed in previous studies (e.g. Wolf *et al.*, 2010; Xu *et al.*, 2008) suggesting that grazing reduces emissions. The negative effect of grazing on  $N_2O$ , likewise on  $CH_4$ , may be attributed to the fact that grazing reduces plant biomass and organic N content (Semmartin *et al.*, 2010), and consequently decreases nitrous oxide emissions. This is confirmed by the positive impact of total aboveground plant biomass on  $N_2O$  fluxes, observed in our results and which conforms to previous findings (Zhang *et al.*, 2015). Greater biomass leads to greater decomposed plant material, thus enhancing N mineralization and continuously  $N_2O$  and simultaneously  $CH_4$  emissions from the soil, as previously observed in our results. However, looking at the subalpine grassland CAST1850, the effect of grazing on  $N_2O$  fluxes was rather positive (Table 4.5). This suggests that the effect of grazing on  $N_2O$  emissions is highly site-specific and more work needs to be done to elucidate the processes behind that effect.

Seasonality, expressed by DOY, did not exhibit the same impact on  $N_2O$  fluxes in different sites. For example,  $N_2O$  fluxes seem to increase along the growing season in BERT1276 and decrease in CAST1850 (Table 4.5). Previous studies indicate that these differences may arise from the different length of the vegetation growing season and the local characteristics of each site, such as management (e.g. Imer *et al.*, 2013; Meurer *et al.*, 2016), C and N availability (e.g. Pilegaard *et al.*, 2006; Signor & Pellegrino Cerri, 2013), soil properties (e.g. Brentrup *et al.*, 2000; Snyder *et al.*, 2009), and microbial community dynamics (e.g. Jones *et al.*, 2013; Regan *et al.*,

2011). In the montane site BERT1276,  $T_s$  exhibited a strong positive influence on  $N_2O$  fluxes (Table 4.4). Our results are in line with previous findings that suggest a strong positive effect of  $T_s$  on  $N_2O$  fluxes (Zhang & Han, 2008; Luo *et al.*, 2013; Merbold *et al.*, 2014; Sosulski *et al.*, 2014), since temperature enhances microbial activity and soil respiration, and consequently denitrification (Signor & Pellegrino Cerri, 2013). In both the montane and the subalpine sites, our results revealed a negative effect of SWC on  $N_2O$  emissions. Liu *et al.* (2011) and Sosulski *et al.* (2014) observed a similar decrease in  $N_2O$  emissions with increasing soil moisture. This may be explained by the fact that very high soil moisture hinders the microbial activity, and the strictly anaerobic conditions may lead to the formation of  $N_2$  rather than  $N_2O$  (Butterbach-Bahl *et al.*, 2013).

#### 4.5.5 Comparison between GHG fluxes under light and dark conditions, with and without vegetation

The question about whether  $CH_4$  and  $N_2O$  fluxes act differently with vegetation or on bare soil, and under light or dark conditions, remains controversial. Our findings, in the case of  $CH_4$ , suggest that fluxes are higher on intact vegetation compared to bare soil at the subalpine grassland CAST1850. This is most likely attributed to the more diverse and denser vegetation compared to the other grassland sites because plants can serve as a conduit of methane (e.g. Nisbet *et al.*, 2009; Whiting *et al.*, 1991). In contrast, NIU2479 revealed a different effect of vegetation presence on  $CH_4$  fluxes. In fact, methane fluxes on bare soil were higher than fluxes on intact vegetation. This is probably the result of: 1) plant injury, which seems to be more pronounced in the alpine site than in the other sites, since several studies suggest that plant injury (cutting the vegetation) enhances emissions of  $CH_4$  (Wang *et al.*, 2009a, 2011; Bruhn *et al.*, 2012), 2) vegetation composition in NIU2479 because it is suggested that changes in vegetation composition may regulate  $CH_4$  exchange (Ribas *et al.*, 2015), due to their effect on soil labile carbon and enzymatic activity (Luan *et al.*, 2016). In the case of  $N_2O$ , there was a tendency that fluxes measured on intact vegetation are lower than those measured on bare soil. This may be attributed to the increased water filled pore space by vegetation cutting, and, in turn, increased soil moisture which can increase  $N_2O$  emissions (Rafique *et al.*, 2012), or to the potential role of vegetation on microbial processes coupled with  $N_2O$  fluxes (Shurpali *et al.*, 2016).

Whereas  $CH_4$  fluxes under light conditions did not vary from fluxes measured in the dark,  $N_2O$  fluxes were lower under light conditions. In fact, Stewart *et al.* (2012) reported that light can shift  $N_2O$  fluxes under dark conditions from sources to sinks because soil moisture – the main driver of  $N_2O$  – is reduced under light conditions. Also, the effect of light on  $N_2O$  fluxes may be connected to resource competition between vegetation and soil microbes in re-

sponse to light-driven changes in O<sub>2</sub> availability (Stewart *et al.*, 2012), or to the photosynthetic activity of the plants through increased root exudation, and hence increased C supply to soil microorganisms during light periods (Blackmer *et al.*, 1982).

## 4.6 Conclusions

**O**verall, the four extensively managed semi-natural grasslands in the Pyrenees were mainly sources of CH<sub>4</sub>, and our flux values were higher than previously reported in grasslands using chamber techniques. Yet, our CO<sub>2</sub> and N<sub>2</sub>O values were comparable to studies in European grasslands. The grasslands at low altitudes were generally small sources of N<sub>2</sub>O, while the subalpine and the alpine grasslands were small sinks. We also found that GHG fluxes decrease with altitude in extensively managed grasslands in the Pyrenees; the higher the grassland altitude (hence the colder) the lower the fluxes. Additionally, not only did CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O fluxes change along the altitudinal gradient, but also along different seasons, with the highest GHG emissions occurring in the summer period.

**W**e identified aboveground biomass and temperature as the main drivers for fluxes of CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O in the Pyrenean grasslands. Additionally, grazing proved to have an important role on both CH<sub>4</sub> and N<sub>2</sub>O in the subalpine grassland CAST1850, most likely through its effect on vegetation composition and plant biomass reduction. Seasonality and soil water content also demonstrated a strong influence on N<sub>2</sub>O fluxes in both the montane and the subalpine grasslands.

**O**ur findings on the effect of vegetation removal on CH<sub>4</sub> and light/darkness conditions on N<sub>2</sub>O fluxes underline the need to investigate more in depth these factors and better understand their role in GHG fluxes, notably the controversial role of vegetation in grasslands as emitter or conduit of methane to the atmosphere. We also suggest continuous or more frequent GHG measurements on grasslands in the Pyrenees. The importance of studying these particularly vulnerable ecosystems lies in the fact that they can act as carbon sinks and mitigate GHG emissions, especially at high altitudes as our results indicate. Moreover, future management practices in these semi-natural grasslands should focus on regulating the intensity of grazing and the animal type, and preventing the abandonment, degradation or conversion of these ecosystems in order to reduce C losses and increase C sequestration.

## Acknowledgments

We would like to thank Helena Sarri, Esther Iñiguez, Mercedes Ibañez, and Teresa Marí for their assistance in the sampling and in processing the vegetation biomass. We would also like to thank

Mari Pihlatie from the University of Helsinki for fruitful discussions and suggestions. This work was funded by the Spanish Science Foundation (FECYT) through the projects CAPAS (CGL2010-22378-C03-01) and BIOGEI (CGL2013-49142-C2-1-R). The project was also developed within the project CAPACITI supported by a Marie Curie Intra European Fellowship within the 7th European Community Framework to NA (PIEF-GA-2010-275855), HD was supported by a FPI fellowship from the Spanish Ministry of Economy and Competitiveness (BES-2011-047009). Work within the project CAMELEON (PIM2010ECR-00709) funded by an ERA-Net CIRCLE2 Mountain contributed to the development of many ideas about carbon dynamics in mountain ecosystems included in this paper. Funding through the project “Potential for capture or emission of greenhouse gases in agro-pastoral systems” (2012-AGEC-00080) by the Catalan Government is also acknowledged.

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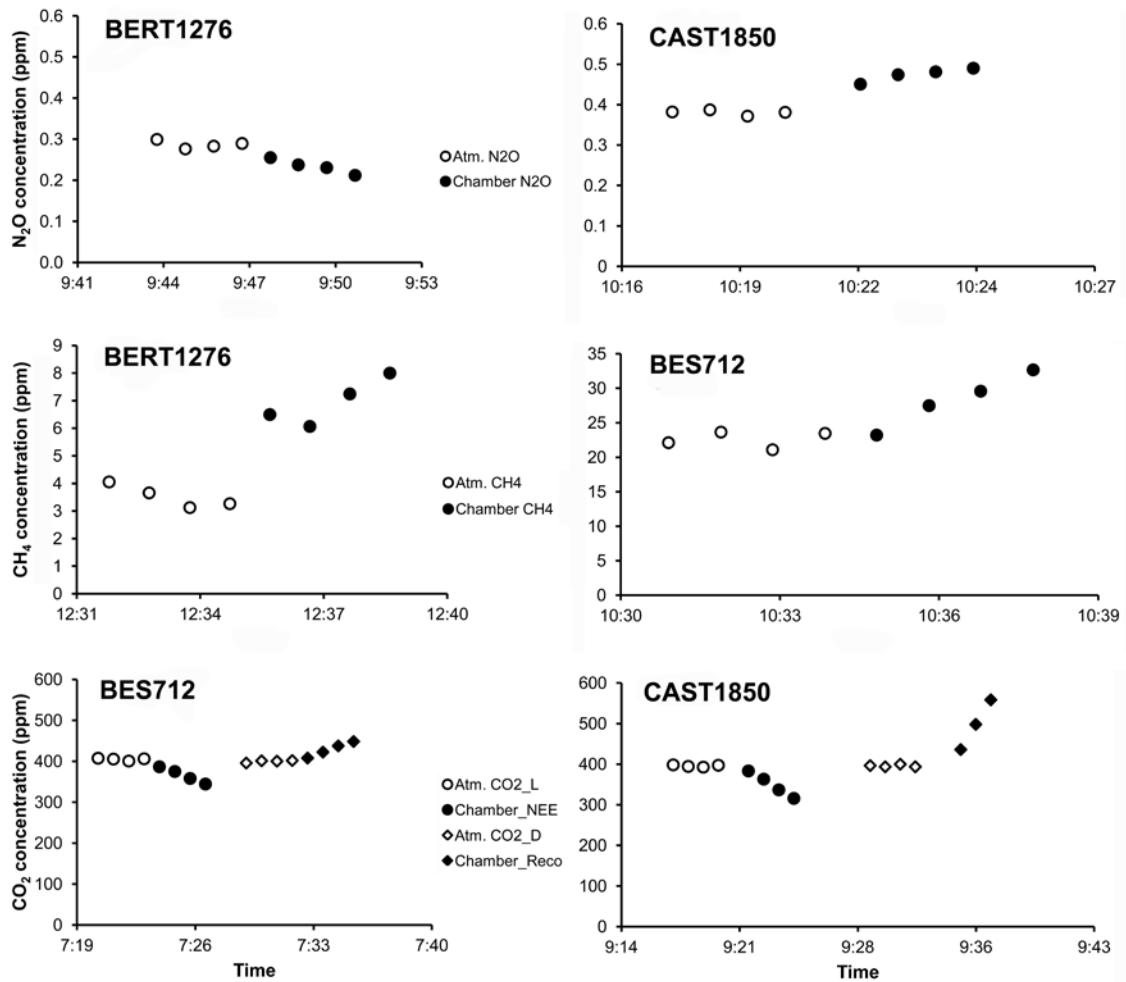
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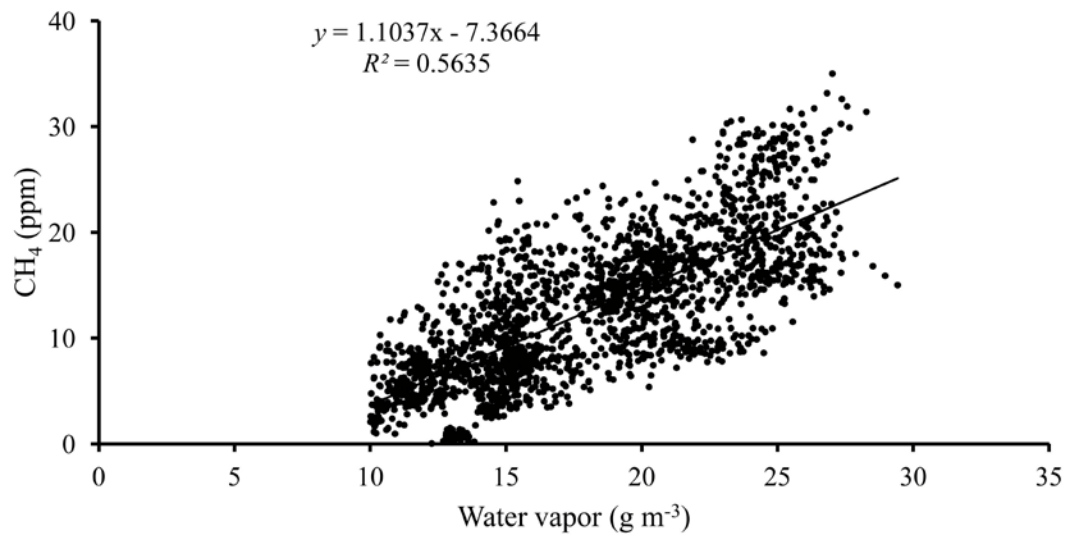
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## 4.8 Supporting information

### Supplementary figures

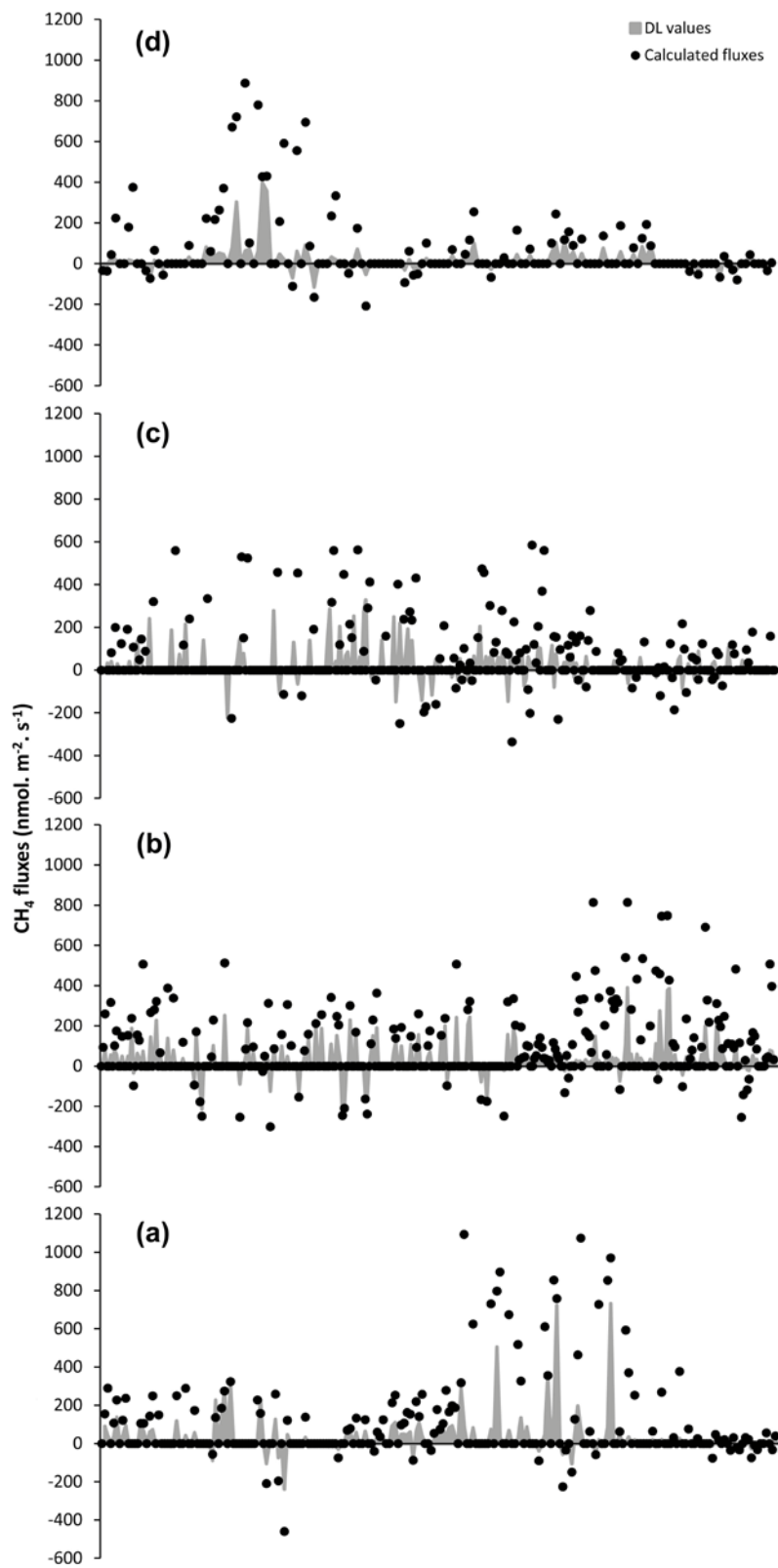


**Figure S4.1** | Examples of atmospheric/ambient and chamber closure concentrations of CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O along time. Atm. CO<sub>2</sub>\_L and Atm. CO<sub>2</sub>\_D = ambient CO<sub>2</sub> concentrations before chamber closure measurements under light (L) and dark (D) conditions; Chamber\_NEE = net ecosystem exchange inside the chamber; Chamber\_Reco = ecosystem respiration inside the chamber; Atm. CH<sub>4</sub> = ambient CH<sub>4</sub> concentrations; Chamber CH<sub>4</sub> = CH<sub>4</sub> concentrations inside the chamber; Atm. N<sub>2</sub>O = ambient N<sub>2</sub>O concentrations; Chamber N<sub>2</sub>O = N<sub>2</sub>O concentrations inside the chamber.

