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Faculdade de Ciências
Departamento de Biologia Vegetal

Effects of increased Nitrogen availability on the structure and functioning of a Mediterranean Basin maquis

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Doutoramento em Biologia
Especialidade de Ecofisiologia

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“Rien ne se perd, rien ne se crée, tout se transforme.”

Antoine Laurent de Lavoisier

Resumo

Foi desde a Revolução Industrial que as acções humanas se tornaram o principal motor da mudança ambiental global, ameaçando assim os processos químicos, biológicos e físicos do nosso planeta. Directa ou indirectamente, a humanidade deu origem às alterações climáticas e dos ciclos biogeoquímicos do carbono (C), azoto (N) e fósforo (P); reduziu a qualidade e quantidade de água potável; e conduziu à perda de biodiversidade e degradação dos serviços dos ecossistemas. Pensa-se que as alterações no ciclo global do N já terão excedido o limite de segurança da Terra.

O aumento do N reactivo (todas as formas de N, excepto o azoto molecular - N_2), resultante da intensificação das actividades agro-industriais, tem uma quimera de efeitos directos e indirectos sobre a atmosfera, solo, água e organismos. Dado que a estabilidade dos ecossistemas naturais e semi-natural depende da baixa fertilidade do solo, o aumento da deposição de N é reconhecido como uma ameaça à biodiversidade. Embora o efeito do N reactivo que chega aos ecossistemas varie de acordo com as condições edafo-climáticas, o tipo de ecossistema e/ou a sua fase da sucessão, a maioria dos estudos sobre os efeitos do aumento da disponibilidade de N foi realizada em ecossistemas florestais temperados (norte da Europa e América). Recentemente, em 2011, os impactos da deposição de N em ecossistemas terrestres foram revistos e as cargas críticas de N (uma estimativa quantitativa da exposição à deposição de N abaixo da qual não ocorrem, de acordo com o conhecimento actual, efeitos prejudiciais significativos nos elementos sensíveis do ambiente) foram reavaliadas para habitats europeus e norte-americanos. No entanto, ainda persistem graves lacunas no conhecimento da resposta de outros tipos de ecossistemas. Os ecossistemas de tipo Mediterrânico encontram-se na 'lista dos ecossistemas negligenciados'.

Apesar de o bioma Mediterrâneo (todas as regiões de tipo Mediterrânico - Califórnia, região central do Chile, Bacia do Mediterrâneo, sul da região do Cabo e sudoeste e sul da Austrália) cobrir apenas 2% da área terrestre do planeta, abriga 20% da diversidade conhecida de plantas vasculares, transformando assim a sua conservação numa prioridade global a par com as florestas tropicais. As características mais distintivas dos ecossistemas do tipo Mediterrânico em termos do controlo da resposta ao aumento da disponibilidade de N são: a sazonalidade da precipitação, a regulação do ciclo do N pelo fogo, o predomínio da deposição seca de N e a assoncronia entre a disponibilidade de N e a actividade biológica. A Califórnia e a Bacia do Mediterrâneo são as regiões Mediterrânicas mais ameaçados pela

deposição de N e embora existam muitos estudos nos ecossistemas Californianos, há muito poucos nos da Bacia do Mediterrâneo. Devido às suas diferenças geográficas, geológicas e históricas, estas regiões Mediterrânicas diferem em características importantes (por exemplo, a fertilidade do solo, disponibilidade de água, histórico de intervenção humana e ameaças presentes) que podem controlar a resposta aos motores de mudança globais e, dada a baixa fertilidade dos seus solos, ao aumento da disponibilidade de N em particular.

Por outro lado, muitos trabalhos têm abordado aspectos individuais dos efeitos do aumento da disponibilidade de N em ecossistemas do tipo Mediterrânico: deposição antropogénica de N, alterações na biodiversidade, impacto nos fluxos de gases entre o solo e a atmosfera, lixiviação de nitrato, microbiologia do solo e decomposição da matéria orgânica. No entanto, os impactos das mudanças globais nos ecossistemas Mediterrânicos Europeus são mal compreendidos e têm sido prejudicados pela falta de integração dos estudos ao nível do sistema. Assim, o objectivo geral desta tese é desenvolver uma estudo integrado das respostas ao aumento da disponibilidade de N de um mato pobre em nutrientes da Bacia do Mediterrâneo. As hipóteses fundamentais são:

1. Os matos da Bacia do Mediterrâneo são muito limitados pela biodisponibilidade de N, o que os torna muito sensíveis a incrementos de N.
2. Embora as formas de N sejam inter-convertíveis, a forma de N adicionada irá influenciar a resposta do ecossistema, uma vez que a diversidade e funcionalidade dos biota (plantas e microrganismos) é modelada pela limitante disponibilidade de N.
3. A resposta do ecossistema ao aumento da disponibilidade de N será detectada através de biotransformações chave que regulam o ciclo do N.
4. Estas biotransformações irão mediar os efeitos combinados do N nos compartimentos do ecossistema: biótico (plantas e microrganismos) e abiótico (solo), determinando assim a capacidade de retenção do N do ecossistema.

Esta investigação foi desenvolvida num local da rede Natura 2000 (PTCON0010 Arrábida/Espichel) que corresponde a um mato da Bacia do Mediterrâneo. Em 2007 (quatro anos após um fogo) foi montada uma experiência de campo de manipulação de N (dose e forma) num local com baixa deposição de N estimada (5,2 kg ha^{-1} ano^{-1}). As doses de N aplicadas foram menores do que a deposição de N noutras áreas de clima Mediterrânico (145 kg N ha^{-1} ano^{-1}) mas suficiente altas para estabelecer os 'piores' cenários de aumento de disponibilidade de N neste tipo de habitat. As formas de N foram escolhidos para simular os cenários mais prováveis de poluição de N na Bacia do Mediterrâneo. A disponibilidade de N foi modificada pela adição de 40 e 80 kg N ha^{-1} ano^{-1}, sob a forma de NH_4NO_3 (tratamentos designados por 40AN e 80AN) e 40 kg N ha^{-1} ano^{-1} como uma mistura 1:1 de NH_4Cl e $(NH_4)_2SO_4$ (designado por 40A). As parcelas controlo não foram fertilizadas.

Durante os 4,5 anos seguintes monitorizaram-se efeitos seleccionados sobre a estrutura e funcionalidade da vegetação e solo.

O aumento da disponibilidade de N promoveu a diversidade de plantas: após apenas um ano, as espécies mudaram mais com a dose de N mais alta (80AN), enquanto na quinta Primavera, as mudanças ainda estavam a decorrer principalmente nas parcelas que receberam mais N reduzido (40A e 80AN). O aumento da diversidade das plantas devido ao N, que contrasta com a maioria dos estudos, reflectiu a redução da condição de stress (limitação de N), permitindo assim a coexistência das espécies características do local com a entrada de espécies mais oportunistas. As espécies beneficiadas pelo N foram plantas pequenas, muitas vezes anuais, e típicas de áreas perturbadas e/ou eutrofizadas, enquanto que as afectadas pelo N foram arbustos perenes característicos de locais muito pobres em nutrientes. Algumas espécies de plantas responderam à dose de N e/ou à forma e algumas destas respostas foram compatíveis com o habitat dessas espécies e/ou com as suas respostas noutros estudos de aumento da disponibilidade de N.

Os tratamentos de adição de N com mais espécies de plantas (40A e 80AN) não produziram mais biomassa aérea e aumentaram a percentagem de solo nu. No entanto, o tratamento de N combinado com menos NH_4^+ (40AN) aumentou a biomassa aérea, diminuindo assim a percentagem de solo nu, mas à custa das pequenas plantas que foram incapazes de tolerar o crescente ensombramento. Nestas comunidades vegetais, parece haver um compromisso entre a diversidade de plantas e a produtividade/protecção do solo. Os dados sugerem que a agricultura, a maior fonte de NH_4^+-N, pode afectar a estrutura do mato diminuindo a cobertura vegetal permanente potencialmente aumentando a erosão do solo. Por outro lado, as actividades industriais e urbanas, fontes de NO_3^--N, podem promover a acumulação de biomassa, aumentando assim o risco de fogo. Este conhecimento é de importância significativa para a gestão do uso da terra em ecossistemas biodiversos e fragmentado como os do Mediterrâneo, especialmente em locais da rede Natura 2000.

Dada a interligação entre as comunidades vegetal e microbiana do solo, a resposta destas comunidades ao aumento do N foi semelhante. Ao fim de um ano de experiência, o aumento da disponibilidade de N (especialmente a dose mais elevada de N) também promoveu a diversidade de bactérias e fungos do solo. Os tratamentos de adição de N também mudaram a composição das espécies de esporos de Fungos Micorrízicos Arbusculares (FMA), com algumas respostas sendo compatíveis com aquelas obtidas noutros estudos de aumento da disponibilidade de N. Se as mudanças observadas na composição de espécies de plantas e FMA forem robustas a longo prazo, estas poderão ser indicadores estruturais sensíveis do aumento da disponibilidade de N, pelo menos em ecossistemas Mediterrânicos.

Independentemente dos tratamentos de adição de N e da duração da experiência, os solos no local experimental tinham concentrações mais baixas de C e N (~2% e ~0,1%, respectivamente) do que as descritas para a Bacia do Mediterrâneo. Não houve efeito dos tratamentos de adição de N na razão C/N e pH do solo. Os efeitos do N na concentração de matéria orgânica e na disponibilidade de N inorgânico no solo dependeram do tempo de tratamento/dose, ou seja, na Primavera do primeiro e segundo anos da experiência (2007 e 2008, respectivamente), a matéria orgânica e o N inorgânico não estavam relacionados com os tratamentos de adição de N. Mas na quinta Primavera (2011), detectou-se mais matéria orgânica e N inorgânico nas parcelas submetidas à dose de N mais elevada (80AN), sugerindo assim que o cumulativo de adições de N aumentou o 'estatuto de N' do ecossistema.