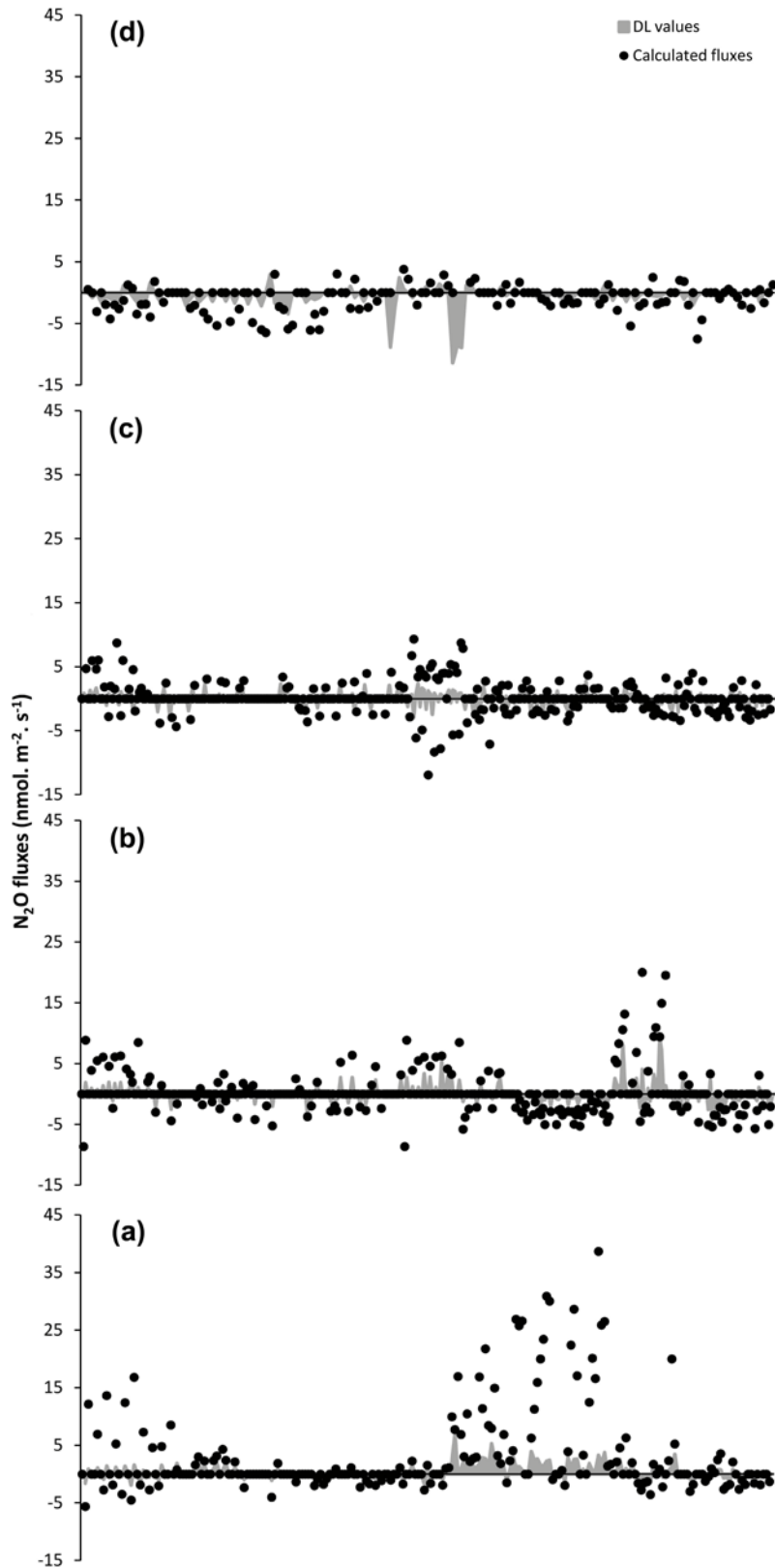


**Figure S4.2** | Relationship between CH<sub>4</sub> concentrations (ppm) measured with the PAS from ambient air and water vapor (g m<sup>-3</sup>) for all the four grassland sites.





**Figure S4.3** | Scatterplot presenting the total calculated CH<sub>4</sub> fluxes compared to the detection limit values (DL; gray-shaded area) in the four grassland sites along time, along the altitudinal gradient: a) Besora (BES712), b) La Bertolina (BERT1276), c) Castellar de n'Hug (CAST1850), d) Niu de l'Àliga (NIU2479).



**Figure S4.4** | Scatterplot presenting the total calculated N<sub>2</sub>O fluxes compared to the detection limit values (DL; gray-shaded area) in the four grassland sites along time, along the altitudinal gradient: a) Besora (BES712), b) La Bertolina (BERT1276), c) Castellar de n'Hug (CAST1850), d) Niu de l'Àliga (NIU2479).



# Chapter 5



# Effect of plant functional types on greenhouse gas fluxes in grasslands along a climatic gradient



An edited version of this chapter is in preparation for submission.

Debouk H., Llorba R., Altimir N., Ribas A., Mari T., Sebastià, M-T. 2017.

## 5.1 Abstract

**G**rasslands are expected to witness strong shifts in plant diversity and vegetation composition under climate change. Such changes may thus modify the feedback of grassland ecosystems to the atmosphere. Changes in composition of plant functional types (PFTs) can alter greenhouse gas (GHG) fluxes through the impact of vegetation on the biological, physical and chemical processes in the soil. However, a lot of uncertainties remain concerning the overall effects of single PFT and PFT interactions on GHG exchange. Also, the extent to which these effects are altered by environmental conditions is not fully known. We studied the effects of dominant PFTs on fluxes of carbon dioxide (CO<sub>2</sub>), methane (CH<sub>4</sub>), and nitrous oxide (N<sub>2</sub>O) in 14 grassland sites, all extensively managed. The locations are distributed along a climatic gradient in the North-Eastern Iberian Peninsula, representing six climatic zones: semi-arid, Mediterranean, montane, Atlantic, subalpine, and alpine. We measured the GHG fluxes at the peak of the vegetation biomass using transparent static chambers. We then collected vegetation samples and separated them into PFTs to calculate their proportions. Overall we found uptake of CO<sub>2</sub> and N<sub>2</sub>O, and emissions of CH<sub>4</sub> at the peak of the vegetation biomass in all the grassland sites. Only Atlantic grasslands, characterized by moist climate, exhibited N<sub>2</sub>O emissions instead of uptake. PFT composition significantly affected GHG fluxes, with the effect of PFT being strongly mediated by climatic conditions. The interaction between PFTs increased CH<sub>4</sub> and N<sub>2</sub>O uptake compared to single PFTs (identity effects). We provide evidence that plant functional type composition can alter GHG exchange, and that this alteration is mediated by the environmental conditions of the grasslands. Our findings also highlight the importance of functional diversity and its positive effect on CH<sub>4</sub> and N<sub>2</sub>O uptake, and consequently on the feedback from the ecosystem to the atmosphere.

## 5.2 Introduction

**U**nder climate change conditions, grasslands are expected to experience strong shifts in biodiversity and vegetation composition (Sala *et al.*, 2000; Sebastià *et al.*, 2008). These ecosystems can, in turn, alter climate change through direct and indirect impacts on carbon (C) and nitrogen (N) cycles (Owensby, 1998). In fact, it is suggested that changes in land cover and plant community composition in grasslands can play an important role in regulating gas exchange (Epstein *et al.*, 1998; Smith *et al.*, 2014). This is attributed to the fact that different plant functional types (PFTs) with different nutrient and water use efficiencies, and nutrient allocation into plant tissues (Hobbie, 1992, 1996; Fry *et al.*, 2013), alter differently the soil processes, such as soil organic matter (SOM) accumulation and decomposition, and C and N cycling (Vitousek *et al.*, 1987; Chapin *et al.*, 1996).

**G**reenhouse gas (GHG) emissions in grasslands – carbon dioxide (CO<sub>2</sub>), methane (CH<sub>4</sub>) and nitrous oxide (N<sub>2</sub>O) – mainly originate from soil processes (Bremner, 1997; Topp & Pattey, 1997; Le Mer & Roger, 2001; Butterbach-Bahl *et al.*, 2013). CO<sub>2</sub> is assimilated by the plants during photosynthesis and transformed into plant biomass. Once the biomass decays, it is transferred into soil organic matter, which in turn decays and releases CO<sub>2</sub> to the atmosphere (Brevik, 2012). Methane (CH<sub>4</sub>), the second most important GHG after CO<sub>2</sub> (IPCC, 2007), is produced by soil organisms mostly under anaerobic but also aerobic conditions. In soils where aerobic conditions dominate such as in grasslands, methanotrophic bacteria oxidize CH<sub>4</sub> into CO<sub>2</sub> (Le Mer & Roger, 2001). In submerged soils where anaerobic conditions dominate, methanogens utilize easily degradable carbon sources to produce methane – a process known as methanogenesis. CH<sub>4</sub> is thus either produced or consumed in the soil by methanogenic or methanotrophic bacteria respectively, which makes soils either sources or sinks of CH<sub>4</sub> (Le Mer & Roger, 2001). As for N<sub>2</sub>O, it is formed in the soil by a series of complex microbial metabolic pathways, known mainly as nitrification and denitrification processes (Schlesinger 1996; Butterbach-Bahl *et al.*, 2013). Nitrification is a two-step process that occurs mainly under aerobic conditions, and in which ammonium (NH<sub>4</sub><sup>+</sup>) is converted to nitrite (NO<sub>2</sub><sup>-</sup>), which is subsequently oxidized to nitrate (NO<sub>3</sub><sup>-</sup>) by autotrophic bacteria (Venterea *et al.*, 2012). Denitrification, on the other hand, is the reduction of NO<sub>3</sub><sup>-</sup> to NO, N<sub>2</sub>O, and N<sub>2</sub> by heterotrophic bacteria and is the main source of N<sub>2</sub>O under anaerobic conditions (Venterea *et al.*, 2012). Chemo-denitrification of soil nitrite and abiotic decomposition of ammonium nitrate are other processes contributing to N<sub>2</sub>O formation in soils, which occur in the presence of light, humidity and reacting surfaces (Bremner 1997; Butterbach-Bahl *et al.*, 2013).

Any alteration in vegetation composition will have consequences on GHG fluxes, because of the strong interactions between the functional composition of plant communities and nutrient inputs for soil processes involved in GHG exchange (Niklaus *et al.*, 2006, 2016). Yet, great uncertainties remain regarding the effects of vegetation composition change and its consequences on GHG exchange, notably on CH<sub>4</sub> and N<sub>2</sub>O, and more importantly regarding how these effects vary along environmental gradients. The effects of vegetation composition on ecosystem functions such as GHG fluxes include the effects of individual species or individual PFT (identity effects), and those of pairwise interactions between species or PFTs based on their relative abundance (Kirwan *et al.*, 2007). Since climate change is expected to alter vegetation composition in grasslands and reduce diversity in these ecosystems, the effects of different PFTs and their interactions with environmental conditions on GHG emissions need to be better understood. Particularly, not much is known about the effects and interactions of PFTs on GHG fluxes in extensively managed grassland ecosystems compared to wetlands and peatlands (e.g. Kuiper *et al.*, 2014; Robroek *et al.*, 2015; Ward *et al.*, 2013), and sown pastures and croplands (e.g. Dusenbury *et al.*, 2008; Ribas *et al.*, 2015; Rochette and Janzen, 2005). Furthermore, legumes are known to enhance N<sub>2</sub>O emissions due to their N<sub>2</sub> fixing properties (Jensen *et al.*, 2012; Lüscher *et al.*, 2014), which leads to higher N contents in the soil (Whitehead, 1995). Grasses, on the other hand, are believed to cause less N<sub>2</sub>O emissions and can be potential sinks of nitrogen (Ribas *et al.*, 2015). However, less attention has been paid to the effects of other PFTs in grassland ecosystems such as non-legume forbs, sedges or subshrubs/woody plants. Investigating woody plants is, in fact, important because their encroachment into European grasslands is expected to accelerate under climate and land use changes (Komac *et al.*, 2014); a process that alters ecosystem structure and functioning (Briggs *et al.*, 2005; Eldridge *et al.*, 2011).

In addition to PFT identity effects, a great uncertainty also surrounds the effects of the interactions between PFTs on the highly variable CH<sub>4</sub> and N<sub>2</sub>O fluxes, due to the complexity and large number of processes involved. For instance, interactions between grasses and legumes in a cropland seem to enhance N<sub>2</sub>O uptake and to a certain extent reduce CH<sub>4</sub> emissions compared to monocultures, as observed by Ribas *et al.* (2015). However, the effects of further interactions between PFTs on GHG exchange are still not fully known. What is known is that plant diversity can alter the processes involved in uptake or emission of CH<sub>4</sub> and N<sub>2</sub>O through its positive effects on productivity, water use, and root density and architecture (Niklaus *et al.*, 2016). For example, increased productivity as a result of increased diversity provides soil microbial communities with more organic substrates which could increase heterotrophic activity and oxygen consumption (Sextone *et al.*, 1985). Increased water use with increased diversity, on the other hand, may reduce soil moisture and increase soil diffusivity (Leimer *et al.*, 2014; Spehn *et al.*, 2000,) and consequently inhibit denitrification. Soil aeration and carbon and nitrogen availability may also

increase with increased diversity due to the effects of the latter on root density and architecture (Niklaus *et al.*, 2016).

A thorough examination of plant functional types (PFTs) in grasslands, their interactions with climatic conditions, and the consequences of such interactions on soil biological, physical and chemical processes are therefore needed to determine ecosystem responses, particularly GHG exchange, in a changing environment. Here we present a study investigating the effects of PFTs and climatic conditions, in combination with their interactions on fluxes of CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O. The particular aims of the study were to investigate: 1) The effects of PFT composition, including both identity effects and pairwise interactions, on greenhouse gas fluxes: CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O; 2) if the climatic conditions modify the relationship between PFTs and GHG fluxes.

## 5.3 Material and Methods

### 5.3.1 Site description

Our study was carried out in a total of 14 grassland sites (Fig. 5.1). The sites are located in the North-Eastern Iberian Peninsula along a climatic gradient, ranging from semi-arid to subalpine grasslands, all characterized by a low-intensity management of extensive seasonal grazing. The 14 grassland sites have different climatic conditions, varying from warm continental in the low-altitude sites, to cold temperate conditions in the high-altitude sites. Since several grassland sites have similar climatic (mean annual precipitation and temperature) and vegetation (dominant PFT and species) characteristics (Table 5.1), we grouped the 14 grasslands in six climatic zones: Mediterranean (PRAT, BEL, BLL), semi-arid (MON, ALG, FAR), montane (BES, BERT, PALL1, PALL2), Atlantic (IRA1, IRA2), subalpine (CAST), and alpine (NIU). The climatic data of the 14 grassland sites were obtained from local climatic atlases (Ninyerola *et al.*, 2005, 2003, 2000; Pons, 1996).

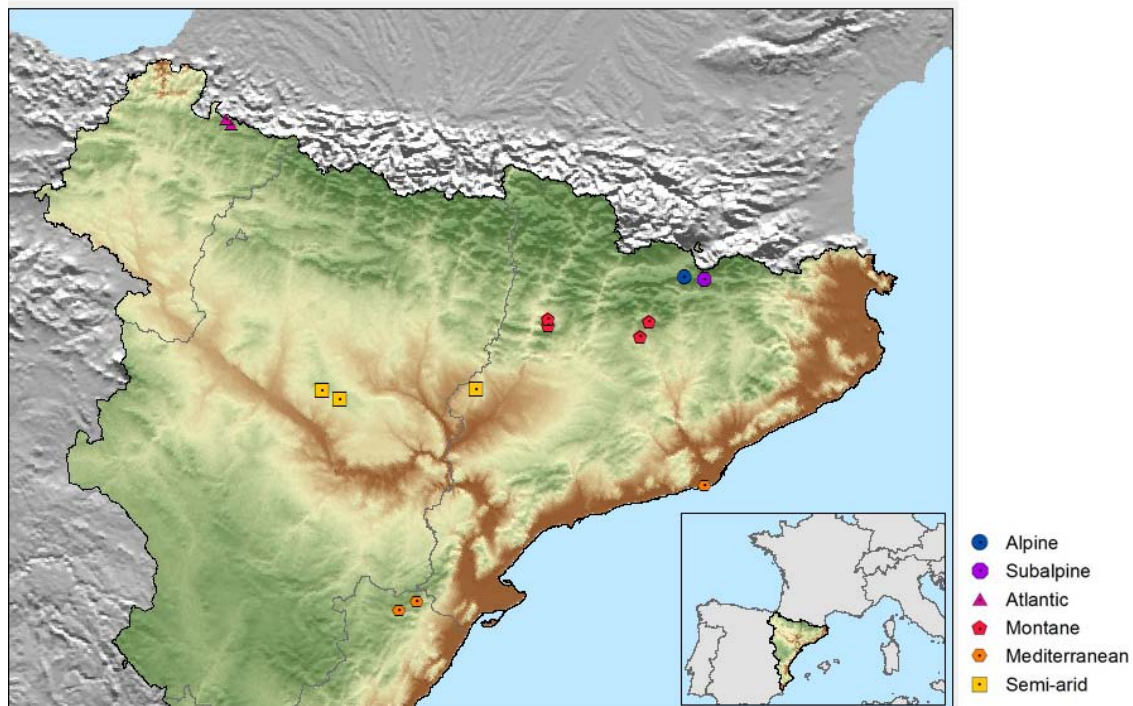
The vegetation in the grassland sites is dominated by grasses and forbs, the latter including legumes and non-legumes except for the semi-arid sites where, in addition to grasses and non-legume forbs, woody plants are abundant (see Figure S5.1 in Supplementary material for average proportions of PFT in each climatic zone). The woody plants correspond to subshrubs, such as *Thymus vulgaris* L. and *Lavandula angustifolia* Mill. In the alpine grassland site NIU, sedges are more dominant than legumes. They are also common in the montane grassland site PALL1 and the subalpine site CAST.

**Table 5.1** | General characterization of the 14 grassland sites; MAT = mean annual temperature; MAP = mean annual precipitation; PFT = plant function type.

Site	Altitude (m)	MAP (mm)	MAT (°C)	Climatic zone	Soil pH	Live-stock	Dominant PFTs	Dominant species
PRAT	5	664.90	15.60	Mediterranean	8.62	Sheep	F,G,L	<i>Galium</i> sp., <i>Vicia</i> sp.
ALG	334	430.50	13.90	Semi-arid	8.54	Sheep	F,G,W	<i>Plantago lanceolata</i> L., <i>Thymus vulgaris</i> L.
FAR	488	396.40	13.60	Semi-arid	8.14	Sheep	G,W	<i>Stipa parviflora</i> Desf., <i>Thymus vulgaris</i> L.
PALL2	622	666.40	11.90	Montane	7.94	Sheep	F,G,W	<i>Eryngium campestre</i> L., <i>Thymus vulgaris</i> L.
BLL	636	714.90	13.60	Mediterranean	7.99	Mixed	F,G,L,W	<i>Medicago</i> sp., <i>Eryngium campestre</i> L.,
MON	657	225.20	12.60	Semi-arid	8.19	Sheep	F,G,W	<i>Papaver rhoeas</i> L., <i>Bromus</i> sp., <i>Thymus vulgaris</i> L.
BES	712	720.50	11.70	Montane	8.19	Cattle	F,G,L	<i>Bromus hordeaceus</i> L., <i>Plantago lanceolata</i> L.
BEL	860	800.80	12.30	Mediterranean	7.94	Mixed	F,G,L,W	<i>Eryngium campestre</i> L., <i>Trifolium</i> sp., <i>Lavandula angustifolia</i> Mill.
PALL1	937	775	10.40	Montane	7.78	Sheep	F,G,S,L,W	<i>Festuca</i> sp., <i>Carex</i> sp., <i>Plantago media</i> L.
IRA2	1014	1594.20	10.60	Atlantic	4.76	Mixed	F,G,L	<i>Trifolium alpinum</i> L., <i>Festuca</i> sp.
IRA1	1064	1413	9.50	Atlantic	5.19	Mixed	F,G,L	<i>Trifolium alpinum</i> L., <i>Festuca</i> sp.
BERT	1276	954.80	8.70	Montane	8	Cattle	F,G,L	<i>Festuca arundinacea</i> Schreb., <i>Trifolium repens</i> L.
CAST	1850	1199	5.40	Subalpine	5.42	Mixed	F,G,L,S	<i>Festuca nigrescens</i> Lam., <i>Carex caryophylla</i> Latourr.
NIU	2479	1302.10	2.40	Alpine	6.09	Mixed	F,G,S	<i>Festuca airoides</i> Lam., <i>Carex caryophylla</i> Latourr.

\*MAP and MAT are acquired from the "Atlas climatic digital de Catalunya" (Pons 1996; Ninyerola et al. 2000, 2003) and from the "Atlas Climático Digital de la Península Ibérica" (Ninyerola et al. 2005).





**Figure 5.1** | Map showing the locations of the 14 grassland sites and their six corresponding climatic zones: semi-arid (Alguaire, Monegrillo, Farlete), Mediterranean (El Prat, Bel, Ballestar), montane (Pallars Jussà 1 and 2, Besora, La Bertolina), Atlantic (Irati 1 and 2), subalpine (Castellar de n'Hug), and alpine (Niu de l'Aliga).

### 5.3.2 Experimental design

The common sampling strategy aimed to collect chamber-based flux data of CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O, and aboveground biomass. The sampling was carried out in the year 2013, during one day in each site at the peak of the vegetation, when there is the maximum live biomass and vegetation activity. The measurements took place during daylight (between 8 am and 4 pm approximately; solar time). Sampling points were spread over a grid in the grassland, covering an area of about 50-70 m radius. We chose the points to represent the dominant plant functional types (PFT), -grasses, sedges, legumes, non-legume forbs, and woody-, with three replicates per PFT. Therefore, we had a total of nine to twelve sampling points per measurement site, depending on whether the site was dominated by three or four PFT. In the case of the semi-arid site FAR (dominated by two PFT) and the montane site PALL1 (dominated by 5 PFT), we had a total of six and 15 sampling points, respectively. We sampled the points by placing collars (height = 8 cm; diameter = 25 cm) 3 cm deep into the ground.

We used a self-assembled portable gas-exchange system to perform *in-situ* field surveys. The system consisted of a cylindrical uncoated transparent methacrylate chamber (20L nominal volume), connected to a multi-gas analyzer through Teflon tubing (see chapter 4 for details). We measured all gases simultaneously with a photoacoustic spectroscopy (PAS) analyzer (INNOVA 1412, LumaSense Technologies, Denmark). The PAS was calibrated prior to the field

campaigns by the vendor in the customary way (Moody *et al.*, 2008). The analyzer was used in the cross-interference and the water-interference modes, to take into account the cross interference between gases and the interference of water vapor in the measure of gases (for more details on PAS modus-operandi and comparability see Iqbal *et al.*, 2013).

**D**uring measurements, the chambers were moved manually between sampling points. Flux measurements were done by placing the chamber around the collar to enclose the vegetation and soil for about four minutes. The chamber was always left open for four minutes before each flux measurement to ensure ventilation of the chamber headspace, and to obtain the values of ambient gas concentrations. We measured fluxes of intact vegetation and undisturbed soil under light conditions. Afterwards, once the GHG measurements were completed, we harvested the plants at ground level. We later separated the vegetation samples in the laboratory into plant functional types. We placed the samples in the oven at 60°C for 48 hours to obtain the dry weight of each PFT, and we calculated afterwards the proportion of each PFT per site. A soil sample of approximately 250 g representative of each study site was also collected at 10 cm depth for further soil analyses. The soil samples were first placed in the oven and dried at 60°C to a constant weight. Soil was later sieved through a 2 mm sieve in order to remove all the roots and stones present. A representative subsample of 100-150 g of the obtained fine soil from each study site was then packed and sent to the laboratory for soil analyses, to determine: pH in the supernatant of a 1:2.5 solid-to-liquid (g/ml) ratio suspension of soil sample in water, soil organic nitrogen (SON) with the Kjeldahl acid-digestion method (Bremner & Mulvaney, 1982), soil organic matter (SOM) with the Walkley-Black method (Walkley & Black, 1934), and carbon-to-nitrogen ratio (C:N) with total C and N determined by the elemental auto-analyzer.

### 5.3.3 Flux calculations

**T**he flux was computed using the change in gas concentration monitored during the closure. When the chamber is placed in the collar, the plant-soil system is forced into a dynamic state where light, temperature, humidity and gas concentrations change due to the activity of the soil-plant system, which in turn responds to the change. The flux of interest is the rate of concentration change since the time of closure. We used a linear estimation to calculate the flux as the slope of the relationship of gas concentration versus time:

$$F = \frac{V\delta C}{A\delta t}$$

where  $F$  is the flux in mol/s,  $V$  is the chamber volume in  $\text{m}^3$ ,  $A$  is the chamber surface area in  $\text{m}^2$ ,  $\delta C$  is the gas concentration in  $\text{mol}/\text{m}^3$ , and  $t$  is the time in s. Positive flux values refer to gas

emissions to the atmosphere, and negative values represent uptake of the gas by the vegetation and/or the soil. The CO<sub>2</sub> flux represents the net exchange as the balance between assimilation and respiration processes of soil and vegetation, and we will refer to it as net ecosystem exchange (NEE). For more details on flux calculations and data quality, see chapter 4.

### 5.3.4 Data analysis

We carried out general linear models (GLM) to test the effects of climatic and soil conditions, and PFT composition (identity effects, pairwise interactions, evenness) on net ecosystem exchange (NEE), and fluxes of CH<sub>4</sub> and N<sub>2</sub>O in 14 grassland sites along a climatic gradient. The climatic variables included: mean annual temperature (MAT), mean annual precipitation (MAP), Lang's rain factor (Lang 1920), and mean daily air temperature during the sampling date (T<sub>a</sub>). The soil variables included: pH, soil organic nitrogen (SON), soil organic matter (SOM), and carbon-to-nitrogen (C:N) ratio). We selected the best models according to the Akaike information criterion (AIC). Based on the best models for each gas, we calculated the coefficient estimates of the diversity-flux functions per climatic zone as a result of a change in PFT proportions. The experimental setup followed a simplex design (Kirwan et al. 2007; 2009), with PFT proportions and evenness ranging between 0 and 1, and the response variable regressed against these. The simplex design was illustrated with ternary plots. The latter depict with a triangle the proportions of three variables (in our case three PFTs) which sum to a total value of 1. The GLMs were conducted using SAS 9.1 software (SAS Institute Inc., Cary, NC, USA), and the ternary plots were built using the packages: "rsm" (Lenth, 2009), "lattice" (Sarkar, 2008), "lme4" (Bates *et al.*, 2012), "arm" (Gelman & Su, 2013), "ez" (Lawrence, 2012), and RcolorBrewer" (Neuwirth, 2011) of R software (R Core team, 2013).

## 5.4 Results

### 5.4.1 Magnitude and range of fluxes

The absolute values of NEE and fluxes of CH<sub>4</sub> and N<sub>2</sub>O varied along the climatic gradient and with different soil conditions (Fig. S5.2). Mean NEE values were negative in all climatic zones, indicating CO<sub>2</sub> uptake/assimilation, and ranged between  $-2.94 \pm 0.83 \mu\text{mol m}^{-2} \text{s}^{-1}$  and  $-10.16 \pm 1.19 \mu\text{mol m}^{-2} \text{s}^{-1}$  in the Atlantic and the subalpine grasslands, respectively. As for methane, fluxes in all the climatic zones were positive, indicating emissions to the atmosphere, with values ranging between  $72.97 \pm 108.33$  in the subalpine grassland and  $499.38 \pm 262.04 \text{ nmol m}^{-2} \text{s}^{-1}$  in the semi-arid grasslands. N<sub>2</sub>O fluxes were, however, negative in all sites indicating mainly uptake, except in the Atlantic grasslands where the values were positive. N<sub>2</sub>O values ranged be-

tween  $-3.06 \pm 0.42 \text{ nmol m}^{-2} \text{ s}^{-1}$  in Mediterranean grassland sites and  $1.15 \pm 0.77 \text{ nmol m}^{-2} \text{ s}^{-1}$  in the Atlantic sites (Table 5.2).

1 **Table 5.2** | General characterization of the six climatic grassland zones; MAT = mean annual temperature; MAP = mean annual precipitation; PFT = plant function type; NEE = net  
 2 ecosystem exchange.

Site	MAP (mm)	MAT (°C)	Lang	Soil pH	SON	C:N ratio	SOM	Dominant PFTs	NEE ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	CH <sub>4</sub> ( $\text{nmol m}^{-2} \text{s}^{-1}$ )	N <sub>2</sub> O ( $\text{nmol m}^{-2} \text{s}^{-1}$ )
Mediterranean	727	14	52.56	8.18	0.32	24.85	6.52	F,G,L	-6.73±0.69	284.76±70.15	-3.06±0.42
Semi-arid	328	13	24.74	8.37	0.15	65.58	2.6	F,G,W	-7.98±1.03	499.38±262.04	-1.63±0.35
Montane	779	11	72.94	8	0.27	28.2	4.44	F,G,L	-5.26±0.53	425.45±46.29	-1.54±0.38
Atlantic	1500	10	150	5	0.5	11.64	8.8	F,G,L	-2.94±0.83	453.74±120.76	1.15±0.77
Subalpine	1199	5	222	5.42	0.68	12.19	9.88	F,G,L,S	-10.16±1.19	72.97±108.33	-0.16±1.19
Alpine	1302	2	542.54	6.09	0.57	13.98	9.14	F,G,S	-3.62±0.73	131.81±50.36	-2.86±0.43

3 \*The presented values correspond to the average values in the grassland sites of each climatic zone.

## 5.4.2 Effects of climatic conditions and PFT composition on GHG fluxes

### 5.4.2.1 Net ecosystem exchange

In general, mean NEE values in the warmer and drier zones (Mediterranean, semi-arid and montane) were higher than in the colder and more humid grasslands (Atlantic and alpine), except for the subalpine grassland which exhibited the highest CO<sub>2</sub> uptake among all sites despite its cold and moist climatic conditions (Table 5.2 and Fig. S5.2). Indeed, the NEE model showed that sites with higher MAT, instantaneous air temperature ( $T_a$ ), soil pH, soil C:N ratio and lower MAP had higher CO<sub>2</sub> uptake (Table 5.3). Plant functional types had also a significant effect on CO<sub>2</sub> uptake, and this effect depended on Lang's rain factor (Table 5.3). For instance, patches of forbs exhibited the highest CO<sub>2</sub> uptake, but only in cold and humid grasslands characterized by high Lang's rain factor (Table S5.1 and Fig. 5.2). Meanwhile in warmer and drier grasslands with low Lang, legume patches were the ones exhibiting the highest CO<sub>2</sub> uptake with coefficient estimates of  $-10.21 \mu\text{mol m}^{-2} \text{s}^{-1}$  ( $P < 0.001$ ) in the Mediterranean sites, and  $-9.34 \mu\text{mol m}^{-2} \text{s}^{-1}$  ( $P < 0.001$ ) in the montane sites. In semi-arid systems, the highest CO<sub>2</sub> uptake (most negative NEE) was detected in woody species patches with a coefficient estimate of  $-11.60 \mu\text{mol m}^{-2} \text{s}^{-1}$  ( $P < 0.001$ ), showing higher uptake compared to grass and forb patches (Figs. 5.2 and S5.3). In mixed forb/grass patches, we detected lower CO<sub>2</sub> uptake (Table 5.3) compared to patches consisting of either forbs or grasses separately, however only in colder and more humid sites with higher Lang's rain factor, and not in the warmer and more arid sites (Table S5.1 and Fig. 5.2).

**Table 5.3** | GLM testing the effect of diversity, individual climatic and soil variables and their interactions on NEE fluxes of the 14 grassland sites in 2013; *p* values are given for the individual drivers ( $T_a$  = mean air temperature; MAP = mean annual precipitation; MAT = mean annual temperature; Lang = MAP/MAT; F = forb; G = grass; L = legume; S = sedge; W = woody).