Dado que as plantas e os microrganismos do solo evoluíram sob determinados ambientes azotados, existem preferências por determinados padrões de disponibilidade de N, padrões esses que foram alterados pelos tratamentos de adição de N. Assim, o ambiente azotado dos dois principais grupos de plantas Mediterrânicas (semi-decíduas de Verão e esclerófilas sempre verdes) estava ligado com as suas estratégias de uso do N. Embora as espécies semi-decíduas de Verão ocupassem solos mais ricos em nitrato, a sua capacidade de o utilizar (i.e., actividade da nitrato reductase das folhas) foi significativamente menor que o das espécies esclerófilas sempre verdes. O padrão de disponibilidade de nitrato e amónio ao longo do ano também distinguiu estes dois grupos de plantas. Os traços distintivos de cada grupo de plantas (fenologia e sistema radicular) foram os principais determinantes das respectivas estratégias de uso do N, o que pode ter implicações para o nicho ecológico que estes grupos de plantas ocuparão com o estimado aumento da deposição de N.

As mudanças no padrão temporal da disponibilidade de N inorgânico pode explicar as mudanças a curto prazo na diversidade das plantas e dos microrganismos do solo. Os dados sugerem que, sob a condições da Primavera Mediterrânica, o N extra que foi adicionado foi absorvido pelas plantas e microrganismos e portanto retida pelo compartimento biótico do ecossistema. De acordo, a espécie vegetal dominante, *Cistus ladanifer*, mudou significativamente a sua cobertura e aumentou a concentração de N nas folhas e folhada. Durante o Verão, uma proporção significativa do N foi devolvida ao solo através de queda da folhada e no Outono, a sua decomposição fez com que o N voltasse à forma inorgânica. Assim, a disponibilidade de N inorgânico apresentou um pico no Outono, muito semelhante ao total cumulativo de N que tinha sido adicionado. Os dados sugerem que o N adicionado ao longo do primeiro ano de experiência na forma de NH_4NO_3 (40AN e 80AN) foi retido pelo sistema e reflectiu-se na disponibilidade de N inorgânico no Outono. Contudo, para o N adicionado como NH_4^+ (40A), uma proporção significativa não foi detectada no solo. A disponibilidade de N inorgânico (nitrato em particular), medido no

Outono parece ser um indicador funcional sensível do aumento da disponibilidade de N para os ecossistemas Mediterrânicos. Por outro lado, dado que o N adicionado foi maioritariamente armazenado na vegetação, a razão C/N do solo pode não ser um bom indicador da retenção do N nestes ecossistemas.

Considerar o efeito das mudanças integradas da comunidade vegetal (e não apenas de espécies individuais) devido ao aumento da disponibilidade de N na decomposição constitui uma abordagem inovadora e mais ecológica do que a que tem sido utilizada até agora. Assim, após 2,5 anos dos tratamentos de adição de N, solo das parcelas controlo e folhada dos quatro tratamentos (controlo, 40A, 40AN e 80AN) foram recolhidos na experiência de campo de manipulação de N para um estudo de decomposição em condições controladas (laboratório). Apenas a folhada produzida sob a dose de N mais elevada (80AN) tinha o dobro da quantidade de folhada de escleróﬁlas sempre verdes, em relação aos restantes tratamentos. Consequentemente, a folhada 80AN tinha maior concentração de lenhina e de N, resultando em menores razões C/N e lenhina/N, que são poderosos determinantes das taxas de decomposição. Como resultado, a decomposição da matéria orgânica nos microcosmos contendo folhada 80AN foi mais lenta (com concomitante redução na biomassa e actividade microbianas) do que nos tratamentos com folhada 40 kg N ha^{-1} ano^{-1} (40A e 40AN). Isto implica que se o aumento da disponibilidade de N nos matos Mediterrânicos seleccionar espécies de plantas com características de folhada que atrasem a decomposição pode, potencialmente, aumentar a acumulação de matéria orgânica do solo. Embora também seja importante quantificar a decomposição da folhada *in situ*, usando por exemplo sacos de folhada, o que também incluiria as alterações na comunidade microbiana do solo, no final da experiência de campo de manipulação de N, a maior acumulação de matéria orgânica nas parcelas 80AN corrobora as taxas mais lentas de decomposição observadas no laboratório. As características da folhada (concentrações de N e lenhina e a respectiva razão) parecem ser indicadores funcionais sensíveis do aumento da disponibilidade de N nos ecossistemas do Mediterrâneo.

Os dados permitiram estabelecer uma primeira estimativa de carga crítica de N para este habitat Europeu, entre 20 e 30 kg N ha^{-1} ano^{-1}. Para o habitat equivalente Californiano, 'coastal sage scrub', a carga crítica de N definida foi muito mais baixa (<10 kg N ha-1 ano^{-1}). No entanto, dada a curta duração desta experiência e a falta de outros estudos, talvez este novo valor venha a precisar de ser revisto em baixa para o valor da Califórnia, e passe a incluir a importância da forma de N.

Com base neste estudo, e no que é conhecido do 'coastal sage scrub' e 'chaparral' Californianos, as implicações das mudanças estruturais e funcionais para a sustentabilidade e funcionalidade do ecossistema foram avaliados, tendo em consideração que a dinâmica das comunidades vegetais Mediterrânicas está intimamente relacionada com a ocorrência

de fogos. Assim, desenvolveram-se cenários conceptuais para os efeitos do aumento da disponibilidade de N (principalmente NH_y ou NO_x) nos ecossistemas da Bacia Mediterrânica, de acordo com a fase de sucessão pós-fogo. Os dados sugerem que os ecossistemas Mediterrânicos no início da sucessão são mais sensíveis ao aumento da disponibilidade de N do que os mais avançados. Isto está relacionado com uma maior capacidade de retenção do N nos ecossistemas mais ‘jovens’. Dado que nos ecossistemas mais avançados há uma diminuição da taxa de crescimento das plantas, a sua capacidade de retenção do N diminui. Assim, há uma relação entre a retenção de N e a fase de sucessão do ecossistema, ou seja, o N reactivo ou é retido e altera a estrutura e funcionamento do ecossistema ou é perdido e vai alterar um outro sistema mais 'a jusante, nomeadamente os ecossistemas aquáticos. Embora os processos não sejam claros, as bacias hidrográficas do Mediterrâneo têm uma elevada capacidade de retenção do N, o que é evidenciado pelo facto a quase totalidade do N (~ 90%) que entra na Bacia do Rio Ebro (Espanha) ser retida, não atingindo o Mar Mediterrâneo.

Os resultados desta tese fornecem novos e importantes ‘insights’ sobre o efeito do N na estrutura e funcionamento dos ecossistemas da Bacia do Mediterrâneo. No entanto, e reconhecendo as suas limitações, um novo projecto foi proposto e financiado: In-Nitro. Os objectivos do In-Nitro são: (i) manter a experiência de campo de manipulação de N e os tópicos de investigação desenvolvidos no presente estudo, para obter resultados de longo prazo (ii) incorporar a importância da co-limitação por água e P na resposta integrada dos ecossistemas Mediterrânicos ao aumento da disponibilidade de N, e (iii) integrar os resultados obtidos nos distintos projectos para melhorar o quadro conceptual das respostas dos ecossistemas Mediterrânicos ao aumento da disponibilidade de N.

Palavras-chave: Plantas; *Cistus ladanifer*; Microrganismos do solo; Solo; Nitrato; Amónio; Retenção de N; Recirculação do N; Folhada; Decomposição

ABSTRACT

Increased N availability, resulting from agro-industrial activities, affects ecosystems' stability. Mediterranean Basin ecosystems are biodiversity hotspots threatened by N deposition, however, little or no information is available on their responses to increased N. The general aim of this thesis was to develop an integrated system-level approach to study the responses of a Mediterranean Basin maquis to increased N availability. Since 2007, the form and dose of N available at the experimental site (ambient N deposition 5.2 kg ha^{-1} yr^{-1} and soil N 0.1%) was modified by addition of 40 kg NH_4^+-N ha^{-1} yr^{-1}, 40 and 80 kg NH_4NO_3-N ha^{-1} yr^{-1}. The structure and functioning of above- and below-ground communities were monitored for 4.5 years.

Over the first year, the added N was retained by the system and detected in the autumn as soil inorganic N. The increased N promoted plant and soil microbial diversity. Exposure to 40 kg NH_4^+-N ha^{-1} yr^{-1} (solely or with nitrate) increased plant diversity but not productivity, while exposure to 40 kg NH_4NO_3-N ha^{-1} y^{-1} increased productivity but not diversity. The N-benefited species were small short-lived plants, from disturbed and/or eutrophied sites, while N-affected plants were perennial shrubs characteristic of very poor sites.

After 2.5 years of treatments, soils from the control and litter from the four treatments were collected for a laboratory decomposition study. The leaf litter produced under the high N treatment was enriched in N and lignin. This study suggested that increasing N in Mediterranean maquis selected for plants with lower litter decomposability, potentially increasing soil organic matter. In agreement, after 4.5 years, soil organic matter and inorganic N had increased under the higher N dose. The dataset permitted the first estimation of an N critical load for this habitat (20-30 kg N ha^{-1} yr^{-1}).

Data suggest that agriculture, a source of NH_4^+-N, may decrease the permanent plant cover and increase soil erosion, while industrial and urban activities, sources of NO_3^--N, may promote productivity and increase fire risk. This is of significant importance for land-use management in biodiverse and fragmented ecosystems such as the Mediterranean ones, especially in Natura 2000 sites. However, longer-term studies are needed to explore the applicability of the findings and the interaction of N availability with other global change drivers.