Model	R <sup>2</sup> adj.	Estimate	Std. Error	<i>P</i>
***	0.84			
F		42.09	7.88	***
G		41.32	8.05	***
L		35.43	8.88	***
W		31.41	7.53	***
S		32.59	8.19	***
$T_a$		-0.16	0.05	**
MAP		0.005	0.003	*
MAT		-0.96	0.32	**
pH		-2.88	0.71	***
C:N ratio		-0.07	0.02	***
Lang		0.02	0.05	n.s.
F*G		-11.80	9.89	n.s.
Lang*F		-0.21	0.06	***
Lang*G		-0.18	0.05	***
Lang*L		-0.16	0.05	**
Lang*F*G		0.21	0.10	*

n.s.: not significant. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

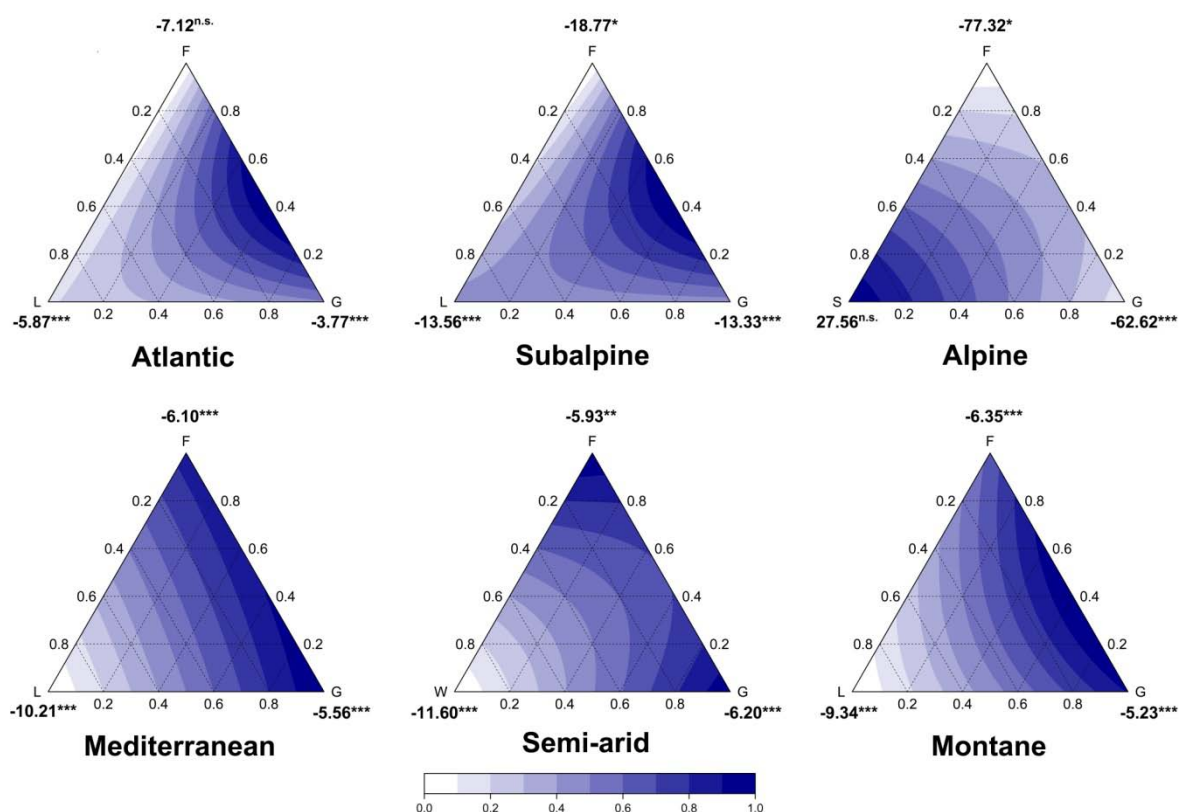
#### 5.4.2.2 CH<sub>4</sub> fluxes

The range of CH<sub>4</sub> fluxes changed along the climatic gradient, where sites with higher MAT and SON, and lower  $T_a$  and SOM, had lower CH<sub>4</sub> fluxes (Table 5.4). Methane fluxes were positive for all patches, regardless of the dominant PFT. Also, CH<sub>4</sub> emissions from PFT patches varied with Lang's rain factor. For instance, in colder and more humid grasslands characterized by higher Lang (Table 5.2), patches of forbs exhibited the highest CH<sub>4</sub> emissions with a coefficient estimate of 434.97 nmol m<sup>-2</sup> s<sup>-1</sup> (*P* < 0.05) in montane, and 588.33 nmol m<sup>-2</sup> s<sup>-1</sup> (*P* < 0.01) in Atlantic (Table 5.4 and Fig. 5.3) grasslands. These emissions were higher than those of forb patches in warmer and more arid grassland sites with estimates of 305.64 nmol m<sup>-2</sup> s<sup>-1</sup> (*P* = 0.09) in semi-arid and 412.69 nmol m<sup>-2</sup> s<sup>-1</sup> (*P* < 0.05) in Mediterranean (Table 5.4 and Fig. 5.3) grasslands. In the semi-arid and Mediterranean grasslands where woody plants are more dominant than in other sites, CH<sub>4</sub> emissions were higher under woody patches (*P* < 0.001) than under other PFT patches (Table 5.4 and Figs. 5.3 and S5.4). In patches of legumes and woody plants, the interaction between the two PFTs, despite being non-significant (*P* = 0.13; Table 5.4), reduced CH<sub>4</sub> emissions (Fig. 5.4). In an alternative model (see Table S5.2 in Supplementary material), we observed that the summed interactions of woody plants with the rest of PFTs decreased CH<sub>4</sub> emissions with an estimate of -2751.17 nmol m<sup>-2</sup> s<sup>-1</sup> (*P* < 0.05), thus offsetting the positive contribution of woody plants to methane emissions.

**Table 5.4** | GLM testing the effect of diversity, individual climatic and soil variables and their interactions on CH<sub>4</sub> fluxes of the 14 grassland sites in 2013; *p* values are given for the individual drivers (*T*<sub>a</sub> = mean air temperature; MAT = mean annual temperature; SON = soil organic nitrogen; SOM = soil organic matter; Lang = MAP/MAT; F = forb; G = grass; L = legume; S = sedge; W = woody).

	Model	R <sup>2</sup> adj.	Estimate	Std. Error	<i>P</i>
	***	0.41			
F			1371.44	707.95	n.s.
G			1236.49	728.72	n.s.
L			1323.90	817.23	n.s.
S			997.26	790.63	n.s.
W			2984.56	816.52	***
<i>T</i> <sub>a</sub>			24.10	9.53	*
MAT			-125.65	48.97	*
SON			-4834.12	1815.17	**
SOM			282.87	93.37	**
Lang			-24.84	8.65	**
L*W			-5310.10	3496.14	0.13
Lang*F			23.50	8.84	**
Lang*G			23.05	8.68	**
Lang*L			22.81	9.37	*
Lang*S			22.69	8.86	*

n.s.: not significant. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.



**Figure 5.2** | Ternary plots of NEE ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) of the PFT proportions (F: forb; L = legume; G = grass; S = sedge; W = woody), in grasslands of six climatic zones. Ternary plots are scaled to have a maximum of 1. Color intensity shows a gradient from the most negative flux value/highest uptake (white) to the lowest uptake (dark blue). *P*-values of estimates: n.s. not significant. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.



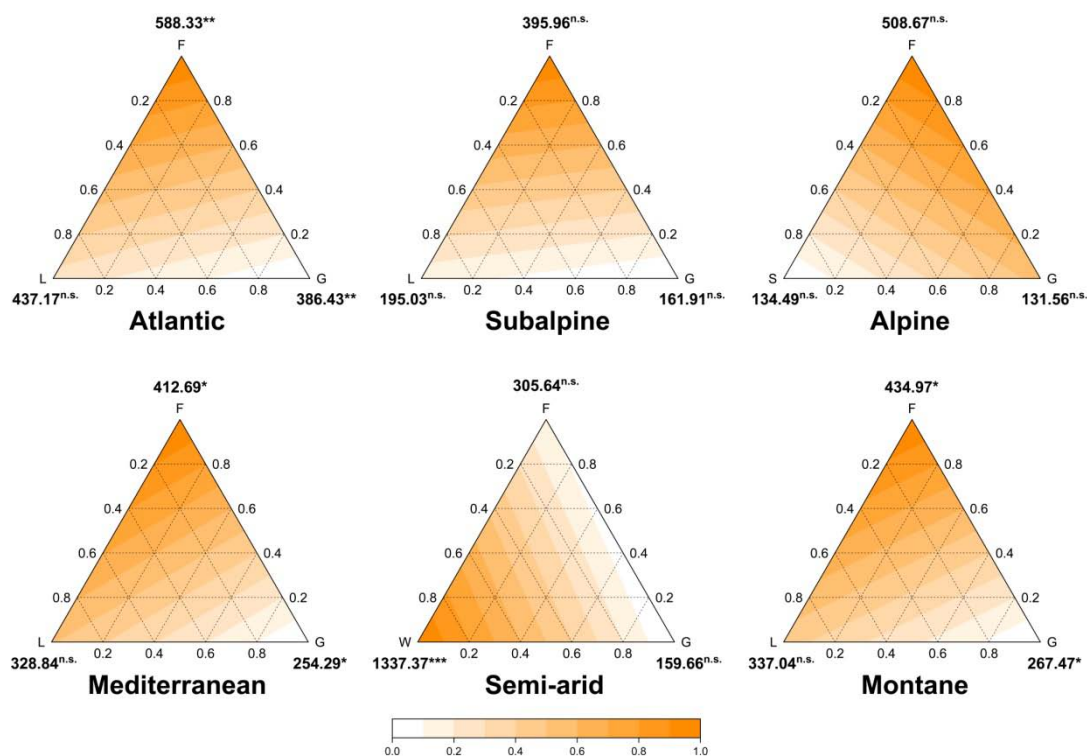
### 5.4.2.3 N<sub>2</sub>O fluxes

**N**itrous oxide fluxes changed with climatic and soil conditions, where grassland sites with higher  $T_a$  and lower SON had lower uptake of N<sub>2</sub>O (Table 5.5). We observed uptake of N<sub>2</sub>O in all the grassland sites, except the Atlantic ones, which exhibited mainly emissions. The highest emissions of N<sub>2</sub>O with an estimate of 8.33 nmol m<sup>-2</sup> s<sup>-1</sup> ( $P < 0.001$ ) occurred in legume patches in Atlantic grasslands, characterized by high precipitation rates and humid conditions (Tables 5.1 and 5.2). In the subalpine and alpine grasslands, legume patches exhibited also emissions of N<sub>2</sub>O compared to uptake by other PFTs, with coefficient estimates of 3.21 nmol m<sup>-2</sup> s<sup>-1</sup> ( $P < 0.05$ ) and 3.20 nmol m<sup>-2</sup> s<sup>-1</sup> ( $P < 0.05$ ) in the subalpine and alpine grasslands respectively (Figs. 5.5 and S5.5). In the warmer and drier grasslands (semi-arid, Mediterranean, montane), we observed N<sub>2</sub>O uptake even in legume patches (Table 5.5). Interactions between different PFTs, namely between legumes and grasses, and legumes and woody plants, had a negative effect on N<sub>2</sub>O fluxes regardless of the climatic zone, suggesting higher uptake or lower emissions of N<sub>2</sub>O in these patches (Table 5.5). Indeed in all the climatic zones, excluding the Atlantic and the alpine sites, the combination of grasses and legumes showed the highest N<sub>2</sub>O uptake (Figs. 5.5 and S5.5), with estimates of -7.11 nmol m<sup>-2</sup> s<sup>-1</sup> in semi-arid, -5.69 nmol m<sup>-2</sup> s<sup>-1</sup> in Mediterranean, -5.13 nmol m<sup>-2</sup> s<sup>-1</sup> in montane, and -3.19 in subalpine grasslands ( $P < 0.001$  for all estimates). Further, we observed that the pairwise interaction of forbs and sedges decreased N<sub>2</sub>O emissions while the interaction of grasses with sedges enhanced it, but only in the alpine site, characterized by high MAP and a dominance of sedges rather than legumes (Table 5.2 and Fig. 5.5).

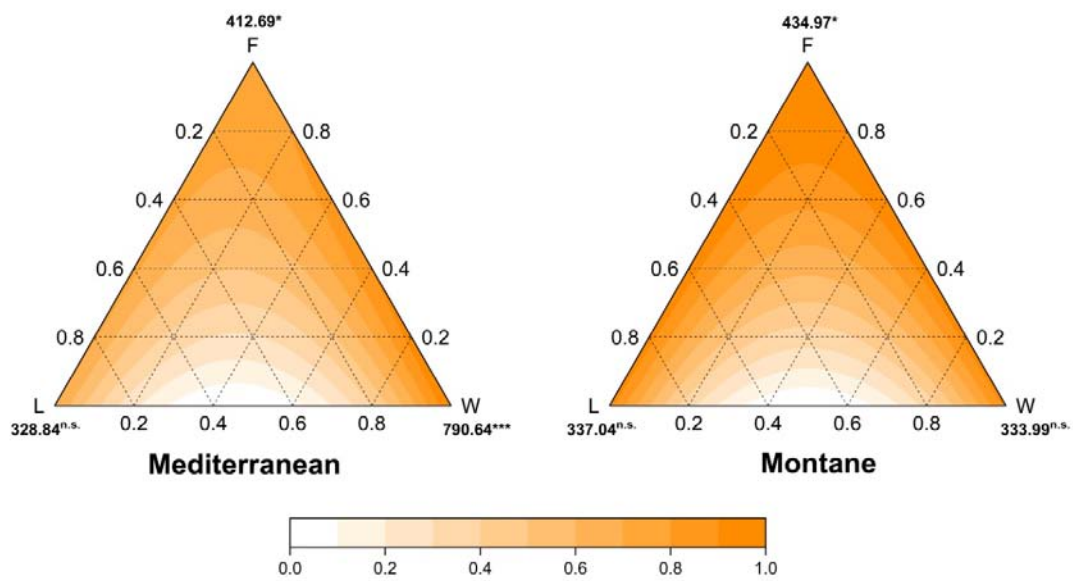
**Table 5.5** | GLM testing the effect of diversity, individual climatic and soil variables and their interactions on N<sub>2</sub>O fluxes of the 14 grassland sites in 2013; *p* values are given for the individual drivers (*T*<sub>a</sub> = mean air temperature; SON = soil organic nitrogen; MAP = mean annual precipitation; F = forb; G = grass; L = legume; S = sedge; W = woody).

Model	R <sup>2</sup> adj.	Estimate	Std. Error	<i>P</i>
***	0.58			
F		2.64	5	n.s.
G		3.04	5.25	n.s.
L		-6.86	6.84	n.s.
S		15.36	15.74	n.s.
W		2.93	4.61	n.s.
<i>T</i> <sub>a</sub>		0.15	0.03	***
pH		-0.85	0.51	n.s.
SON		-3.62	1.60	*
MAP		0.01	0.004	**
F*G		-7.98	8.53	n.s.
F*S		353.80	134.70	**
G*L		-11.93	4.24	**
G*S		-128.60	56.81	*
L*S		44.91	25.29	n.s.
L*W		-26.31	12.28	*
S*W		59.77	31.37	n.s.
MAP*F		-0.01	0.004	**
MAP*G		-0.01	0.003	***
MAP*S		-0.02	0.01	n.s.
MAP*W		-0.01	0.005	*
MAP*F*G		0.02	0.01	n.s.
MAP*F*S		-0.46	0.17	**
MAP*G*S		0.14	0.05	**

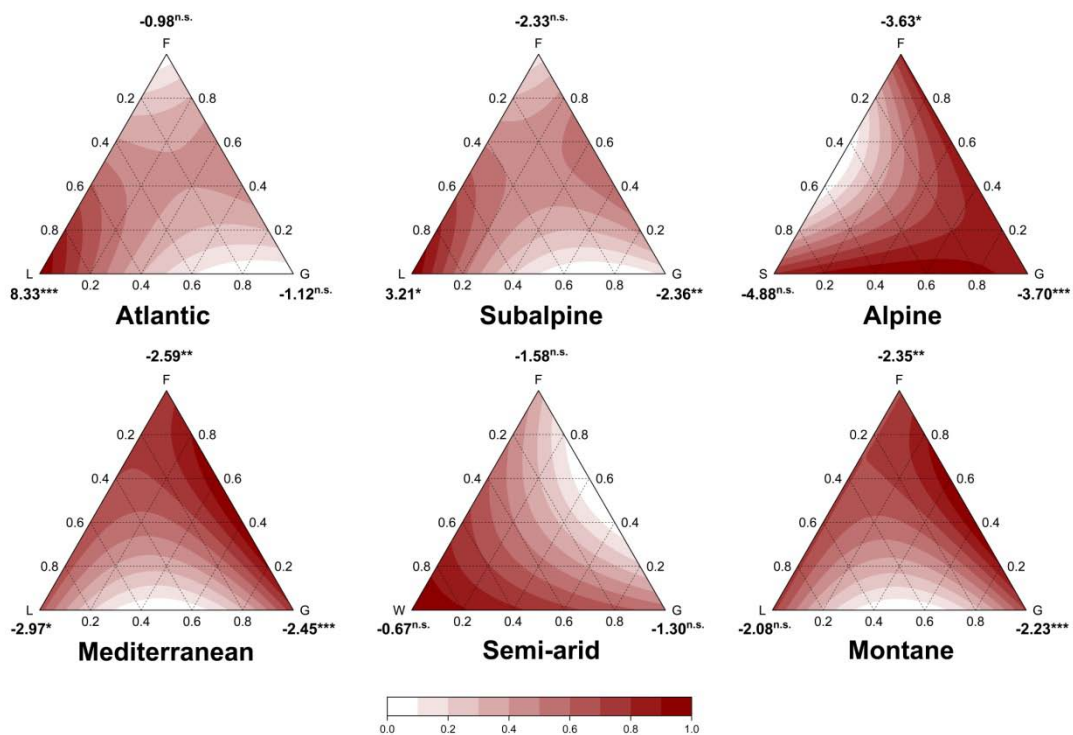
n.s.: not significant. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.



**Figure 5.3** | Ternary plots of CH<sub>4</sub> fluxes (nmol m<sup>-2</sup> s<sup>-1</sup>) of the PFT proportions (F: forb; L = legume; G = grass; S = sedge; W = woody), in grasslands of six climatic zones. Ternary plots are scaled to have a maximum of 1. Color intensity shows a gradient from the minimum (white) to the maximum gas emission (dark orange). *P*-values of estimates: n.s. not significant. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.



**Figure 5.4** | Ternary plots of CH<sub>4</sub> fluxes (nmol m<sup>-2</sup> s<sup>-1</sup>) of the PFT proportions (F: forb; L = legume; W = woody), in the Mediterranean and the montane grassland sites. Ternary plots are scaled to have a maximum of 1. Color intensity shows a gradient from the minimum (white) to the maximum gas emission (dark orange). *P*-values of estimates: n.s. not significant. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.



**Figure 5.5** | Ternary plots of N<sub>2</sub>O fluxes (nmol m<sup>-2</sup> s<sup>-1</sup>) of the PFT proportions (F: forb; L = legume; G = grass; S = sedge; W = woody), in grasslands of six climatic zones. Ternary plots are scaled to have a maximum of 1. Color intensity shows a gradient from the maximum gas uptake/minimum emission (white) to the minimum gas uptake/maximum emission (dark red). *P*-values of estimates: n.s. not significant. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

## 5.5 Discussion

There exist large uncertainties regarding the contribution of grasslands to climate change and their potential role as sinks or sources of GHGs. In this context, a lively debate continues as to which extent changes in vegetation composition under climate change alter GHG exchange. With this study, we aimed to shed light on the interactive effects of plant functional types, soil processes and climatic factors on greenhouse gas fluxes in grasslands. In the following, we will discuss these interactive effects responsible for NEE and fluxes of CH<sub>4</sub> and N<sub>2</sub>O, and the possible soil processes involved.

### 5.5.1 Climatic gradient

Our results show that GHG fluxes changed notably along the climatic gradient with different temperature and moisture conditions. For instance, CO<sub>2</sub> uptake and CH<sub>4</sub> emissions in warmer and drier sites were generally higher than in cold and moist sites, except for the subalpine grassland where the highest CO<sub>2</sub> uptake occurred. As for N<sub>2</sub>O, while the highest uptake was observed in the warm and dry Mediterranean zone, the highest emissions took place in the cold and humid Atlantic sites (Table 5.2). The different patterns between these two climatic zones correspond to different processes involved in N<sub>2</sub>O emissions or uptake. For instance, the highest emissions in the Atlantic sites are most likely the result of denitrification which is favored in such high moist conditions (Linn & Doran, 1984; Butterbach-Bahl *et al.*, 2013), whereas the patterns of N<sub>2</sub>O fluxes observed in the warm and dry Mediterranean zone (Table 5.2) can be attributed to nitrification (Freney *et al.*, 1979; Linn & Doran, 1984). At the peak of the vegetation, all grassland sites showed uptake of CO<sub>2</sub> and N<sub>2</sub>O, and emissions of CH<sub>4</sub>. Only Atlantic grasslands, characterized by high precipitation rate (Tables 5.1 and 5.2) and humid conditions exhibited N<sub>2</sub>O emissions instead of uptake, resulting most likely from denitrification (Linn & Doran, 1984; Butterbach-Bahl *et al.*, 2013). Although climatic and soil conditions were significant drivers, plant functional types also played an important role on GHG exchange.

### 5.5.2 Effects of PFTs and climate interactions on GHG exchange

Investigating plant functional types is crucial to explain patterns of GHG exchange because they control microbial composition and soil physical and chemical properties, including organic C and N and inorganic N forms (Wullschleger *et al.*, 2014; Gould *et al.*, 2016). Climate may play an overriding role because of its direct and indirect effects on soil processes; indirect effects representing how climate can drive plant diversity effects on soil (e.g. Neilson *et al.* 2005; Pold &

DeAngelis 2013; Classen *et al.* 2015), and consequently on GHG fluxes. This study provides a comprehensive overview, first, on the overall effect of each analyzed PFT (identity effect) and second, on the effect of PFT interactions (pairwise interaction) on GHG exchange. We found that the impact of identity effects and pairwise interactions on GHG exchange was strongly mediated by the environmental conditions of the grassland sites. This will be discussed for each GHG separately in the following section.

### 5.5.2.1 NEE

**O**verall, CO<sub>2</sub> uptake in the alkaline, warmer and drier zones (Mediterranean, semi-arid and montane) was higher than in the acidic, colder and more humid grasslands (Atlantic and alpine), except for the subalpine site, which exhibited the highest CO<sub>2</sub> uptake among all sites despite its relatively cold and moist climatic conditions (Table 5.3). Lower CO<sub>2</sub> uptake in the Atlantic and alpine grasslands compared to the warmer sites may be attributed to the lower temperatures in these cold grasslands which lead to slower growth and lower C:N ratio, and thus lower carbon flux (Gill & Jackson 2000; Leifeld *et al.*, 2015). Also moist soil conditions, as found in the Atlantic and alpine grasslands, are favorable for soil microbial respiration, and consequently the ecosystem respiration may be higher than CO<sub>2</sub> assimilation. As for the maximum uptake of CO<sub>2</sub> in the cold subalpine grassland, it is most likely the result of the more dense vegetation found in this site compared to all the other grasslands, since the higher the live above-ground biomass the higher the NEE. Also, subalpine grasslands are characterized by high productivity at the peak of the vegetation because of the corresponding optimal growth conditions in these grasslands, such as light, temperature, and precipitation. In addition, increased temperatures (warming) in the recent years could be releasing nutrients from the characteristically rich deep soils in subalpine grasslands (Sebastià, 2007), thus making them highly productive.

**F**orb patches showed maximum CO<sub>2</sub> uptake compared to other PFTs in cold and humid climates, while legume patches were the ones exhibiting the highest NEE in the warmer Mediterranean and montane sites. As for the semi-arid grasslands, we found the highest CO<sub>2</sub> uptake in woody patches (Fig. 5.2). This stresses that PFTs perform differently depending on the climatic conditions. In other words, functional traits of forb patches made them most efficient in the use of carbon and in the carbon assimilation-respiration balance under cold and humid conditions, but not in warm and dry climates where legume patches performed best. However, our sampling was selective and thus does not represent the overall composition of these grasslands. As for woody patches in the semi-arid grasslands, their corresponding highest CO<sub>2</sub> uptake may be attributed to two factors. First, these grasslands have the strongest dominance of woody plants among all other study sites, and second, woody plants are generally characterized by

higher aboveground biomass (Montané *et al.*, 2007), and hence higher photosynthetic and respiration rates (Ramachandra Reddy & Das, 1986) and supposedly higher assimilation/respiration balance in our case, compared to the other PFTs.

As for diversity, no effect was detected in NEE except for the pairwise interaction between forbs and grasses in cold and humid grasslands, which resulted in lower CO<sub>2</sub> uptake than in patches of forbs or grasses separately (Table 5.3 and Fig. 5.2). This is most likely not the result of the direct effect of the pairwise interaction per se, but the result of the interplay of the pairwise interaction with the cold climatic conditions. Low temperatures normally lead to lower resource availability and slower plant growth, and consequently lower photosynthetic rates (Haferkamp, 1988; Gill & Jackson, 2000) compared to warmer Mediterranean and semi-arid grasslands. This negative effect of low temperatures, however, seemed to be more pronounced in forb-grass patches compared to other mixed patches, particularly forb-legume and grass-legume patches. Previous studies have observed that grass-legume mixtures were more productive than monocultures, and that grasses had higher photosynthesis and respiration rates, and leaf N content when mixed with legumes (e.g. Ribas *et al.*, 2015; Liu *et al.*, 2016). The positive effect of grass-legume or forb-legume mixtures on photosynthesis and productivity can be attributed to the strong effect of legumes on associated PFTs and the performance of the grassland community. In other words, legumes are nitrogen fixers, and some of the fixed N is transferred to the associated non-fixers such as forbs and grasses (Ledgard & Steele, 1992; Temperton *et al.*, 2007). Consequently, the increased N stimulates plant growth and photosynthesis of the vegetation community (Lawlor, 1995; Joel *et al.*, 1997).

#### 5.5.2.2 CH<sub>4</sub>

Our results showed that CH<sub>4</sub> fluxes in warmer grassland sites were lower than in colder sites (Table 5.4). This is most likely attributed to the fact that colder high-altitude grasslands have moister soils, and thus more favorable conditions for CH<sub>4</sub> production compared to the semi-arid and Mediterranean sites. We also found that local temperature and organic matter are important drivers of CH<sub>4</sub> emissions in grasslands (Table 5.4), confirming previous studies (e.g. Le Mer & Roger 2001; Mosier *et al.* 2004; Luo *et al.* 2013). In fact, temperature drives CH<sub>4</sub> emissions by controlling microbial activities (Le Mer & Roger, 2001), and increased organic matter decomposition in the soil increases methane production by increasing soil C and N pools (Gregorich *et al.*, 2005). Soil organic N, considered as a proxy for soil organic matter, had a negative effect on CH<sub>4</sub> emissions (Table 5.4). This shows that soils with high availability of SON do not necessarily emit more CH<sub>4</sub>. In fact, soil inorganic N is the form of nitrogen that affects soil-atmosphere fluxes of methane, particularly through stimulating CH<sub>4</sub> consumption by methanotrophic bacteria (Sylvia *et al.* 2005; Bodelier and Laanbroek 2004), and not the organic

one. We shall also keep in mind that we used average values of SON from each study site and SON may vary considerably between different patches of the same site (Xi, 2015). Therefore, the variability in SON in our results may have not depicted the exact effect of SON on CH<sub>4</sub> fluxes in these grasslands.

All PFT patches exhibited methane emissions and these emissions were higher in colder and more humid sites compared to warmer and more arid sites (Table 5.2). First, the positive role of vegetation on CH<sub>4</sub> emissions is a well established fact because plants provide substrates for methanogenic bacteria through root exudation or decay (Raimbault *et al.*, 1977). Furthermore, it has been suggested that plants enhance emissions because they serve as CH<sub>4</sub> conduit (Whiting *et al.*, 1991; Nisbet *et al.*, 2009). Second, grasslands characterized by higher soil moisture (montane, Atlantic, subalpine, alpine) emit more CH<sub>4</sub> because soil moisture plays a crucial role in the induction of methanogens activity (Lessard *et al.*, 1997; Van Den Pol-van Dasselaar *et al.*, 1998). In contrast, in the semi-arid and Mediterranean grasslands where temperatures are higher and soils are drier, CH<sub>4</sub> values were generally lower. Among these semi-arid grasslands, patches of woody plants showed the highest CH<sub>4</sub> emissions compared to other PFTs (Fig. 5.3). These highest emissions are most likely the result of the generally higher biomass of woody plants compared to other PFTs, which may lead to more substrates available for methanogenic bacteria through root exudation or decay (Raimbault *et al.*, 1977).

Interestingly, when woody plants were mixed with other PFTs we observed CH<sub>4</sub> uptake instead of emissions. This pattern points towards an offsetting effect of diversity on the higher CH<sub>4</sub> emissions in patches of woody plants (Fig 5.4; Table S5.2 in Supplementary material). The same trend was observed by Ribas *et al.* (2015) in sown mixed forage swards. Niklaus *et al.* (2006) highlighted that plant diversity can be a strong deterrent of CH<sub>4</sub> emissions, responsible for shifting the ecosystem from production to consumption of methane. The positive effect of diversity on CH<sub>4</sub> uptake may be attributed to lower accumulation of inorganic N, which consequently inhibits denitrification and nitrification associated with N<sub>2</sub>O and CH<sub>4</sub> exchange (Tilman *et al.*, 1996; Niklaus *et al.*, 2001). Also, higher diversity enhances water use efficiency, which in turn reduces soil moisture and increases soil diffusivity (Spehn *et al.*, 2000; Leimer *et al.*, 2014). Lastly, a more diverse plant community alters belowground density and composition, and as a result enhances soil aeration and modifies C and N deposition (Niklaus *et al.*, 2016), which are crucial factors for methanotrophs.

### 5.5.2.3 N<sub>2</sub>O

We observed mainly uptake of N<sub>2</sub>O in all grassland sites, except for the Atlantic zone, where we found emissions. This could be related to the high precipitation rates and

moist soil conditions in the Atlantic grasslands (Tables 5.1 and 5.2). Soil moisture is a major driver of N<sub>2</sub>O emissions because high soil water content restricts oxygen availability, hence leading to denitrification – an important source of N<sub>2</sub>O (Butterbach-Bahl *et al.*, 2013; Soussana *et al.*, 2010).

When looking at the effect of PFTs, the highest N<sub>2</sub>O emissions in the Atlantic, as well as in the subalpine and alpine grasslands, occurred in legume patches (Fig. 5.4). This is attributed to the fact that legumes increase soil N content (Whitehead, 1995) and consequently the denitrification potential (Nieder & Benbi, 2008), especially when combined with moist soils. This underscores that not only climate but also PFTs can modify N<sub>2</sub>O emissions. In this context, we observed that temperature enhanced N<sub>2</sub>O emissions (Table 5.5), as observed in previous studies (e.g. Brentrup *et al.*, 2000; Luo *et al.*, 2013) because it can increase the activity of soil microorganisms and their use of organic matter (Frey *et al.*, 2013; Classen *et al.*, 2015). We expected a positive effect of SON on N<sub>2</sub>O emissions, since a number of studies suggest that the accumulation of plant-derived organic matter, and simultaneously SON, increases N content in the soil, and consequently enhances N<sub>2</sub>O emissions through nitrification and denitrification processes (e.g. Macdonald *et al.*, 2016; Qiu *et al.*, 2015). Our results, however, showed the opposite: lower soil organic N increased N<sub>2</sub>O emissions (Table 5.5). This implies that fertile soils with higher SON do not necessarily produce more N<sub>2</sub>O but instead can emit less N<sub>2</sub>O than poor soils. This is most likely related to the availability of inorganic N in these soils, a form of nitrogen (related to ammonium and nitrate) that is available for uptake and that is the main driver of N<sub>2</sub>O emissions (Butterbach-Bahl *et al.*, 2013; Millar *et al.*, 2014). Therefore, a soil with high organic N can lead to lower N<sub>2</sub>O emissions if the availability of the inorganic N form is low, and vice versa.

Our findings show that diverse patches enhanced N<sub>2</sub>O uptake (Table 5.5 and Fig. 5.5). Particularly, legume-grass, legume-woody, and forb-sedge interactions enhanced N<sub>2</sub>O uptake, and the highest N<sub>2</sub>O uptake in all sites was observed in mixtures of legumes and grasses. These findings are in line with previous results by Ribas *et al.* (2015) showing lower N<sub>2</sub>O emissions and higher uptake under legume-grass mixtures where N is used more efficiently, compared to legume monocultures. In fact, it has been suggested that more diverse plant communities lead to lower accumulation of inorganic N, and consequently inhibit denitrification and nitrification associated with N<sub>2</sub>O exchange (Tilman *et al.*, 1996; Niklaus *et al.*, 2001). This stresses the importance of diversity in grassland ecosystems and its potential role in mitigating N<sub>2</sub>O, as well as CH<sub>4</sub> emissions as discussed above.



## 5.6 Conclusions

Overall, we found uptake of CO<sub>2</sub> and N<sub>2</sub>O, and emissions of CH<sub>4</sub> at the peak of the vegetation biomass in all the grassland sites. Only Atlantic grasslands, characterized by moist climate, exhibited N<sub>2</sub>O emissions instead of uptake. PFT composition significantly affected GHG fluxes, and interactions between PFTs increased the uptake of CH<sub>4</sub> and N<sub>2</sub>O compared to single PFTs (identity effects). Our study provides evidence that plant functional type composition can alter GHG exchange, and that this alteration is mediated by the environmental conditions of the grasslands. Our findings also highlight the importance of functional diversity and its positive effect on CH<sub>4</sub> and N<sub>2</sub>O uptake, and consequently on the feedback from the ecosystem to the atmosphere.

We also observed that specific PFTs perform differently in different climatic conditions. This is most likely driven by plant functional traits, which play an important role in the structure and stability of grassland communities. Therefore, we believe that further studies on the relationship between specific traits and GHG exchange need to be conducted, to determine and predict the feedback of grassland soils to the atmosphere under climate change conditions.

## Acknowledgments

We would like to thank Helena Sarri and Esther Iñiguez for their assistance in the sampling and processing of plant biomass. This work was funded by the Spanish Science Foundation (FECYT) through the projects CAPAS (CGL2010-22378-C03-01) and BIOGEI (CGL2013-49142-C2-1-R). The study was also developed within the projects CAPACITI supported by a Marie Curie Intra European Fellowship within the 7th European Community Framework to NA (PIEF-GA-2010-275855), and CAMELEON funded by an ERA-Net CIRCLE2 Mountain (PIM2010ECR-00709). HD was supported by a FPI fellowship from the Spanish Ministry of Economy and Competitiveness (BES-2011-047009).

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## 5.8 Supporting information

### Supplementary tables

**Table S5.1** | Estimates of net ecosystem exchange (NEE) in  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , and fluxes of  $\text{CH}_4$  ( $\text{nmol m}^{-2} \text{s}^{-1}$ ) and  $\text{N}_2\text{O}$  ( $\text{nmol m}^{-2} \text{s}^{-1}$ ) from the general linear models (GLM), corresponding to plant functional type (PFT) composition: forbs (F), grasses (G), legumes (L), sedges (S), and woody plants (W).

		Semi-arid		Mediterranean		Montane		Atlantic		Subalpine		Alpine	
		Coeff.	P-value	Coeff.	P-value	Coeff.	P-value	Coeff.	P-value	Coeff.	P-value	Coeff.	P-value
NEE	F	-5.93	<0.01	-6.10	<0.001	-6.35	<0.001	-7.12	0.08	-18.77	<0.05	-77.32	<0.05
	G	-6.20	<0.001	-5.56	<0.001	-5.23	<0.001	-3.77	<0.001	-13.33	<0.001	-62.62	<0.001
	L	-11.57	<0.001	-10.21	<0.001	-9.34	<0.001	-5.87	<0.001	-13.56	<0.001	-54.48	<0.001
	S							14.80	<0.01	18.38	<0.05	27.56	0.26
	W	-11.60	<0.001	-5.89	<0.001	-1.84	0.27						
$\text{CH}_4$	F	305.64	0.09	412.69	<0.05	434.97	<0.05	588.33	<0.01	395.96	0.18	508.67	0.25
	G	159.66	0.23	254.29	<0.05	267.47	<0.05	386.43	<0.01	161.91	0.44	131.56	0.82
	L	241.01	0.49	328.84	0.22	337.04	0.16	437.17	0.11	195.03	0.68	86.34	0.96
	S							92.35	0.86	-158.52	0.81	134.49	0.88
	W	1337.37	<0.001	790.64	<0.001	333.99	0.31						
$\text{N}_2\text{O}$	F	-1.58	0.26	-2.59	<0.01	-2.35	<0.01	-0.98	0.71	-2.33	0.19	-3.63	0.05
	G	-1.30	0.06	-2.45	<0.001	-2.23	<0.001	-1.12	0.21	-2.36	<0.01	-3.70	<0.001
	L	-6.96	<0.01	-2.97	<0.05	-2.08	0.06	8.33	<0.001	3.21	<0.05	3.20	<0.05
	S							-4.35	0.39	-2.48	0.43	-4.88	0.16
	W	-0.67	0.40	-0.93	0.31	-0.59	0.58						

**Table S5.2** | GLM testing the effect of diversity, individual climatic and soil variables and their interactions on CH<sub>4</sub> fluxes of the 14 grassland sites in the year 2013; *p* values are given for the individual drivers (*T<sub>a</sub>* = mean air temperature; MAT = mean annual temperature; SON = soil organic nitrogen; SOM = soil organic matter; Lang = MAP/MAT; F = forb; G = grass; L = legume; S = sedge; W = woody).