Keywords: Plants; *Cistus ladanifer*; Soil microorganisms; Soil; Nitrate; Ammonium; N retention; N cycling; Litter; Decomposition

List of abbreviations

40 kg NH_4^+-N ha^{-1} yr^{-1} – 40A
40 kg NH_4NO_3-N ha^{-1} yr^{-1} – 40AN
80 kg NH_4NO_3-N ha^{-1} yr^{-1} – 80AN
Ammonia – NH_3
Ammonium – NH_4^+
Ammonium chloride – NH_4Cl
Ammonium nitrate – NH_4NO_3
Ammonium sulfate – $(NH_4)_2SO_4$
Among other things – inter alia
Arbuscular mycorrhizal fungi - AMF
At sea level – a.s.l.
Bovine serum albumin – BSA
Calcium – Ca
Carbon – C
Carbon dioxide – CO_2
Carbon to nitrogen ratio – C/N ratio
Carbon to phosphorus ratio – C/P ratio
Centimeter – cm
Cubic centimeter – cm^3
Degree celsius – ºC
Dithiothreitol – DTT
Dry weight – Dwt or DW
East – E
Ethylenediaminetetraacetic acid – EDTA
Flavin adenine dinucleotide disodium salt hydrate – FAD
For example – e.g.
Fresh weight – Fwt or FW
Gases of oxidized N – NO_x
Gases of reduced N – NH_y
Gram – g
Hectare – ha
Hour – h
Hydrochloric acid – HCl
Inorganic N – inorgN
International Organization for Standardization – ISO
Kilogram – kg
Kilometer – km
Lignin to N ratio – lignin/N ratio
Liter – l
Litter from 40A – L40A
Litter from 40AN – L40AN
Litter from 80AN – L80AN
Litter from Control – LC
Magnesium – Mg
Magnesium chloride – $MgCl_2$
Manganese – Mn
Meter – m
Microcosms with L40A – M40A
Microcosms with L40AN – M40AN
Microcosms with L80AN – M80AN
Microcosms with LC – MC
Microcosms with soil only – MnL
Microgram – µg
Microlitre – µl
Micromolar – µM
Milimolar – mM
Milligram – mg
Milliliter – ml
Millimeter – mm
Minute – min
Molar – M
Nanometer – nm
Nitrate – NO_3^-
Nitrate reductase activity – NRA
Nitric oxide – NO
Nitrogen – N
Nitrogen to phosphorus ratio – N/P ratio
Nitrous oxide – N_2O
North – N
NR – Nitrate reductase
Organic matter – OM
Percentage – %
Phosphate – PO_4^{3-}
Phospholipid fatty acid – PLFA
Phosphorus – P
Plant Functional Groups – PFG
Polyvinylpyrrolidone – PVP
Potassium – K
Potassium chloride – KCl
Potassium hydroxide – KOH
Potassium nitrate – KNO_3
Rotations per minute – rpm
Sodium – Na
South – S
Square meter – m^2
Sulphur – S
Temperature Gradient Gel Electrophoresis – TGGE
That is – i.e.
Tones – t
Volume per volume – v/v
Weight per volume – w/v
West – W
Year – yr
β-Nicotinamide adenine dinucleotide, reduced dipotassium salt – NADH

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Chapter 1

General Introduction

General Introduction

GLOBAL CHANGE DRIVERS

Although the Earth has undergone many periods of significant environmental change, during the past 10 000 years (the Holocene), our planet's environment has been unusually stable (Rioual *et al.* 2001). This stability has allowed human civilizations to arise, develop and thrive (Rockström *et al.* 2009). However, since the Industrial Revolution human actions have become the main driver of global environmental change, so it is accepted that we are now in a new era, the Anthropocene (Crutzen 2002). In this new era, several global change drivers threaten our planet's biological, chemical and physical processes (Rockström *et al.* 2009; Erisman *et al.* 2011). The human drivers include a growing population and changing per capita consumption patterns both of which affect food demand and quality, livestock and fibre production, energy use (fossil fuel and biofuel), and cause land-use changes. Directly or indirectly, these have given rise to climate change, alteration of the biogeochemical cycles of carbon (C), nitrogen (N) and phosphorus (P), reduced water quality and availability, exacerbated biodiversity loss and degraded ecosystem services (Erisman *et al.* 2011). Thus, these planetary-scale changes also threaten mankind (Sala *et al.* 2000; Rockström *et al.* 2009; Erisman *et al.* 2011). Although the methodologies used to establish the safety boundaries for planetary-scale changes are questionable (e.g. Allen 2009; Bass 2009; Molden 2009; etc), Rockström *et al.* (2009) identified three main interdependent pressures on global sustainability (changes in the global N cycle, biodiversity loss and climate change) as exceeding the planetary safety boundaries. The role of increased N deposition in food and energy security, human health (Erisman *et al.* 2011), biodiversity loss (for review see Bobbink *et al.* 2010) and on alterations to the functioning of some ecosystems (Rockström *et al.* 2009) have been assessed, although the feedbacks to climate change are less well understood (Erisman *et al.* 2011).

INCREASED REACTIVE N

As the human population continues to expand, so do the global impacts of reactive N (all N forms except di-nitrogen – N_2) released into the biosphere as a result of increased agricultural activities and fossil fuel emissions - Galloway *et al.* 2008; Rockström *et al.* 2009; Erisman *et al.* 2008, 2011). Human activities have caused total N loading to the global landmass to nearly double since the pre-industrial era (Galloway *et al.* 2008). This recent input of reactive N has a myriad of effects on waters, soils, and atmosphere (Rockström *et al.* 2009).

Europe's awareness on this issue may be evidenced by the NitroEurope, a project (2005-2011) for integrated European research into the N cycle that was part of the European Union's Sixth Framework Programme for Research and Technological Development. Under the auspices of NitroEurope, a cost-benefit analysis highlighted how the overall environmental costs of all reactive N losses in Europe (estimated as €70-€320 billion per year at current rates) outweighed the direct economical effects of reactive N in agriculture (Sutton *et al.* 2011). Therefore, at least in Europe, besides its scientific worth, the N issue has recently gained socio, economical and political significance.

Because most global biodiversity is contained within natural and semi-natural ecosystems, and their stability depends upon low soil fertility (Bobbink, Hornung & Roelofs 1998), increased reactive N deposition has been acknowledged as a threat to biodiversity (Sala *et al.* 2000; Phoenix *et al.* 2006; Rockström *et al.* 2009). Most research on the effects of increased N availability has been conducted in temperate forest ecosystems (northern Europe and America – Bobbink, Hornung & Roelofs 1998; Phoenix *et al.* 2006; Bobbink *et al.* 2010). But the fate of the reactive N entering ecosystems appears to be strongly dependent on edaphic and climatic conditions, leading to regional differences in N export and use which should not be overlooked in forecasts of N cycle dynamics (Galloway *et al.* 2008). Also, the type of ecosystem and/or its successional stage, appear to influence the impact of the reactive N enrichment (Bobbink *et al.* 2010, 2011; Pardo Robbin-Abbott & Driscoll 2011). As an example, in California, the addition of 50 kg N ha^{-1} yr^{-1} changed the relative dominance of shrubs in a coastal sage scrub, but did not alter the plant community in a post-fire chaparral (Vourlitis & Pasquini 2009).

Despite the effort to understand and characterize the impacts of N deposition on terrestrial ecosystems (Bobbink *et al.* 2010, 2011; Pardo *et al.* 2011), gaps in knowledge for ecosystems other than the temperate ones persist (Bobbink *et al.* 2010). Mediterranean-type ecosystems are on the 'neglected ecosystems list'. The most distinctive features of these ecosystems in terms of controlling the response to increased N availability are: precipitation seasonality, regulation of the N cycle by fire, dominance of dry N deposition and asynchrony between N availability and biological activity (Ochoa-Hueso *et al.* 2011).

- The present thesis will focus on the effects of increased N availability in Mediterranean Basin ecosystems.

MEDITERRANEAN-TYPE ECOSYSTEMS

Although the Mediterranean biome (all Mediterranean-type ecosystems) only covers 2% of the Earth's land area, it hosts 20% of our planet's known vascular plant diversity (Cowling *et al.* 1996; Klausmeyer & Shaw 2009). Therefore, the Mediterranean biome represents a global conservation priority (Myers *et al.* 2000; Klausmeyer & Shaw 2009) that even rivals tropical rainforests (Cowling *et al.* 1996; Klausmeyer & Shaw 2009).

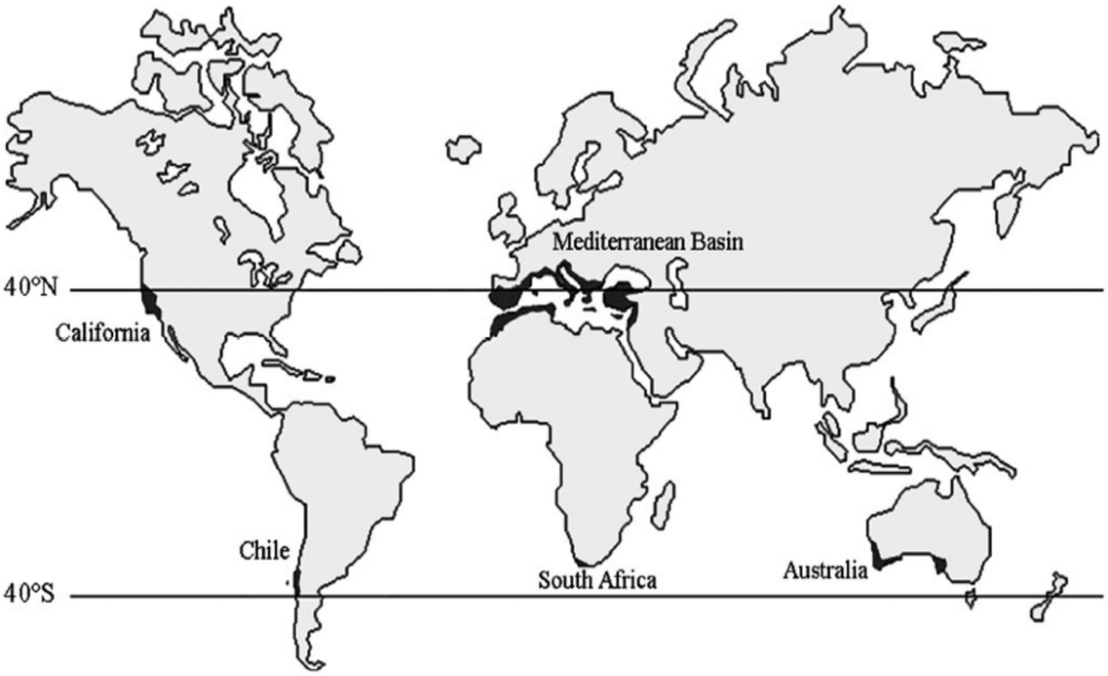

Figure 1.1 – The five regions of the world with a Mediterranean climate (adapted from Cody & Mooney 1978).

Mediterranean-type ecosystems have developed under the influence of the Mediterranean climate, that is a transition between cold temperate and dry tropical; it is highly seasonal with warm and dry summers (seasonal-drought), which contrast with cool and rainy winters (spring and autumn are usually the growing seasons). This bimodal climate only began to appear in the Pliocene (3.2 million years) as part of a global cooling trend, and occurs in five regions of the globe: California, central Chile, Mediterranean Basin, southern Cape region and south western and southern Australia, between 30º and 45º latitude and where land is influenced by cold offshore ocean currents (Fig. 1.1 - Cody & Mooney 1978). As a result of their geographic, geological and historical differences, these Mediterranean regions differ in important characteristics (e.g. soil fertility, water availability, past human intervention and present threats) that may control the response to global change drivers especially, given its low soil fertility, to N inputs. Recently, Ochoa-Hueso *et al.* (2011) reviewed the ecological consequences of N deposition on the five Mediterranean regions of the world. California and the Mediterranean Basin were considered to be the most threatened by N deposition. However, while there are many studies on Californian ecosystems (Table 1.1 – adapted from

Fenn, Allen & Geiser 2011), there are very few on the Mediterranean Basin (Bonanomi, Caporaso & Allegrezza 2006; Ochoa-Hueso & Manrique 2010; Pinho *et al.* 2011, 2012; Ochoa-Hueso *et al.* 2011). Thus, more research is needed to understand and characterize the impacts of N deposition in each Mediterranean region and vegetation type (Ochoa-Hueso *et al.* 2011).

THE MEDITERRANEAN BASIN CONTEXT

The Mediterranean Basin stretches over approximately 3800 km W-E and 100 km N-S, between 30 and 45°N, and borders 24 countries. It is one of the most complex regions of the world and the only case of a large sea surrounded by three continents (Blondel & Aronson 2004). Its complexity is readily perceived by its patchiness, which plays a critical role in maintaining and generating diversity at the scale of species and communities. This patchiness has been generated by topography, climate, geopedology and human intervention (Klausmeyer & Shaw 2009). Most of the topographic diversity derives from the numerous mountain chains that define the Basin’s various continental contours, which also create microclimates with rising altitude. In general, a sharp gradient exists between the colder and wetter north-western and north-eastern extremities of the Basin and the hotter and more arid, south-eastern and south-western parts in North Africa and the Near East. Although many Mediterranean soils and substrates are limestone of marine origin, unusual soil types and discontinuous geological substrates (including volcanic soils) also contribute to the local and regional diversity of the habitats (Blondel & Aronson 2004). The importance of the Mediterranean Basin for human civilizations is evidenced by the Sea *Mediterraneus* that means in the middle of the Earth. Mediterranean Basin ecosystems have experienced intensive human development and impact for millennia, significantly longer than any other biodiversity hotspot (http://www.biodiversityhotspots.org/xp/hotspots/mediterranean/Pages/impacts.aspx). Mediterranean Basin vegetation includes species with origins in almost all known biogeographic zones because it is a tension zone lying between the temperate, arid and tropical biogeographical regions that surround it. As a result, intricate interpenetration and interaction of taxa, hybridization, and speciation have been particularly favoured and fostered, as compared to more homogenous regions further north and south (Quézel 1985). Altogether, the Mediterranean flora is one of the richest in the world, comprising annual grasslands, shrublands, dry woodlands and forests (Cowling *et al*. 1996). Despite its complexity and diversity, the vegetation of Mediterranean ecosystems may be grouped into two main plant functional types: summer semi-deciduous and evergreen-sclerophylls. Each group has been characterized on the basis of its phenology (Correia 1988), water relations, carbon exchange properties (Werner, Correia & Beyschlag 1999), soil surface characteristics (Cruz *et al.* 2008) and fire-regenerating strategies (Trabaud 1981; Keeley 1986; Clemente, Rego & Correia 2005).

Present threats to Mediterranean Basin ecosystems

The mild climate and proximity to the sea make Mediterranean regions very attractive to humans, resulting in disproportionately high conversion of land for agriculture, urban development, tourism and other human uses (Hoekstra *et al.* 2005; Klausmeyer & Shaw 2009). At present, the Mediterranean Basin, together with California, are the most populated and therefore impacted, Mediterranean-type regions. As a consequence, within the Mediterranean Basin, N deposition is estimated to increase three fold by 2050 (Galloway *et al.* 2004; Phoenix *et al.* 2006). Land use changes, namely rural abandonment (Sala *et al.* 2000; Ochoa-Hueso *et al.* 2011), habitat fragmentation (Ochoa-Hueso *et al.* 2011) and biotic exchange (e.g. exotic invasive species – Sala *et al.* 2000) are additional threats in this region. Although fire is a natural element in Mediterranean ecosystems (except in the Chilean matorral), rural abandonment and/or higher N-driven dry biomass accumulation (Ochoa-Hueso *et al.* 2011) may alter the fire regime and its severity (Lavorel *et al.* 1998; Bonanomi, Caporaso & Allegrezza 2006) by creating a continuum of flammable material. This could result in dramatic changes in the landscape and, when fires occur close to populated areas, could potentially impact human well-being and the economy (Ochoa-Hueso *et al.* 2011). The potential for enhanced N deposition to change the landscape appears therefore to be particularly high in the Mediterranean Basin.

Because summer semi-deciduous are seeders (regenerate by seed germination) and evergreen sclerophylls are resprouters (regenerate by resprouting from belowground organs - Trabaud 1981; Keeley 1986; Clemente, Rego & Correia 2005), after a fire summer semi-deciduous plants dominate. However these are progressively eliminated under the canopies of evergreen sclerophylls in later phases of a post-fire succession. Due to recurring disturbance factors or unknown mechanisms, the transition from *Cistus* dominated maquis to forests may be delayed or may never happen. Moreover, under the current and forecasted climatic trends, maquis are likely to expand because *Cistus* shrubs are physiologically well adapted to dry conditions and recurring fires (Acácio *et al.* 2007). Within Europe, the total area covered by shrublands (Mediterranean and temperate) is 14%, while for southern European countries (Andorra, Albania, Bosnia and Herzegovina, Cyprus, Spain, France, Greece, Croatia, Italy, Montenegro, Macedonia, Malta, Portugal, Turkey and Vatican City) is 18% but for Portugal is down to 4% (http://www.eea.europa.eu/data-and-maps/data/data-viewers/land-accounts).

- The present thesis will focus on the effects of increased N availability in Mediterranean Basin shrublands.

MEDITERRANEAN SHRUBLANDS: THE MAQUIS

A Mediterranean maquis is a habitat that comprises closed vegetation, usually with 100% cover, mainly shrubs with few annuals and some geophytes, while trees are nearly always present, some of which may be in shrub form (http://eunis.eea.europa.eu/habitats/1697). Thus the most characteristic feature of maquis is that they contain all the growth forms recognized by plant ecologists (Thompson 2005). As a result of natural and/or human influences (thin soils, forest clearance for timber, grazing by livestock and/or repeated fires) there are several types of shrublands in the Mediterranean Basin. Within the Mediterranean Basin, there are different local names to designate the forms of these natural and semi-natural shrublands and the landscapes they dominate: garrigue, gariga and maquis or machia in France and Italy; Xerovuni and phrygana in Greece; matorral and tomilares in Spain, Choresh or Maquis in Israel, and bath's troughout the Near East are just some of the collection. Comparable terms used in others Mediterranean climate regions, where similar vegetation types are common, include respectively: chaparral and coastal sage in California; matorral and Jaral in Chile, fymbos in south Africa; and Kwongan and mallee in Southern Australia (Blondel & Aronso 2004).

- The present thesis will focus on the effects of increased N in Mediterranean Basin maquis in Portugal (Fig. 1.2).

POTENTIAL EFFECTS OF INCREASED N AVAILABILITY ON MEDITERRANEAN BASIN PLANT COMMUNITIES

As previously mentioned, there are many studies on the effects of increased N deposition in Californian ecosystems but very few in the Mediterranean Basin. Therefore, the following sections will focus on indicators of high N availability identified for Californian ecosystems, especially for the coastal sage scrub that is the equivalent to the Mediterranean maquis (Table 1.1 – adapted from Fenn, Allen & Geiser 2011). Special attention will be paid to the indicators used for developing the N critical loads (a quantitative estimate of exposure to N deposition below which significant harmful effects on sensitive elements of the environment do not occur according to present knowledge - Nilsson & Grennfelt 1988).

N-driven changes on diversity

Assuming that N limitation represents a component of environmental severity, in very poor-soils subjected to increased N availability, plant diversity would be expected to increase (Bobbink, Hornung & Roelofs 1998; Chalcraft *et al.* 2008) based on the revised Grime's humped-back model (Grime 1973; Michalet *et al.* 2006). The increment in plant diversity would reflect the co-existence of the slow growing conservative species (characteristic of sites very poor in nutrients) with the incoming, fast growing, exploitative ones (typical of disturbed and/or eutrophied sites). Therefore, the initial ecosystem N status seems to determine in which zone of the humped-back model (Grime 1973; Michalet *et al.* 2006) the system is at, and therefore the effect that the increased N availability will have on diversity.