	Estimate	Std. Error	<i>P</i>
F	1491.39	703.79	*
G	1350.01	709.68	n.s.
L	1365.93	683.07	*
S	1067.60	729.66	n.s.
W	2380.17	760.56	**
MAT	-133.52	47.84	**
Lang	-2.04	1.05	0.05
<i>T<sub>a</sub></i>	26.75	9.53	**
SOM	270.62	91.13	**
SON	-4748.47	1758.68	**
W*(F+G+L+S)	-2751.17	1292.12	*

n.s.: not significant. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

Supplementary figures

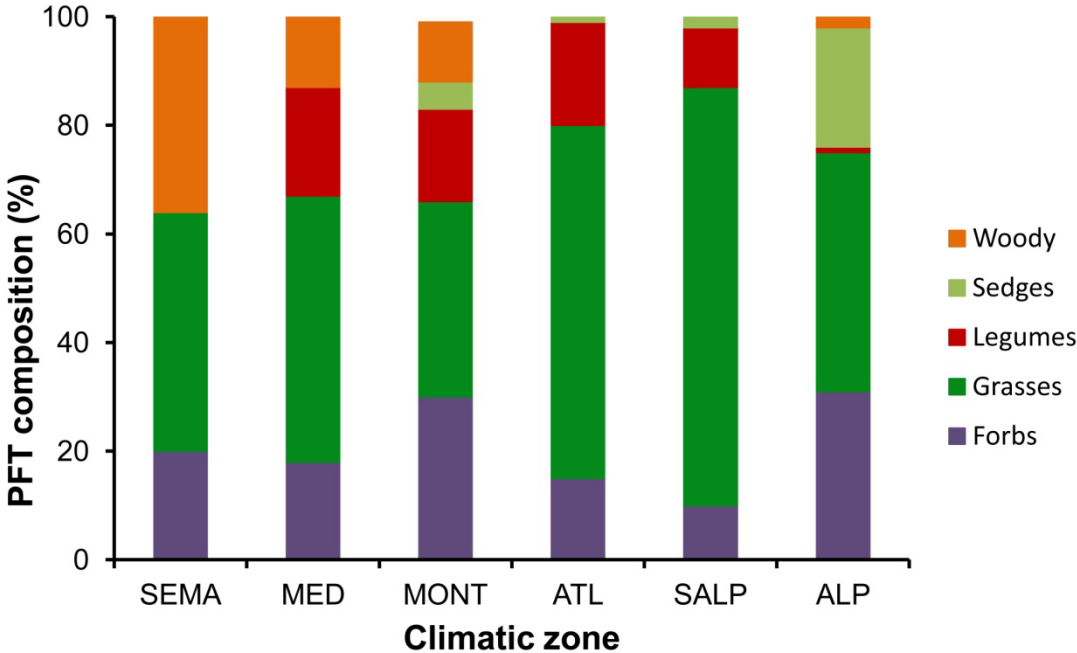
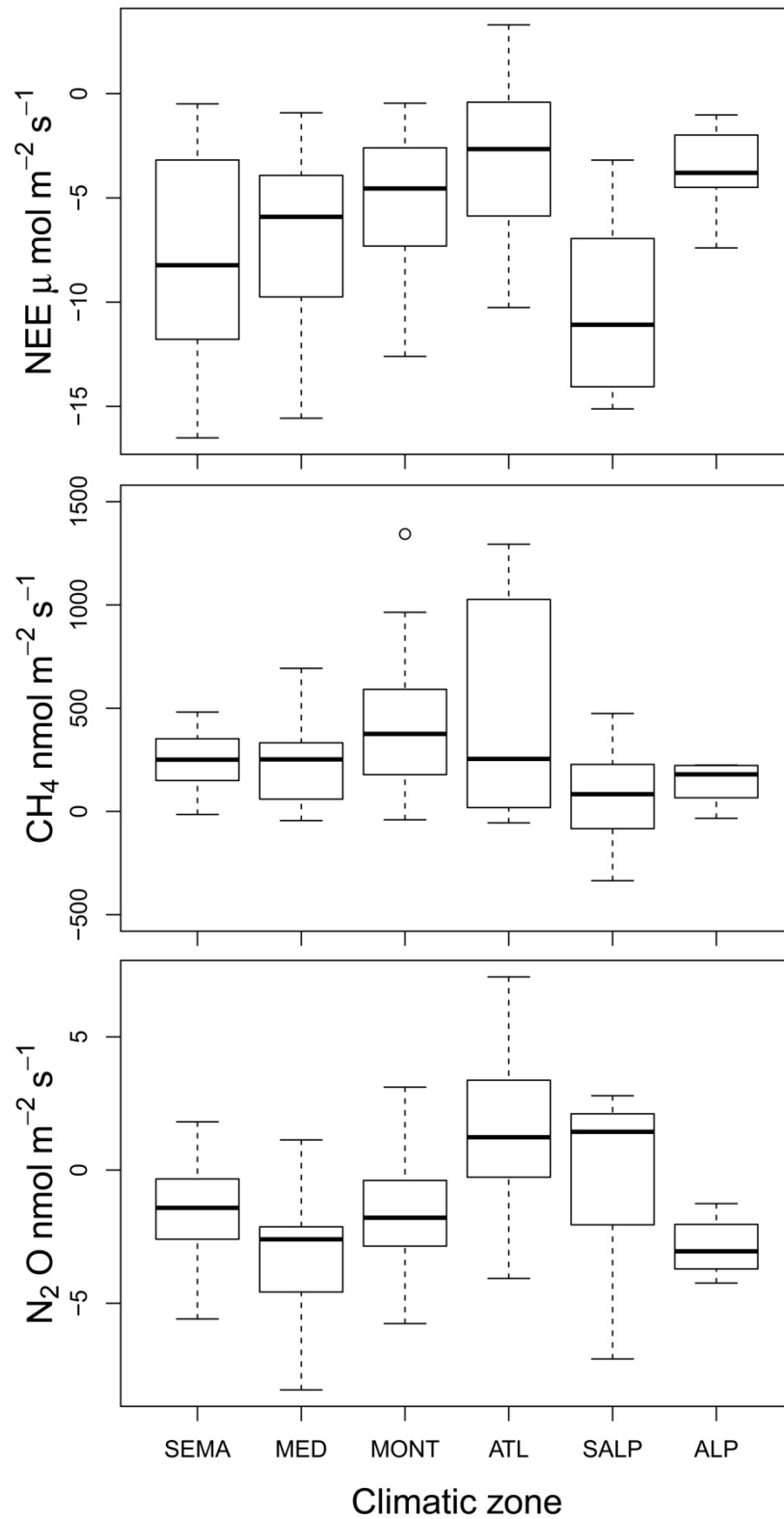
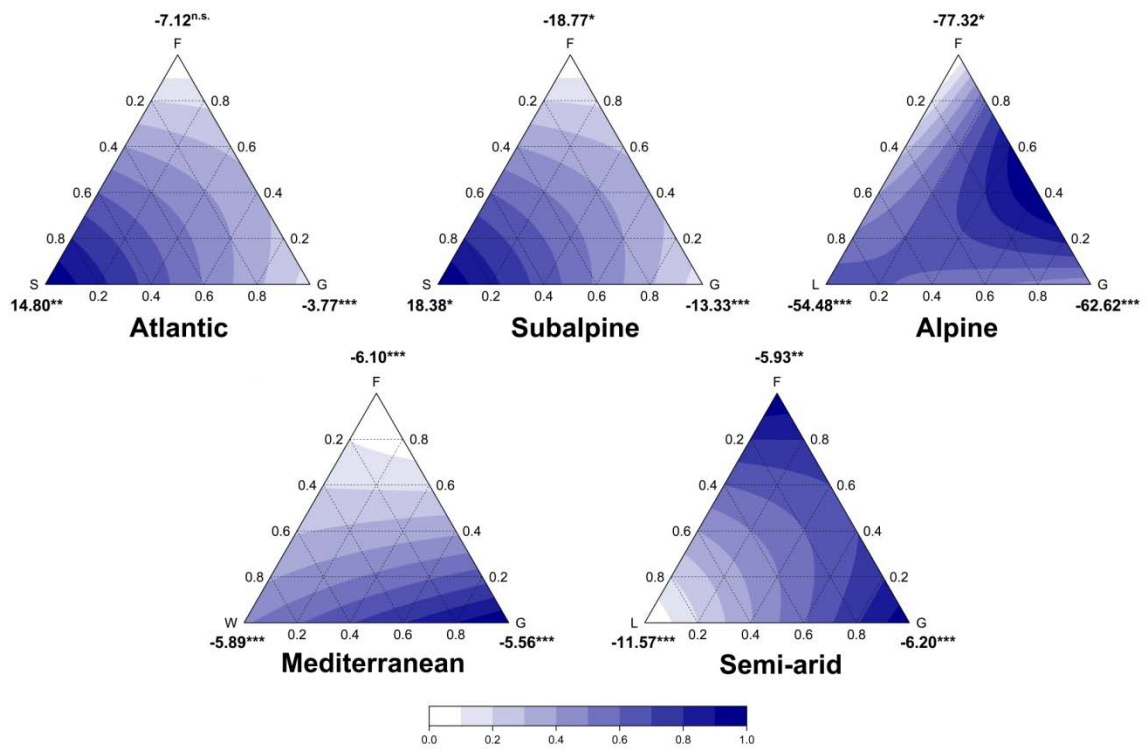


Figure S5.1 | Plant functional type (PFT) composition expressed in terms of percentage of the total sampled above-ground biomass in the six climatic zones: SEMA (semi-arid), MED (Mediterranean), MONT (montane), ATL (Atlantic), SALP (subalpine), and ALP (alpine).

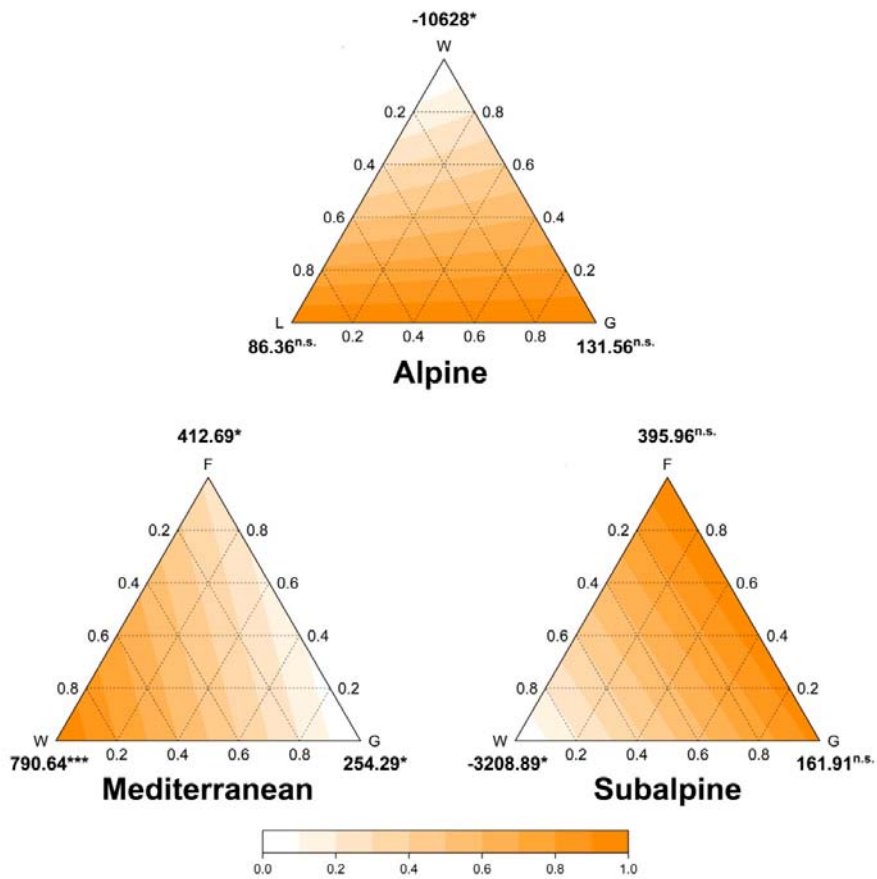




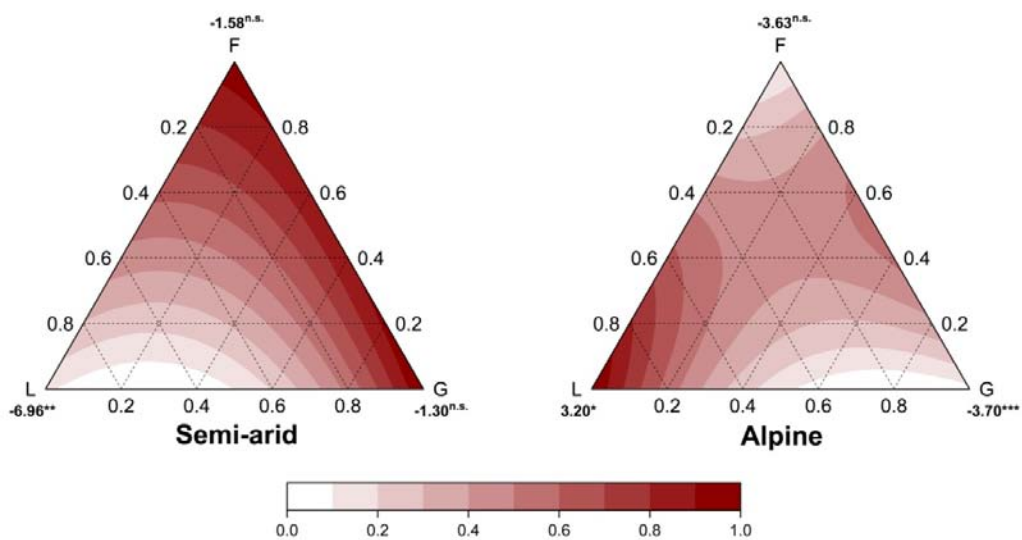
**Figure S5.2** | Mean net ecosystem exchange (NEE) and mean fluxes of CH<sub>4</sub> and N<sub>2</sub>O in grasslands of six climatic zones: SEMA (semi-arid), MED (Mediterranean), MONT (montane), ATL (Atlantic), SALP (subalpine), ALP (alpine).



**Figure S5.3** | Ternary plots of NEE ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) of the PFT proportions (F: forb; L = legume; G = grass; S = sedge; W = woody). Ternary plots are scaled to have a maximum of 1. Color intensity shows a gradient from the most negative flux value/highest uptake (white) to the lowest uptake (dark blue). *P*-values of estimates: n.s. not significant. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.



**Figure S5.4** | Ternary plots of CH<sub>4</sub> fluxes (nmol m<sup>-2</sup> s<sup>-1</sup>) of the PFT proportions (F: forb; L = legume; G = grass; S = sedge; W = woody), in the six climatic zones. Ternary plots are scaled to have a maximum of 1. Color intensity shows a gradient from the minimum (white) to the maximum gas emission (dark orange). P-values of estimates: n.s. not significant. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001.



**Figure S5.5** | Ternary plots of N<sub>2</sub>O fluxes (nmol m<sup>-2</sup> s<sup>-1</sup>) of the PFT proportions (F: forb; L = legume; G = grass; S = sedge; W = woody), in the six climatic zones. Ternary plots are scaled to have a maximum of 1. Color intensity shows a gradient from the maximum gas uptake/minimum emission (white) to the minimum gas uptake/maximum emission (dark red). P-values of estimates: n.s. not significant. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001.





# Chapter 6





**S**ignificant uncertainties remain regarding the effects of climate change on functional and species diversity, and their implications in soil functions and GHG exchange in grasslands. This dissertation investigates in extensive field experiments the role of diversity and functional traits composition on community stability, and how vegetation composition affects soil activity and GHG exchange under changing climatic conditions. In the following sections, the main research findings of each chapter are summarized and discussed (6.1-6.3). Last, we evaluate the implications of our findings and their future prospects (6.4).

## 6.1 Importance of plant functional traits in regulating grassland community stability and productivity under climate change

**C**limate change poses a threat to the stability and functioning of grasslands by inducing strong shifts in productivity, species diversity, and plant functional traits structure (Sebastià *et al.*, 2004, 2008b, de Bello *et al.*, 2005, 2006; Sebastià, 2007). This threat is even more pronounced in high mountain grasslands (Sanz-Elorza *et al.*, 2003; Kohler & Maselli, 2009; Spehn *et al.*, 2010; Gartzia *et al.*, 2016), particularly in the Eastern Pyrenees due to their position at the south-western edge of the semi-natural grassland biome in Europe (Sebastià, 2007). More diverse plant communities are believed to be more stable and productive under changing environmental conditions (MacArthur, 1955; Elton, 1958; Tilman & Downing, 1994; Palmer & Maurer, 1997). Therefore, most studies investigating community stability (resistance and resilience) have focused on species diversity, but the role of plant functional traits in community stability and productivity has been generally overlooked (Klumpp & Soussana, 2009; Májeková *et al.*, 2014; Fischer *et al.*, 2016). Plant functional traits are, however, an ideal indicator of the ecosystem functioning because changes in resource availability are strongly reflected in plant functional traits. In fact, we found **that plant functional traits, compared to diversity effects, had a greater effect on the productivity and stability of the grassland community under short-term warming (Tables 2.2 and 2.2; Figs. 2.1 to 2.3 in chapter 2)**. In the following section, we highlight and discuss in detail the importance of plant functional traits for community stability and productivity.