Table 1.1 – Empirical N critical loads for the Mediterranean Californian habitats. Reliability rating: ## reliable; # fairly reliable (adapted from Fenn, Allen & Geiser 2011).

Ecosystem	N Critical Load (kg N ha^{-1} yr^{-1})	Reliability	Responder/indicator	Study
Coastal sage scrub	7.8-10	#	Exotic invasive grass cover, native forb richness, arbuscular mycorrhizal richness	Egerton-Warburton & Allen 2000; Tonnesen *et al.* 2007
Chaparral	3.1-14	#	NO_3^- leaching; changes in lichen communities	Fenn *et al.* 2003, 2010; Geiser *et al.* 2010; Meixner & Fenn 2004; Meixner *et al.* 2006;
Mixed-conifer forest	3-39	##	Lichen chemistry and community changes; NO_3^- leaching; soil acidification; reduced fine root biomass	Grulke *et al.* 1998; Allen *et al.* 2007; Breiner, Gimeno & Fenn 2007; Fenn *et al.* 2008, 2010;
Serpentine grasslands	6	##	Annual grass invasion, replacing native herbs	Weiss 1999; Fenn *et al.* 2010

But N-driven increased diversity has rarely been observed even in Mediterranean-type ecosystems. The few studies that exist within the Mediterranean Basin have not reported any N-driven increase in plant diversity, probably because those ecosystems in particular have a higher N status: Bonanomi, Caporaso & Allegrezza (2006) conducted a 3-year experiment in a species-poor grassland in Italy, which had been used for agriculture while Ochoa-Hueso (2011) conducted a ~ 3-year experiment in a semi-arid kermes oak thicket close to Madrid (Spain) and therefore with high background N deposition (by current modelling, ~ 22 kg N ha^{-1} yr^{-1} - Mark Theobald, personal communication). By contrast, the few studies reporting N-driven increased diversity in Mediterranean-type (plants – Allen, Rao & Steers 2009; Vourlitis & Pasquini 2009; epiphytic lichens – Pinho *et al.* 2011, 2012) and temperate ecosystems (plants – Calvo *et al.* 2005; epiphytic lichens – Mitchell *et al.* 2005) have been conducted at sites with low N status. Due to the higher N status of most studied ecosystems, increasing N availability pushed these systems even further down the descending 'zone' of the humped-back model, i.e., only loss of plant species was observed (Bobbink *et al.* 2010; De Schrijver *et al.* 2011). Therefore, not much is known about the initial response of an ecosystem with low N status subjected to N enrichment.

Questions to be addressed here include:

- N enrichment in a nutrient poor Mediterranean Basin maquis: will plant diversity increase or decrease (**chapters 2 and 7**)?
- Do effects depend on the form of deposited N (NH_y *versus* NO_x - **chapters 2 and 7**)?

Mechanisms involved in the N-driven changes

Increasing N availability will cause many direct and indirect effects on ecosystem structure and function (Bobbink *et al.* 2010).

Changes in nutrients availability

In Mediterranean-type ecosystems subjected to low N deposition, soil inorganic N pools are highly variable through the year (Padgett *et al.* 1999; Gallardo *et al.* 2000; Cruz *et al.* 2008). Under conditions of increased N deposition (gradient or N-manipulation experiment) soil inorganic N pools remain heterogeneous but the annual pattern of availability changes (Padgett *et al.* 1999; Vourlitis, Pasquini & Mustard 2009). Therefore, plants (and microorganisms) that are 'active' within those periods of increased N availability are more likely to be N-benefited or N-affected. This effect chain can also be influenced by other soil factors, such as P limitation (Bobbink *et al.* 2010). Most semi-natural ecosystems, and Mediterranean ones in particular (Sardans & Peñuelas 2004), are co-limited by N and P. Soil N/P ratio best explained Mediterranean herbaceous plant communities arranged along a gradient of ruderality (Fanelli, Lestini & Sauli 2008).

Questions to be addressed here include:

- Will inorganic N pools reflect increased N availability in a Mediterranean maquis, always or just in specific seasons (**chapters 2, 5 and 7**)?
- Is there a link between N-driven changes in plant community and alterations in soil nutrients availability in a Mediterranean maquis (**chapters 2 and 4**)?
- Does the form of deposited N (NH_y versus NO_x - **chapters 2, 4, 5 and 7**) modify these changes?

Direct ammonium toxicity

Sensitivity to NH_4^+ seems to be a universal phenomenon (ranging from plants to humans - Britto & Kronzucker 2002). But plants differ in their tolerance to ammonium (Cruz, Lips &

Martins-Loução 2003; Kronzucker *et al.* 2003), with early successional species, summer semi-deciduous, preferring nitrate, and being relatively NH_4^+ intolerant, while late successional ones, evergreen sclerophylls, tend to be more NH_4^+ tolerant (Cruz, Lips & Martins-Loução 2003; Kronzucker *et al.* 2003). Summer semi-deciduous species are therefore more likely to suffer from direct NH_4^+ toxicity than evergreen sclerophylls. This may help explain why the response of Mediterranean-type ecosystems to increased N availability depends on where the ecosystem sits with respect to ecological succession, being possibly faster and more dramatic in the early phases following a disturbance (e.g. fire - Ochoa-Hueso *et al.* 2011).

Questions to be addressed here include:

- ❖ How long will it take for a Mediterranean maquis to respond to increased N (**chapter 2**)?
- ❖ Does the form of deposited N (NH_y versus NO_x – **chapters 2 and 7**) modify the response?
- ❖ Do summer semi-deciduous and evergreen sclerophylls differ in their N use (**chapter 4**)?

Soil acidification

Soil acidification is one of the most commonly reported effects of N enrichment and may result from base cation loss and nitrification. However, soil acidification tends to be less pronounced in soils of the Mediterranean Basin than in other ecosystems, due to their high cation exchange capacity and alkaline sands blown in from North Africa (Ochoa-Hueso *et al.* 2011). Nevertheless N-driven acidification has been reported for some Mediterranean-type ecosystems: Californian forests (Breiner, Gimeno & Fenn 2007) and shrublands (Vourlitis, Pasquini & Mustard 2009) and Mediterranean Basin shrublands (Ochoa-Hueso & Manrique, 2010).

Questions to be addressed here include:

- ❖ How atypical is N-driven soil acidification within the Mediterranean Basin (**chapters 2, 5 and 7**)?
- ❖ Does it depend on the form of deposited N (NH_y versus NO_x – **chapters 2, 5 and 7**)?

Increased susceptibility to secondary stress and disturbance factors

A few studies have investigated how the interaction between N and water availability (both limiting in Mediterranean ecosystems) might control plant community responses. For example, Mediterranean Basin grasses and forbs (e.g. *Schismus barbatus, Bromus madritensis* and *Erodium cicutarium*) threaten Californian ecosystems (Allen, Rao & Steers 2009), probably because Californian soils are more fertile than Mediterranean Basin ones

(Cowling *et al.* 1996). These species are especially 'aggressive' under higher water availability (Rao & Allen 2010), which may be due to the fact that they are no longer limited by water or nutrients. Given that N enrichment has been shown to increase plant productivity in many types of ecosystems (Bobbink *et al.* 2010) including Mediterranean-type ones (grasslands - Bonanomi, Caporaso & Allegrezza 2006 - and Californian coastal sage scrub - Fenn *et al.* 2003; Allen *et al.* 2005), it may increase the severity of the summer drought. Summer semi-deciduous and evergreen sclerophylls differ in rooting depths; summer semi-deciduous species tend to have a shallow root system while evergreen sclerophylls have both shallow and deeper roots (Correia 1988; Canadell *et al.* 1996). Evergreen sclerophylls are also more water use efficient (Correia & Catarino 1994; Canadell *et al.* 1996). Therefore, evergreen sclerophylls are less likely to be affected by N-driven summer drought than summer semi-deciduous.

N deposition may affect the biotic and abiotic interactions of the plant community (Throop 2005) and alter decomposition rates (Knorr, Frey & Curtis 2005) due to N-driven changes in the quality and availability of plant tissues. N-driven increased herbivory has been shown for a wide range of species from different types of ecosystems: *Ambrosia artemisiifolia* (Asteraceae, common ragweed) growing in a temperate mid-successional old field (Throop 2005), *Calluna vulgaris* growing in a temperate heathland (Power *et al.* 1998) and *Quercus kellogii* in Californian mixed forests (Jones *et al.* 2008). However, it is not always the case, e.g., N enrichment in a Californian annual grassland community increased seedling N concentrations and growth rates but not herbivory (Cleland *et al.* 2006).

Reported N effects on decomposition are controversial and most studies have only addressed decomposition of individual plant species grown under high N availability (Knorr, Frey & Curtis 2005). The few studies made on the effects of increased N on decomposition of Mediterranean litter (Sirulnik *et al.* 2007; Alarcón-Gutiérrez *et al.* 2008; Kazakou *et al.* 2009) suggest that N enrichment may enable Mediterranean-type ecosystems to retain more soil OM (Ochoa-Hueso *et al.* 2011).

Questions to be addressed here include:

- Will N-driven changes in plant quality differ from N-driven changes in whole plant community? How will it impact organic matter accumulation (**chapters 5 and 6**)?
- Do these processes depend on the form of deposited N (NH_y versus NO_x – **chapters 5 and 6**)?

Which plant species will benefit and which will lose out from increased N?