### 6.1.1 Community stability

**M**ountain areas are characterized by low nutrient availability, where mineralization is often limited by cold temperatures (Schmidt *et al.*, 1999; Weintraub & Schimel, 2003). With our warming experiment, we removed the temperature limitation that led to increased resource availability. This had a strong impact on both species diversity and vegetation composition. Species diversity declined as a result of warming, as observed by Suding *et al.* (2008) and Cantarel *et al.* (2013). The reduction of species in the temporarily nutrient-rich habitat may be the outcome of environmental filtering which excludes species that are less adapted to the warming period. It may also be the result of the out-competition of conservative species by opportunistic species with a faster growth rate (competitive exclusion principle, Gause [Gause 1934]).

**S**pecies with opportunistic use of increased resource availability became dominant, while conservative species became less abundant or disappeared from the system. Opportunistic species were equipped with a set of plant functional traits that enabled a faster resource capture and turnover of organs, and consequently a higher productivity (Grime, 1977; Chapin, 1980; Poorter & De Jong, 1999). For instance, we observed earlier flowering as a response to warming prolonging the vegetation period (Menzel *et al.*, 2006; Amano *et al.*, 2010; Wolkovich *et al.*, 2012). Also, there was an increase of rhizomatous plants (Fig. 2.1 in chapter 2) which have the nutrients more readily available compared with species that need to establish new stems (Grime, 1979). Additionally, rhizomes can store resources from previous years (storage effect; Chesson (Chesson, 2000)) and are therefore the fastest in reacting to improved environmental conditions. The increased proportion of erect plants under warming conditions (Fig. 2.1 in chapter 2) suggests that increased competition for light led to the exclusion of prostrate plants (Zimdahl, 2013). The biggest change in composition occurred in turves that had originally more species with lower SLA. In these turves, species with greater SLA took over because they acquired the resources more efficiently under warming conditions (Fig. 2.3; Tables S2.3 and S2.5 in chapter 2). We thus conclude that the disappearance of species was greater in turves with originally lower SLA. On the contrary, diversity components (species and functional diversity) did not affect the stability of the grassland community under warming conditions. This shows that functional traits, and above all SLA, exerted the major effect on the vegetation stability, but not diversity. Our findings thus highlight the importance of plant functional traits and interspecific variability on population stability (Polley *et al.*, 2013; Jung *et al.*, 2014; Májeková *et al.*, 2014).

### 6.1.2 Community productivity

In our warming experiment, productivity increased and diversity decreased as a response to warming. Interestingly, **plant traits and not diversity were the main factor to explain the increased productivity under warming conditions**. A greater biomass production was the outcome of faster mineralization rate and nutrient acquisition by opportunistic species that were taller, had a greater SLA and erect form. Taller plants are considered more competitive since investment in height improves the access to light (Falster & Westoby, 2003), allowing them to acquire nutrients faster thus leading to a faster growth and a greater productivity.

Communities dominated by fast-growing species have a higher resilience and a lower resistance to extreme events in comparison to slow-growing conservative species (Lepš *et al.*, 1982; MacGillivray *et al.*, 1995). Therefore we expect that, in the long-term, changes in resource availability and climate will lead to a new community at the expense of the old one (Körner & Paulsen, 2004). Climate models for our study region project warmer and drier conditions in the long term (Llebot, 2005). We speculate that some species will acclimate through their functional traits to these conditions while others will simply disappear. We also expect the invasion of grasslands by new species (annuals and woody species) more adapted to such extreme conditions, as suggested by the work of (de Bello *et al.* (2005, 2006, 2007).

Overall, maintaining community stability in grasslands is important because any shifts in vegetation composition and species diversity can modify soil biological, physical and chemical processes, and consequently their feedback to the atmosphere.

## 6.2 Environmental conditions and plant functional types drive soil activity and fertility

Soil activity and fertility in grasslands, particularly in mountain areas, are expected to be strongly affected by climate change (Mills *et al.*, 2014) through changes in organic matter quality and supply from biomass, soil temperature, and soil hydrology (Brinkman & Sombroek, 1996). As a consequence, the ecosystem functioning and greenhouse gas (GHG) exchange would be also altered. Beside climatic factors, soil functions are also affected by plant diversity (Niklaus *et al.*, 2006; Castro *et al.*, 2010), because plants determine the quantity and the quality of residues, soil organic matter, as well as soil structure (Wardle *et al.*, 2004). A number of studies suggest that the effect of diversity on soil processes is driven by climate (Neilson *et al.*, 2005; Pold & DeAngelis, 2013; Classen *et al.*, 2015). However, the relative contribution of the environmental conditions and plant diversity and their interactions on soil activity and fertility are poorly understood. For instance, Classen *et al.* (2015) hypothesized that indirect effects of climate change on soil microbial activity mediated through plants may be stronger than direct effects of climate



on shaping microbial community composition and function. Yet, we found that **the variability in soil activity and fertility was better explained by environmental (regional and local climatic, management and soil) conditions than by diversity**, followed by the combined effects of both variables. The unique effects of diversity were, however, small compared to the interaction effects, thus suggesting that **diversity effects cannot be separated from climatic effects on soil activity and fertility**.

### 6.2.1 Environmental conditions

**E**nvironmental conditions had clearly the strongest impact on soil processes, mainly through soil moisture and temperature (Fierer & Jackson, 2006; Castro *et al.*, 2010; Bru *et al.*, 2011; Husson, 2013; Jiao *et al.*, 2016). Increased soil moisture enhanced total nitrogen (N), ammonium, microbial biomass, dissolved organic carbon (DOC) and nitrogen (DON), and phosphatase activity, while increased temperature reduced them (Fig. 3.3 in chapter 3). Indeed, increased soil moisture enhances microbial activity and nitrogen mineralization (Paul *et al.*, 2003; Garten *et al.*, 2009), which consequently increases soil total nitrogen and microbial biomass. As for ammonium, its positive correlation with soil moisture, as observed by Mueller *et al.* (2013), can be attributed to the positive impact of soil moisture on organic matter mineralization (Zhalnina *et al.*, 2012) which is converted into inorganic forms of nitrogen such as ammonium (Troy & Tang, 2011). Phosphatase activity, on the other hand, was enhanced by increased soil moisture because the latter is a key factor in regulating P in the soil, its uptake by the plants for their growth (Grierson & Adams, 2000; Sardans *et al.*, 2007; Shen *et al.*, 2011), and in controlling microbial activity (Leirós *et al.*, 1999; Sardans *et al.*, 2007). Soil moisture also enhances and supports DOC and DON transport in the soil horizons (Solinger *et al.*, 2001; Kaiser *et al.*, 2002; Kalbitz *et al.*, 2003).

**T**emperature, in addition to soil moisture, generally enhances microbial and enzyme activity, as well as mineralization in an ecosystem. Our results showed however the opposite. This is most likely attributed to the environmental conditions of the grassland study sites along the climatic gradient which varied from warm continental in the low-altitude sites (semi-arid) to cold temperate conditions in the high-altitude sites (subalpine and alpine). For example, the warmest sites in our study, mainly the semi-arid, are also characterized by dry soil conditions. Such conditions are capable of inhibiting and eliminating the positive effect of temperature on mineralization, microbial biomass, and soil phosphatase activity (Sardans & Peñuelas, 2005; Castro *et al.*, 2010). The interplay between soil moisture and temperature also affected nitrate and nitrification potential which were the only soil activity and fertility indices which decreased with soil moisture (Fig. 3.3). Among environmental drivers, soil moisture and temperature are regarded as the most important factors for nitrification processes, as well as nitrate concentra-

tions (Haynes, 1986; Emmett *et al.*, 2004; Butterbach-Bahl *et al.*, 2013). Therefore, this decreasing trend of nitrification potential and nitrate with higher soil moisture levels cannot be separated from temperature, especially as sites with high soil moisture (subalpine and alpine) are characterized by low temperatures which would limit these soil processes. Overall, **our results highlight the importance of the interplay between temperature and soil moisture, and stress that the effect of each factor cannot be separated from the other when investigating soil activity processes.**

**S**oil enzyme activities, such as glucosidase and urease, are indicators of soil organic matter quality (Caldwell, 2005; Burns *et al.*, 2013) which control the availability of nutrients such as carbon, nitrogen and phosphorus, and the rate at which they are produced in the soil for microbial assimilation (Chróst, 1991; Burns & Dick, 2002; Gianfreda, 2015). Plants have a strong influence on enzyme activity because they regulate the quantity and quality of microbial biomass (Sardans *et al.*, 2008; Steinweg *et al.*, 2013). Grazers, on the other hand, can exert strong effects on the plant-soil system through their impacts on vegetation composition, soil microbial communities and enzyme activities (Prieto *et al.*, 2011; Olivera *et al.*, 2014), as observed in our results for glucosidase and urease activities. In fact, glucosidase activity increased in grasslands grazed by sheep and dominated by grasses. Grass communities and sheep grazing seemed to enhance plant litter quantity and quality, a main component of glucosidase activity to produce glucose (Tabatabai, 1994).

### 6.2.2 Diversity effects

**B**eside climatic factors, soil activity and fertility are also affected by plant diversity (Niklaus *et al.*, 2006; Castro *et al.*, 2010). Indeed, diversity effects were observed on microbial biomass nitrogen (MBN), dissolved organic nitrogen (DON), total N, urease and phosphatase, and nitrification potential (Table 3.2 in chapter 3). Several studies investigated the role of vegetation composition in soil microbial N and C. Some detected no change in MBN and MBC with functional group diversity (Wardle *et al.*, 2003; Niklaus *et al.*, 2006), while others suggest that PFT and particularly legumes with their important effect on total nitrogen play a great role in shaping soil microbial community (Cui *et al.*, 2016). We found that the interaction between grasses and forbs enhanced MBN and phosphatase activity, but that diversity had no effect on MBC (Table 3.3 in chapter 3). The increased MBN caused by the interaction between forbs and grasses, is most likely the result of the higher quantity and variety of plant-derived compounds available to microbial communities at high diversity (Strecker *et al.*, 2015). Also among all the soil functions that we assessed, MBN was the only one affected by the interaction between climate and PFT. Particularly, MBN was enhanced by the interaction between grasses and forbs in sites with lower mean minimum temperature. This may be attributed to the high sensitivity of microbial bio-

mass nitrogen to changes in the climate and environmental conditions compared to other soil enzyme activities (Fang *et al.*, 2013). Also, the increased plant productivity resulting from increased diversity can explain the observed increase in DON, because diversity leads to higher soil organic matter and consequently increased organic N pools (Dijkstra *et al.*, 2007) and thus greater DON production. This pattern was particularly observed in grass-legume interactions. Grasses are suggested to cause higher microbial biomass compared to other PFTs, because of their dense root system and high root exudation rates (Eisenhauer *et al.*, 2010; Roscher *et al.*, 2012). Legumes, in turn, favor organic N sources compared to other PFTs, due to their effectiveness in transferring amino acids between nodules and roots (Moran-Zuloaga *et al.*, 2015). When mixed together, some of the fixed N by legumes is transferred to the associated non-fixers; in this case grasses (Ledgard & Steele, 1992; Temperton *et al.*, 2007). Increased N generally stimulates plant growth and photosynthesis (Lawlor, 1995; Joel *et al.*, 1997), and consequently leads to higher soil organic matter and DON production. This may explain why the interaction between legumes and grasses enhanced DON.

**D**iversity also enhanced total nitrogen, nitrification potential, and urease (Table 3.2) which are soil activity variables related mainly to the nitrogen cycle. Our findings are in line with previous studies suggesting that more even plant community increases bacterial abundance, which consequently enhances the potential for bacterial nitrification (Lamb *et al.*, 2011). Also, this positive effect of diversity on total N and urease activity can be explained by the positive effect of diversity on productivity and nitrogen availability (Cardinale *et al.*, 2007; Kirwan *et al.*, 2007; Oelmann *et al.*, 2011; Niklaus *et al.*, 2016). In fact, increased community aboveground biomass as a result of increased diversity leads to higher litter and soil nutrient content which are closely associated with urease activity (Niklaus *et al.*, 2016; Shen *et al.*, 2016). The latter is a key enzyme strongly linked to the processes of nitrogen transformation and availability in the soil (Liang *et al.*, 2003; Zhang *et al.*, 2014).

**The strong influence of PFT composition and interactions on soil activity and fertility suggest that any shifts in vegetation composition in grasslands, as a result of climate change, can have serious effects on the feedback of the soil to the atmosphere.** This is due to the fact that greenhouse gas exchange processes mainly occur in the soil, and thus strongly depend on soil microbial biomass, carbon and nitrogen content and availability, and enzyme activities.

## 6.3 Effects of environmental conditions and plant functional types and their interactions on GHG exchange

### 6.3.1 Magnitude and range of GHG fluxes under different environmental conditions

One of the main objectives of the thesis was to determine the range of GHG fluxes in grasslands under different climatic conditions, and to unravel the main drivers of CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O fluxes between ecosystem and atmosphere. In chapter 3, we investigated the fluxes of CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O in four extensively managed semi-natural grasslands in the Pyrenees along an altitudinal gradient, during two growth periods. In chapter 4, we compared GHG fluxes from 14 different grassland sites covering six climatic zones (semi-arid, Mediterranean, montane, Atlantic, subalpine, and alpine). All grassland sites exhibited mostly negative values of net ecosystem exchange (NEE), suggesting small sinks of CO<sub>2</sub> (Figs. 4.4 and S5.2), and were mainly sources of CH<sub>4</sub> (Figs. 4.5 and S5.2). Our CO<sub>2</sub> flux values were generally comparable to values obtained by Wohlfahrt *et al.* (2008) in the Eastern Pyrenees.

Methane flux values were, however, relatively higher than previously reported fluxes in grasslands using chamber techniques, with an average of 150-200 nmol m<sup>-2</sup> s<sup>-1</sup>. To our knowledge, no studies on CH<sub>4</sub> fluxes in the Pyrenees are found to compare them with our values. The high CH<sub>4</sub> values that we measured may be attributed to our regional climatic conditions with Mediterranean influence; low SWC combined with high temperatures particularly in the summer. In fact, the combination of low SWC with elevated soil temperature inhibits soil respiration (Chang *et al.*, 2014), resulting in the formation of anaerobic conditions in the centre of the soil macropores. Such conditions favor the production of N<sub>2</sub> through denitrification, and eventually favor CH<sub>4</sub> formation instead of oxidation (Butterbach-Bahl *et al.*, 2013). In the alpine grassland where drought stress is less pronounced, the clayey soil texture can contribute to favorable conditions for methane production, because clayey soil is characterized by a small amount of macropores which could increase anaerobic microsites (Signor & Pellegrino Cerri, 2013). Our methane flux values are rather close to the order of magnitude of CH<sub>4</sub> emissions measured with eddy-covariance technique on intensively managed grassland in Switzerland with values up to 400 nmol m<sup>-2</sup> s<sup>-1</sup> (Merbold *et al.*, 2014), and a sheep pasture in Scotland with values up to 150 nmol m<sup>-2</sup> s<sup>-1</sup> (Dengel *et al.*, 2011). Similar range and magnitude of CO<sub>2</sub> and CH<sub>4</sub> fluxes were also observed in chapter 4, in 14 grasslands at the peak of the vegetation biomass, along a climatic gradient.

As for N<sub>2</sub>O (Fig. 4.6), our values (-5 to 15 nmol m<sup>-2</sup> s<sup>-1</sup>) were comparable to values obtained by Soussana et al. (2007) in European grasslands and Laubach et al. (2016) in pastures in New Zealand. All grasslands along the climatic gradient showed mainly uptake of N<sub>2</sub>O, except the Atlantic sites characterized by moist soil conditions which exhibited emissions of N<sub>2</sub>O instead of uptake (Fig. S5.2). Overall, we found that the higher the grassland altitude (hence the colder) the lower the fluxes. We are aware of the necessary caution to be taken when discussing the levels of measured CH<sub>4</sub> and N<sub>2</sub>O fluxes, since they are known to vary between patches of the same grassland. However, despite the limitations of our sampling design to capture the full picture, our measurements provide an overview of occurring flux levels in remote mountain areas in the Pyrenees, where to our best knowledge data on GHG, particularly CH<sub>4</sub> and N<sub>2</sub>O are scarce or even lacking.

Additionally, not only did CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O fluxes change along the altitudinal and climatic gradients, but also along different seasons, with the highest GHG emissions occurring in the summer period. This temporal variability in GHG fluxes was most certainly temperature- and vegetation-driven, as underlined by previous studies observing higher CO<sub>2</sub> (Imer *et al.*, 2013; Oertel *et al.*, 2016) and N<sub>2</sub>O (Imer *et al.* 2013; Mori & Hojito 2011) emissions during summer. For methane, however, the picture is less clear. Some studies conducted in temperate and semi-arid grasslands found lower emissions when soil water decreased in summer (e.g. Imer *et al.*, 2013; Luo *et al.*, 2013). Nonetheless, Guillet *et al.* (2015) observed in temperate grasslands a similar trend as in our study, where CH<sub>4</sub> emissions increased in late summer. They attribute these CH<sub>4</sub> peaks to the deep water-saturated soil layers, which are then conducted to the surface by soil cracks formed in the dry summer period.

As we just have shown, **GHG fluxes are highly variable depending strongly on the site-specific climate**. In the next section, we will thus discuss in detail the main biotic and abiotic drivers of GHG fluxes in grasslands.

### 6.3.2 Main biotic and abiotic drivers of GHG fluxes

Aboveground biomass and temperature were the main drivers for all GHG fluxes in the Pyrenean grasslands enhancing CO<sub>2</sub>, CH<sub>4</sub> and N<sub>2</sub>O fluxes, as observed in previous studies (e.g. Ganjurjav *et al.*, 2015; Raich and Schlesinger, 1992 for CO<sub>2</sub>; Le Mer and Roger, 2001; Raimbault *et al.*, 1977 for CH<sub>4</sub>; Brentrup *et al.*, 2000; Luo *et al.*, 2013 for N<sub>2</sub>O). The effect of aboveground biomass on CO<sub>2</sub> fluxes can be explained by the positive correlations between live shoot and root biomass quantity and both NEE and ecosystem respiration (R<sub>eco</sub>); higher above- and below-ground biomass implies higher photosynthesis and respiration rates, particularly at the peak of the vegetation growth. Temperature, on the other hand, regulates the rate at which CO<sub>2</sub> is produced in the soil (Singh & Gupta, 1977; Raich & Schlesinger, 1992; Flanagan & Johnson, 2005;

Hartley *et al.*, 2006), and thus plays an important role in ecosystem ( $R_{\text{eco}}$ ) and soil ( $R_{\text{soil}}$ ) respiration. As for methane and nitrous oxide, temperature not only affects emissions through its role in controlling microbial activities (Le Mer and Roger, 2001; Butterbach-Bahl *et al.* 2013) but it also regulates the way how plants affect  $\text{CH}_4$  and  $\text{N}_2\text{O}$  fluxes. Plants provide substrates for methanogenic bacteria through root exudation or decay (Raimbault *et al.*, 1977) and a greater biomass leads to greater decomposed plant material, thus enhancing N mineralization (Semmartin *et al.*, 2010). As a consequence, higher emissions of  $\text{CH}_4$  and  $\text{N}_2\text{O}$  occur. This positive effect of aboveground biomass on both  $\text{CH}_4$  and  $\text{N}_2\text{O}$  was confirmed by the negative effect of grazing on their fluxes. Grazing can play an important role through reducing biomass quantity and modifying vegetation composition (de Bello *et al.*, 2005, 2006; Sebastià *et al.*, 2008a). Reduced vegetation biomass, and thus decreased litter production, result in less substrates available for methanogenic bacteria (Zhou *et al.*, 2006) and less nitrogen content (Semmartin *et al.*, 2010). Altered plant species composition by grazing can modify soil nitrogen (N) and pH (Semmartin *et al.*, 2010) which may consequently alter the soil processes behind  $\text{CH}_4$  and  $\text{N}_2\text{O}$  production and oxidation. The importance of vegetation on GHG exchange that we have observed in chapter 3 lead us to investigate further in detail the role of plant functional type (PFT) composition on net ecosystem exchange (NEE),  $\text{CH}_4$  and  $\text{N}_2\text{O}$  fluxes in chapter 4. The results are discussed in the following section.

### 6.3.3 The effects of plant functional types on GHG exchange under different environmental conditions

In the previous section we have shown the importance of vegetation on GHG exchange. In this section, we discuss how vegetation effects on GHG fluxes can be altered according to the composition of plant functional types. We found that **PFT composition (grasses, legumes, non-legume forbs, sedges, and woody plants) significantly affected GHG fluxes, and this effect was strongly mediated by the climatic conditions. Also, the interaction between PFTs lead to enhanced uptake of  $\text{CH}_4$  and  $\text{N}_2\text{O}$  compared to single PFT effects, known as identity effects.** In the following three sections we interpret these findings on NEE,  $\text{CH}_4$  and  $\text{N}_2\text{O}$ , respectively.

**F**orb patches showed maximum  $\text{CO}_2$  uptake (in  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) compared to other PFTs in cold and humid climates, while legume patches exhibited the highest NEE in the warmer Mediterranean and montane sites. As for the semi-arid grasslands, we found the highest  $\text{CO}_2$  uptake in woody patches (Fig. 5.2 in chapter 5). This stresses that **PFTs perform differently depending on the climatic conditions.** In other words, forbs possess functional traits that made them most efficient in the use of carbon and in the carbon assimilation-respiration balance under cold and humid conditions, but not in warm and dry climates where legumes performed best. As for

woody patches in the semi-arid grasslands, their corresponding highest CO<sub>2</sub> uptake may be attributed to two factors. First, these grasslands are the only sites with a dominance of woody plants. Second, woody plants are generally characterized by higher aboveground biomass (Montané *et al.*, 2007), hence higher photosynthetic and respiration rates (Ramachandra Reddy & Das, 1986) compared to the other PFTs.

**A**s for diversity, no effect on NEE was detected except for the interaction between forbs and grasses in cold and humid grasslands, which showed lower CO<sub>2</sub> uptake than in patches of forbs or grasses separately (Figure 5.2). This is most likely not the result of the direct effect of the pairwise interaction per se, but the result of the interplay of the pairwise interaction with the cold climatic conditions. Low temperatures normally lead to lower resource availability and slower plant growth, and consequently lower photosynthetic rates (Haferkamp, 1988; Gill & Jackson, 2000) compared to warmer Mediterranean and semi-arid grasslands. This negative effect of low temperatures, however, seemed to be more pronounced in forb-grass patches compared to other mixed patches, particularly forb-legume and grass-legume patches. We can attribute this to the presence of legumes in these patches and their effect on CO<sub>2</sub> uptake. In other words, legumes are nitrogen fixers, and some of the fixed N is transferred to the associated non-fixers such as forbs and grasses (Ledgard & Steele, 1992; Temperton *et al.*, 2007). Increased N generally stimulates plant growth and photosynthesis (Lawlor, 1995; Joel *et al.*, 1997).

**A**mong all PFTs, patches of woody plants showed the highest CH<sub>4</sub> emissions compared to other PFTs (Fig. 5.3). This is most likely the result of the generally higher biomass of woody plants compared to other PFTs, which may lead to more substrates available for methanogenic bacteria through root exudation or decay (Raimbault *et al.*, 1977). Interestingly, when woody plants were mixed with other PFTs we observed CH<sub>4</sub> uptake instead of emissions. This pattern points towards an **offsetting effect of diversity on the higher CH<sub>4</sub> emissions** in patches of woody plants (Fig. 5.4). The same trend was observed by Ribas *et al.* (2015) in sown mixed forage swards. Niklaus *et al.* (2006) highlighted that plant diversity can be a strong deterrent of CH<sub>4</sub> emissions, responsible for shifting the ecosystem from production to consumption of methane. The positive effect of diversity on CH<sub>4</sub> uptake may be attributed to lower accumulation of inorganic N, which consequently inhibits denitrification and nitrification associated with N<sub>2</sub>O and CH<sub>4</sub> exchange (Tilman *et al.*, 1996; Niklaus *et al.*, 2001). Also, higher diversity enhances water use efficiency, which in turn reduces soil moisture and increases soil diffusivity (Spehn *et al.*, 2000; Leimer *et al.*, 2014). Lastly, a more diverse plant community alters below-ground density and composition, and as a result enhances soil aeration and modifies C and N deposition (Niklaus *et al.*, 2016), which are crucial factors for methanotrophs.

**A**s for N<sub>2</sub>O, the highest emissions in the Atlantic, as well as in the subalpine and alpine grasslands, occurred in legume patches (Fig. 5.4). This is attributed to the fact that legumes increase soil N contents (Whitehead, 1995) and consequently the denitrification potential (Nieder & Benbi, 2008), especially in moist soils. This underscores that not only climate but also PFTs can increase N<sub>2</sub>O emissions. Our findings also show that **diverse patches enhanced N<sub>2</sub>O uptake** (Table 5.5 and Fig. 5.5 in chapter 5). Particularly, legume-grass, legume-woody, and forb-sedge interactions enhanced N<sub>2</sub>O uptake, and **the highest N<sub>2</sub>O uptake in all sites was observed in mixtures of legumes and grasses**. These findings are in line with previous results by Ribas et al. (2015) showing lower N<sub>2</sub>O emissions and higher uptake under legume-grass mixtures compared to legume monocultures. In fact, it has been suggested that more diverse plant communities lead to lower accumulation of inorganic N, and consequently inhibit denitrification and nitrification associated with N<sub>2</sub>O exchange (Tilman *et al.*, 1996; Niklaus *et al.*, 2001). This stresses **the importance of diversity in grassland ecosystems and its potential role in mitigating N<sub>2</sub>O, as well as CH<sub>4</sub> emissions** as discussed above.

## 6.4 Implications of our findings and future prospects

**A**ccording to future climate change scenarios, grasslands, especially in mountain areas, are expected to witness strong shifts in vegetation communities. Our findings suggest that such changes may have dramatic effects on soil activity and fertility, and consequently GHG exchange because of the strong effects of vegetation composition on the former factors. Thus, **climate change can alter the feedback from the soil to the atmosphere not only through direct effects, but also through indirect effects via shifting vegetation composition and diversity**.

**B**ased on our findings, we urge the need to conduct continuous GHG measurements, especially of CH<sub>4</sub> and N<sub>2</sub>O, in extensively managed semi-natural grasslands in the Pyrenees in particular, due to the scarcity of data in these ecosystems and the ongoing uncertainties. This will contribute to a better understanding of these ecosystems and their role in global warming potential and climate change mitigation. Notably, the combination of such measurements with measurements of plant functional traits will also allow us to determine the response of grassland communities and the expected feedback from the ecosystem to the atmosphere under future climate change scenarios.



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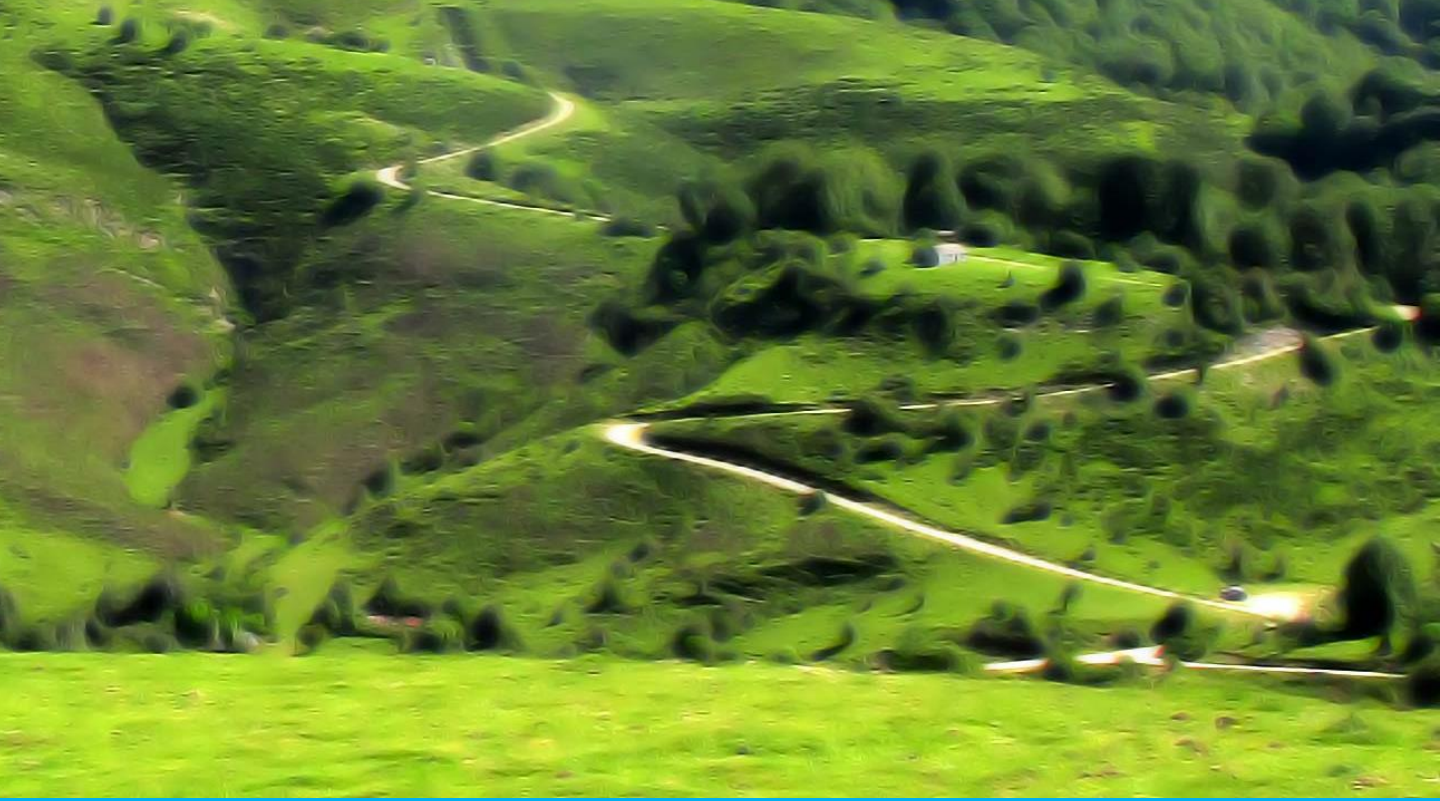
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# Chapter 7



# General conclusions



## Chapter 2

We found that a short-term warming enhanced productivity and reduced diversity significantly. A change in vegetation composition, manifested by a shift in dominance towards acquisitive fast-growing species, was also observed. Our results also advocate that a higher diversity did not contribute to a greater stability of the community under stress; instead plant functional traits, particularly high SLA, were responsible for the stability of the vegetation to increased temperature. Also, the changes in CWM of certain traits (% rhizomes, growth form, and start of first flowering) seem to be the main drivers of increased biomass production under climate warming. The relationship between productivity and species richness remained positive within both the highland and the lowland. We postulate that seasonal climate change strongly affects functional traits and diversity. On the long term, however, knowledge of sensitivity of grasslands to climate change is scant and thus more experiments over longer periods are needed. Particularly, complementary observational studies and reciprocal transplanting (from the lowland back to the highland) could be useful tools to better understand the observed patterns. Also, we suggest that further studies should address the recovery of the vegetation after a short period of climate warming, in terms of functional diversity and plant functional traits.

## Chapter 3

We found that total variability of soil activity and fertility were best explained by environmental variables, followed by the combined effects of environmental and diversity variables. The unique effects of diversity were, however, small compared to the interaction effects, thus suggesting that diversity effects cannot be separated from climatic effects on soil activity and fertility. Among the environmental variables, soil moisture and sheep grazing were the best predictors. This highlights the relative importance of management as a driver of soil activity, suggesting a relevant role of management to attenuate global change effects on soil activity. We also found that diversity (interactions between PFTs and evenness) strongly influence soil activity and fertility, and that these effects were mediated by environmental (regional and local) variables. In particular, diversity enhanced microbial biomass nitrogen (MBN), dissolved organic nitrogen (DON), total N, urease and phosphatase, and nitrification potential. These findings stress the



importance of maintaining community stability in grasslands because any shifts in vegetation composition resulting from climate change can modify soil activity and fertility, and consequently the soil and belowground community feedback to the atmosphere.

## Chapter 4

Extensively managed semi-natural grasslands in the Pyrenees were mainly sources of CH<sub>4</sub>, and flux values were higher than previously reported in grasslands using chamber techniques. Yet, our CO<sub>2</sub> and N<sub>2</sub>O values were comparable to studies in European grasslands. The grasslands at low altitudes were generally small sources of N<sub>2</sub>O, while the subalpine and the alpine grasslands were small sinks. We also found that GHG fluxes decrease with altitude in extensively managed grasslands in the Pyrenees; the higher the grassland altitude (hence the colder) the lower the fluxes. Additionally, not only did CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O fluxes change along the altitudinal gradient, but also along different seasons, with the highest GHG emissions occurring during the summer period. We identified aboveground biomass and temperature as the main drivers for fluxes of CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O in the Pyrenean grasslands. Additionally, grazing proved to have an important role on both CH<sub>4</sub> and N<sub>2</sub>O in the subalpine grassland CAST1850, most likely through its effect on vegetation composition and plant biomass reduction. Seasonality and soil water content also demonstrated a strong influence on N<sub>2</sub>O fluxes in both the montane and the subalpine grasslands. Our findings on the effect of vegetation removal on CH<sub>4</sub> and light/darkness conditions on N<sub>2</sub>O fluxes underline the need to investigate more in depth these factors and better understand their role in GHG fluxes, notably the controversial role of vegetation in grasslands as emitter or conduit of methane to the atmosphere. We also suggest continuous or more frequent GHG measurements on grasslands in the Pyrenees.

## Chapter 5

We found uptake of CO<sub>2</sub> and N<sub>2</sub>O, and emissions of CH<sub>4</sub>, at the peak of the vegetation biomass in all the grassland sites. Only Atlantic grasslands, characterized by moist climate, exhibited N<sub>2</sub>O emissions instead of uptake. PFT composition significantly affected GHG fluxes, and interactions between PFTs increased the uptake of CH<sub>4</sub> and N<sub>2</sub>O compared to single PFTs (identity effects). Our study provides evidence that plant functional type composition can alter GHG exchange, and that this alteration is mediated by the environmental conditions of the grasslands. Our findings also highlight the importance of functional diversity and its positive effect on CH<sub>4</sub> and N<sub>2</sub>O uptake, and consequently on the feedback from the ecosystem to the atmosphere. We also observed that specific PFTs perform differently in different climatic conditions. This is most likely driven by plant functional traits, which play an important role in the structure and stability of grassland communities. Therefore, we believe that further studies on the relationship between

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