In temperate ecosystems, N-benefited species are fast growers, such as the grasses *Eriophorum vaginatum* (Sheppard *et al.* 2011), *Molinia caerulea* and *Deschampsia flexuosa* and the tree *Betula pubescens* (Bobbink *et al.* 2011), while N-affected are slow growing conservative species, such as *Calluna vulgaris*, *Sphagnun capillifolium* and *Cladonia portentosa* (Sheppard *et al.* 2011). As for Mediterranean-type ecosystems, in California the N-benefited species are often invasive grasses and forbs from the Mediterranean Basin (*Schismus barbatus, Bromus madritensis* and *Erodium cicutarium*) while N-affected are slow growing conservative, native species (Allen *et al.* 2007; Allen, Rao & Steers 2009). Most N-benefited species are annuals, or if not with short life cycles while N-affected, are usually perennials (Allen *et al.* 2007). Being short-lived may enable N-benefited species to 'escape' the previously mentioned summer drought in the form of seeds, which allows them to be benefited from the increased N (Suding *et al.* 2005).

Questions to be addressed here include:

- Will N enrichment influence annuals positively and perennials negatively (**chapters 2 and 7**)?
- Will there be consistent plant responders to N enrichment (**chapter 7**)?
- Will it depend on the form of deposited N (NH_y versus NO_x – **chapters 2 and 7**)?

SOIL MICROBIAL N-DRIVEN CHANGES

It is known that increased N availability changes plant diversity, and that above and below ground communites are interconnected (Klironomos 2002). However, and despite their importance for ecosystem response's to increased N availability, studies of soil microbiology have not kept pace with those above ground. This knowledge gap applies especially to Mediterranean Basin ecosystems (Ochoa-Hueso *et al.* 2011). Nevertheless, studies made in other types of ecosystems and in California, established that mycorrhizal colonization and soil microbial activity can be used as 'early' biological indicators of increased N availability (Chung *et al.* 2007).

Mycorrhizal fungi

Mycorrhizal root colonization may indicate inter alia the extent to which plants depend on mycorrhizae for their nutrient uptake. Mycorrhization is typically reduced by high nutrient availability, while spore density is an indication of the amount of carbon flowing from the plant to the fungus and indicates the strength of the mutualistic relationship (Fenn, Allen & Geiser 2011). Within Europe, mycorrhizal fungi, especially their fruiting bodies (or absence - Kottke & Wallenda 1998) have been used to indicate increased N availability. In general, increased N availability altered species composition of ectomycorrhizal fungi (fruit bodies production and below ground mycelia - Brandrud 1995; Lilleskov *et al.* 2002; Treseder 2004). Ericoid mycorrhizal fungi (also present in Mediterranean ecosystems) appear less sensitive to N enrichment than ectomycorrhizal fungi associated with trees (Ishida & Nordin

2010).

As far as we are aware, there are no studies on the effect of increased N availability on arbuscular mycorrhizal fungi (AMF) in temperate ecosystems, but there are for Californian coastal sage scrub (N critical load, 10 kg N ha^{-1} yr^{-1} - Fenn, Allen & Geiser 2011). Within Californian coastal sage scrub (habitat dominated by semi-deciduous shrubs), both mycorrhizal root infection (hyphal and vesicular) and mycorrhizal spore species density (and timing of sporulation) decreased with N enrichment (Egerton-Warburton & Allen 200; Tonnesen *et al.* 2007; Fenn *et al.* 2010). Moreover, increased N induced a shift in AMF community composition, i.e., higher N was associated with the displacement of the larger-spored species of *Scutellospora* and *Gigaspora* (due to a failure to sporulate) with a concomitant proliferation of small-spored *Glomus* species (e.g., *Glomus aggregatum, Glomus leptotichum* - Egerton-Warburton & Allen 2000). These changes indicated that AMF species could be sensitive indicators of N enrichment for that Mediterranean habitat. But the effect of N enrichment on AMF appears to depend on the form of deposited N. As an example, data from Yoshida & Allen (2001) suggested that in coastal sage scrub under low N deposition, mycorrhizae are more important in controlling plant growth in soils dominated by NH_4^+ rather than by NO_3^-. Similar results were obtained by Ochoa-Hueso (2011) in a kermes oak thicket in Spain.

Questions to be addressed here include:

- Can mycorrhizal fungi be used as sensitive indicators of N enrichment in Mediterranean maquis (**chapter 3**)?
- Will it depend on the form of deposited N (NH_y versus NO_x – **chapter 3**)?
- Will there be consistent mycorrhizal responders to N and/or NH_4^+ enrichment (**chapter 3**)?

Soil microbial activity and greenhouse gas emissions

Along an N gradient, N mineralization was found to explain by far the highest variation in species composition and species degradation, from the nutrient enriched border zone to the intact central calcareous grassland (in Europe – Neitzke 2001). Also, in a field study on 600 Swedish deciduous forests, N mineralization and nitrification rates increased (40-80% and 20-90%, respectively) while the C/N ratio decreased by 10-25%, compared to the low N region (Falkengren-Grerup, Brunet & Diekmann 1998). Therefore N-driven changes in soil microbial community may increase nitrification, which in turn may result in NO_3^- leaching.

Increased stream water NO_3^- concentrations have been recorded in Californian habitats in response to N deposition (Fenn *et al.* 2010). Soils from coastal sage scrub and chaparral (dominated by evergreen sclerophylls) sites were collected along an N deposition gradient. Under controlled conditions, N mineralization was shown to increase linearly as a function of

N deposition (Vourlitis *et al.* 2007). Manipulating N availability (+ 50 kg N ha^{-1} yr^{-1}) in the same habitats for four years showed that the N-driven increase in net nitrification was higher in chaparral soil than coastal sage scrub. As a result, NO_3^- leaching was higher in chaparral soil than coastal sage scrub (Vourlitis, Pasquini & Mustard 2009). The high nitrification capacity of chaparral soils is evidenced by virtually complete net nitrification of mineralized N (Fenn, Allen & Geiser 2011).

Questions to be addressed here include:

- How much N will the Mediterranean maquis retain (**chapter 5**)?
- Will it depend on the form of deposited N (NH_y versus NO_x – **chapter 5**)?

AIMS AND OUTLINE

Many studies have addressed single aspects of the effects of increased N availability on Mediterranean-type ecosystems: anthropogenic N deposition (Fenn *et al.* 2003, 2008, 2010) changes in species diversity (Bonanomi, Caporaso & Allegrezza 2006; Pinho *et al.* 2011, 2012; Ochoa-Hueso 2011; for Californian ecosystems see Fenn, Allen & Geiser 2011), impact on soil-atmosphere gaseous fluxes (Rosenkranz *et al.* 2006; Shvaleva *et al.* 2011), NO_3^- leaching (see Fenn, Allen & Geiser 2011), soil microbiology (Egerton-Warburton & Allen 2000; Yoshida & Allen 2001) and organic matter decomposition (Sirulnik *et al.* 2007; Alarcón-Gutiérrez *et al.* 2008; Kazakou *et al.* 2009). However, the impacts of driver changes across European Mediterranean ecosystems are poorly understood and have been hampered by a lack of integrated system-level studies (Phoenix *et al.* 2006).

Overall thesis aim:

Develop an integrated system-level approach to study the responses of a nutrient poor Mediterranean Basin maquis to increased N availability.

Overarching hypotheses:

1. Mediterranean Basin maquis have very low N status (very N limited), which will make them very responsive to increased N.
2. Although the N forms are inter-convertible, the form of N will influence the ecosystem's response, since biota (plants and microorganisms) have evolved under specific nitrogenous environments.
3. The ecosystem's response to N enrichment will be detected through key biotransformations that regulate the N cycle.
4. These biotransformations will mediate concerted effects of N on the ecosystem's compartments, biotic (plants and microorganisms) and abiotic (soil), and will determine the N retention capacity of the ecosystem.

This research was undertaken at a Natura 2000 site (PTCON0010 Arrábida/Espichel) located in the Arrábida Natural Park (Portugal). Estimated background N deposition is 5.2 kg ha^{-1} yr^{-1} (2.9 kg NO_x + 2.3 kg NH_y - http://webdab.emep.int/Unified_Model_Results/AN/). In 2007 the field manipulation experiment (N dose and form) was established in a Mediterranean maquis (Eunis class F5.2). Due to the patchiness of the Mediterranean Basin landscape (Blondel & Aronson 2004) and the importance of the form of inorganic N (Cruz *et al.* 2008), two forms of N were tested: only reduced N (NH_4^+) and a combination of reduced and oxidised N (NH_4NO_3). Thus the most likely N pollution scenarios within the Mediterranean Basin were mimicked, i.e., predominantly agricultural sources (NH_4^+) and combined inputs from urban/industrial sites and agriculture (NH_4NO_3). The N doses were lower than the N deposition reported for other areas in Mediterranean-type ecosystems (145 kg N ha^{-1} yr^{-1} - Fenn *et al.* 2003; Meixner & Fenn 2004) but high enough to establish 'worst case' scenarios of N enrichment in this type of habitat. N availability was modified by the addition of 40 kg N ha^{-1} yr^{-1} as a 1:1 mixture of NH_4Cl and $(NH_4)_2SO_4$ (designated 40A) and 40 and 80 kg N ha^{-1} yr^{-1} in the form of NH_4NO_3 (designated 40AN and 80AN). Control plots were not fertilized. Each treatment was replicated three times, 3 plots each of 400 m^2. In order to restrict boundary effects and dilution processes, all measurements, analyses and sample collection were performed within an internal 100 m^2 square. To prevent N 'contamination' through runoff from N-plots, the experimental plots were distributed in three rows along the 5% slope, with the controls being located in the top row (Fig. 1.2). Over the next 4.5 years selected effects on the structure and functioning of above and below ground fractions were monitored, paying special attention to the N form in determining the overall response to N enrichment.

Given that the studied European Mediterranean ecosystems (Bonanomi, Caporaso & Allegrezza 2006; Ochoa-Hueso 2011) apparently had already reached a threshold where N addition lead to a diversity loss, great effort was placed at studying the initial response of this nutrient poor ecosystem. Thus, after the general introduction (**chapter 1**), the **second and third chapters** aimed to capture the 'theoretical' N-driven increases in the diversity (richness and evenness) of vascular plants (**chapter 2**) and soil fungi (**chapter 3**). Given the interconnectivity between above and below ground communites, the response of these communites to increased N is hypothesized to be fully integrated. Also, in Mediterranean ecosystems, the magnitude of the temporal heterogeneity becomes as important as spatial heterogeneity (Cruz *et al.* 2008). Therefore the N-driven changes in above and below ground communities were hypothesized to be related to the changes in soil inorganic N availability. The changes in vascular plant diversity (individual species and the community) that occurred between the first and second springs of the experiment (2007 and 2008, respectively) were assessed. Due to the complexity of the studied ecosystem (high heterogeneity and biodiversity), plant diversity (Fig. 1.2) was approached on a functional group basis: perennials *versus* annuals (**chapters 2 and 7**). Concerning soil fungi, Temperature Gradient Gel Electrophoresis fingerprinting was applied to monitor the impact of the N addition treatments on the structure of its community (**chapter 3**). Another aim was to identify arbuscular mycorrhizal fungi that could be used as sensitive indicators of increased N availability, so that AMF spores were identified to species from the subcellular structures forming the asexual spores (**chapter 3**).

Because plants and soil biota evolved under specific nitrogenous environments they show preferences for specific patterns of N availability (Cruz, Lips & Martins-Loução 2003; Cruz *et al.* 2008), which the N addition treatments altered. Therefore, **chapter 4** links the nitrogenous environment of the two main plant groups (summer semi-deciduous and evergreen sclerophylls) with their N use strategies. This was done in the same study area but at a site where the plant community is in a more advanced phase of succession, i.e., co-dominance of summer semi-deciduous with evergreen sclerophylls. The availability of soil inorganic N, leaf nitrate concentrations and nitrate reductase activity were measured during the year in three plant species from each group. The distinctive traits of each plant group (phenology and root system) will be important determinants of the respective N use strategies.

Given that N availability was being manipulated, in **chapter 5** the aim was to understand the role of each ecosystem compartment in the cycling of increased N. Soil inorganic N pools from the abiotic compartment were determined seasonally and related to the cumulative N added. While for the biotic compartment, the response of the dominant plant species, *Cistus ladanifer* (Fig. 1.2), was followed with respect to plant cover and N concentrations in the leaves and litter. Quantification of the N that cycled between the two compartments enabled the ecosystem's short-term N retention capacity to be estimated.

Chapter 6 aims to account for the integrated effects of N enrichment on litter decomposability arising from the changes in the diversity of plant communities, and also alterations in the quality of the plant material (leaves and litter). An innovative and more ecological approach was taken, which considered not just N-driven changes in individual species but in the whole plant community. The decomposition experiment was made under controlled laboratory conditions: one type of soil (from the control) and four types of leaf litter (from the four N addition treatments) were collected from the N-manipulation field experiment. Distinct leaf litter traits were quantified (N and lignin concentration and C/N and lignin/N ratios) and correlated with organic matter decomposition, soil microbial biomass and CO_2 and N_2O emissions.

A recent meta-analysis on the effects of N additions (different habitats, soils, temperate climatic conditions and N deposition levels) on plant diversity highlighted the importance of considering cumulative N over time (De Schrijver *et al.* 2011). As a result, short- and longer-term effects of increased N on plant diversity can be distinct. Therefore the aim of **chapter 7** is to compare the short- (one year) and medium-term (4.5 years) effects of increased N on vascular plant diversity, thus enabling the identification of consistent plant responders to increased N. As such, on the fifth spring of experiment the plant community was re-surveyed and soil was collected and analyzed. Confirming the relation between cumulative N and plant richness within a Mediterranean ecosystem, paying special attention to cumulative NH_4^+, is another aim of this chapter. Also, the functional relationship between the N-driven changes in plant diversity and productivity/soil protection will be discussed.

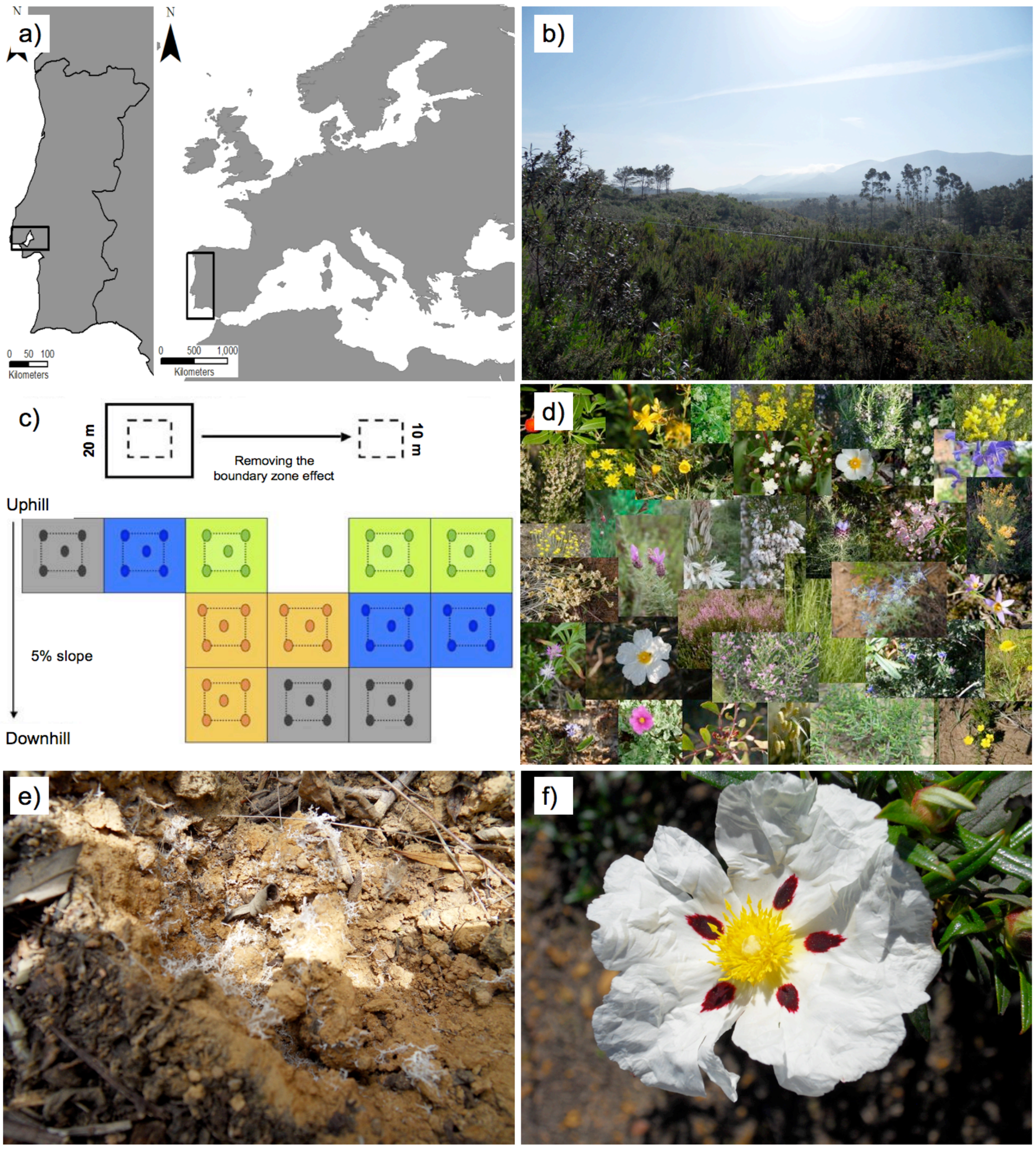

Figure 1.2 – General location of the study site (a); landscape view (b); schematic representation of the experimental design and the relative location of the experimental plots (green, Control; orange, 40A; blue, 40AN; and grey, 80AN – c); example of some of the existing plant species (d); view of surface soil and fungal mycelium (e) and *Cistus ladanifer*, the dominant plant species (f). Photos by Ana Paula Rosa, Márcia Bacelar, Sara Sousa and Teresa Dias.

Finally, in **chapter 8** a general discussion is presented, integrating the key results and achievements based on the N-driven structural and functional changes that were studied. These data were used to make recommendations concerning N critical loads, indicators of N deposition (structural and functional) and the implications of these changes for ecosystem sustainability and functioning, bearing in mind that the dynamics of Mediterranean plant communities is closely related to the occurrence of fires. Conceptual scenarios for the effects of increased N (mainly NH_y or NO_x) on Mediterranean Basin ecosystems, according to the phase of post-fire succession, have been developed.

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Chapter 2

Linking N-driven biodiversity changes with soil N availability in a Mediterranean ecosystem

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Chapter 2

Linking N-driven biodiversity changes with soil N availability in a Mediterranean ecosystem

ABSTRACT

Nitrogen (N) enrichment has been pinpointed as a main driver for biodiversity change. Most of our knowledge of effects of increased N availability on ecosystems comes from northern Europe and America. Most other ecosystem types have been neglected. In contribution to filling this gap, our study examined the short-term effects of N enrichment in a N-manipulation (doses and forms) field study of a severely nutrient-limited Mediterranean ecosystem located in a Natura 2000 site in Portugal. Our aims were to (i) understand the effects of N enrichment on plant diversity, and to (ii) link N-driven plant community changes with changes in soil inorganic N availability. In general, the standing plant community responded to short-term N enrichment with increased richness and evenness. Changes in the plant community occurred through changes in species composition and cover, and were correlated with soil N, and N and phosphorus availability. Fertilization with 80 kg NH_4NO_3 ha^{-1} yr^{-1} was the treatment which changed plant composition the most, while geophytes, hemicryptophytes and therophytes were the biological types more responsive to N enrichment. *Dittrichia viscosa* was the only species that responded significantly to increased N, i.e., its cover decreased in control plots, but increased in fertilized plots, suggesting that it could be used as an indicator of N enrichment in Mediterranean maquis. Changes in plant richness and evenness were correlated with the mean and/or the variation (standard deviation) of soil inorganic N parameters (e.g. nitrate concentration in the soil solution and the soil's ratio of bioavailable N and phosphorus) measured along the time between the two plant community assessments. However, short- and long-term effects can be quite distinct, thus highlighting the need for further studies.

Keywords: Biodiversity changes; Mediterranean; Soil N availability; Plant groups; N-limited ecosystems

INTRODUCTION

Increased nitrogen (N) deposition has been acknowledged as a main driver of biodiversity change (Sala *et al.* 2000), constituting a threat to biodiversity (Phoenix *et al.* 2006; Clarisse *et al.* 2009). Bobbink, Hornung & Roelofs (1998) reviewed the effects of increased N availability on biodiversity of various natural and semi-natural ecosystems pointing out that although most global biodiversity is contained within natural and semi-natural ecosystems, most research has focused on forests and trees. They highlighted serious gaps in knowledge of other ecosystems. More than a decade later, Bobbink *et al.* (2010) acknowledged that ecosystems other than those in northern Europe and America have received insufficient attention. Mediterranean-type ecosystems are included among the 'neglected ecosystems list' although they are biodiversity hotspots. In particular, the Mediterranean-type ecosystems located in the Mediterranean Basin constitute one of the most important biodiversity hotspots for vascular plants (Phoenix *et al.* 2006). Presently, Mediterranean ecosystems are thought to be experiencing the greatest proportional biodiversity change (Sala *et al.* 2000), with N deposition being estimated to increase three fold by 2050 (Galloway *et al.* 2004; Phoenix *et al.* 2006).

Most studies of the effects of increased N availability on Mediterranean-type ecosystems have been made in California (e.g. Fenn *et al.* 2003; Vourlitis, Pasquini & Zorba 2007; Allen, Rao & Steers 2009), while very few have been made on the Mediterranean Basin (Bonanomi, Caporaso & Allegrezza 2006; Pinho *et al.* 2009). This paper describes an integrated field study of short-term effects of increased N availability on a Mediterranean ecosystem located in a Natura 2000 site in southern Portugal (PTCON0010 Arrábida/Espichel). N availability was manipulated in reduced and/or oxidized forms in three equal applications throughout the year corresponding to distinct biological demands and activities. Our aims were:

(i) Understand the effects of N enrichment on plant biodiversity in a severely N-limited Mediterranean ecosystem. Given that biodiversity is a complex variable that includes taxonomic, functional, spatial and temporal aspects of organism diversity, we focused on species richness (the number of species) and evenness (their relative abundance), which are considered to be the most important indicators (Wilsey & Potvin 2000) of changes in biodiversity;

(ii) Link N-driven plant community changes with soil inorganic N availability. Biodiversity changes reflect changes in communities that occur in response to gradients of temperature, moisture and soil chemistry (McGill *et al.* 2006). Besides the spatial heterogeneity, in systems with a marked seasonality such as Mediterranean ecosystems (Grove & Rackham 2001), the magnitude of the temporal heterogeneity becomes as important as spatial heterogeneity (Cruz *et al.* 2008). Therefore we attempted to link observed N-driven plant community changes with changes in soil inorganic N availability measured along the time between the two plant community assessments.

MATERIALS AND METHODS

Study site

The present study was conducted at Serra da Arrábida in the Arrábida Natural Park, in a Natura 2000 site south of Lisbon, Portugal (PTCON0010 Arrábida/Espichel). The study site (38º 29' N - 9º 01' W) is located within a region belonging to the sub-humid thermomediterranean bioclimatic domain (Clemente 2002). According to the climatic normal (1971-2000) mean annual precipitation was 730 mm; mean maximum temperature, 27.8ºC (August); and mean minimum temperature, 8.1ºC (January). Reported data refer to the nearest climatic station (Setúbal, 15 km distance – Instituto Nacional de Meteorologia e Geofísica). The study site is located on a southeast-facing slope (5%) at 130 m a.s.l. that is protected from public access and has not suffered human intervention in the recent decades. Soil is skeletal (topsoil layer of approximately 15 cm) and true profiles cannot be discerned. Silt predominates in the soil (57%), while clay and sand contents are 28% and 15%, respectively (silt-sand-loam – Correia, 1988). In Specht *et al.* (1988), the soils of Serra da Arrábida are classified as calcic rhodo-chromic luvisols and calcareous chromic cambisols (FAO System). The vegetation consists of a dense maquis (Eunis class F5.2, Mediterranean maquis), which developed after a fire event four years before the beginning of N additions (summer 2003). The vegetation is dominated by *Cistus ladanifer* L., an obligate seeder species (after a fire, there is a massive seedling recruitment from a dormant seed bank – Clemente, Rego & Correia 1996). Other abundant plant species include *Erica scoparia* L., *Calluna vulgaris* (L.) Hull, *Genista triacanthos* Brot., *Ulex densus* Welw. ex Webb, *Dittrichia viscosa* L., and *Myrtus communis* L. Herbaceous species comprise ≈ 10% of the total plant cover and many are annual plants which survive the unfavourable season in the form of seeds and complete their life-cycle during favourable seasons (therophyte species).

Experimental design and fertilization schedule

The experimental design consisted of 12 plots, of 400 m^2 each, but to prevent the boundary effect and dilution processes, all measurements and analyses were performed within an internal 100 m^2 square. Each treatment had three replicates (3 plots). To prevent N 'contamination' through runoff from fertilized plots, the experimental plots were distributed in three rows along the slope, with the controls being located in the top row.

Estimated background N deposition is 5.2 kg ha^{-1} yr^{-1} (2.9 kg NO_x + 2.3 kg NH_y - http://webdab.emep.int/Unified_Model_Results/AN/). In Portugal, scattered protected areas represent 21% of the continental territory, making the Portuguese landscape very patchy, with distinct land uses in close proximity. Therefore the chosen N doses were lower than the N deposition reported for other areas in Mediterranean-type ecosystems (145 kg N ha^{-1} yr^{-1} - Fenn *et al.* 2003; Meixner & Fenn 2004) but high enough to assure that this study can be useful to establish 'worst case' scenarios for N enrichment in this type of habitat. Given that the study site is close (< 30km) to urban and industrial areas and that important agricultural activities overlap partially with the Natura 2000 site, we decided to test the effects of reduced and oxidized N (mimicking combined inputs from urban/industrial sites and agriculture) and of only reduced N (mimicking close proximity to an agricultural source). N availability was modified by the addition of 40 and 80 kg N ha^{-1} yr^{-1} in the form of NH_4NO_3-N (40AN and 80AN, respectively) and 40 kg N ha^{-1} yr^{-1} as a 1:1 mixture of NH_4Cl-N and $(NH_4)_2SO_4$-N (40A). Beginning in January 2007, the fertilizer was homogenously added, by hand, in three equal applications throughout the year: middle autumn/winter, spring and summer. Fertilizer granules dissolved rapidly (1-7 days depending on the fertilization period) after application. N additions took place in January, April and August 2007 and January 2008.

Soil sampling

Five soil-sampling locations were identified per plot, corresponding to the four corners and the centre. From each sample location, soil samples (2 cm diameter and 15 cm depth) were removed. Soil samples were sieved and stored at 4ºC until analyses. Sampling took place in May, August and October 2007 and February and April 2008, corresponding to the distinct seasons. Individual soil samples (five per plot) were used to determine soil moisture and concentrations of nitrate, ammonium, inorganic N and phosphate. Bulk soil samples (equal mixtures of the five soil samples from each experimental plot) collected in April 2008 were analyzed for soil characterization (Table 2.1).

Table 2.1 – Response of soil chemical composition (N, nitrogen; $[NO_3^-\text{-N}]$, extractable nitrate; $[NH_4^+\text{-N}]$, extractable ammonium; [inorgN], sum of extractable nitrate and ammonium; pH; OM, organic matter; P, phosphorus; K, potassium; and Mg, magnesium) to distinct N availabilities (Control, 40A, 40AN and 80AN). Soil sampling took place in April 2008 (one year after the beginning of the N additions). There were no significant differences between treatments (ANOVA $p<0.05$ followed by a Bonferroni test). Values represent the mean (n = 3 experimental plots per treatment) ± SE.

Soil properties	Control	40A	40AN	80AN
N (%)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
$[NO_3^-\text{-N}]$ ($\mu g\ g^{-1}$)	7.7 ± 1.7	8.2 ± 1.5	5.3 ± 1.7	9.2 ± 5.0
$[NH_4^+\text{-N}]$ ($\mu g\ g^{-1}$)	1.2 ± 0.4	1.2 ± 0.7	2.0 ± 0.6	2.3 ± 0.8
[inorgN] ($\mu g\ g^{-1}$)	8.9 ± 1.6	9.4 ±1.9	7.3 ± 1.8	11.5 ± 4.4
pH (H_2O)	5.0 ± 0.0	5.6 ± 0.5	5.7 ± 0.3	6.3 ± 0.6
OM (%)	2.8 ± 0.4	2.6 ± 0.3	3.1 ± 0.1	3.1 ± 0.9
P ($\mu g\ g^{-1}$)	5.0 ± 1.2	3.3 ± 0.3	4.0 ± 0.6	4.7 ± 1.2
K ($\mu g\ g^{-1}$)	98 ± 21	118 ± 30	132 ± 22	141 ± 25
Mg ($\mu g\ g^{-1}$)	77 ± 13	95 ± 7	89 ± 4	88 ± 1

Vascular plant community composition assessments

The vegetation at the study site was sampled within one 5x5m square per experimental plot (within the internal 100 m^2). Percentage of vascular plant species cover (herbaceous included), and of bare soil were recorded. Each species' cover was calculated from the total projected crown area (calculated from two perpendicular diameters, assuming elliptical shape). The vascular plant community composition was assessed in the Spring (June 2007, May 2008).

Soil chemical analysis

From each individual soil sample (five per plot), 10 g fwt of soil were used to gravimetrically determine soil water content (Kern EG300 3M). Five g (fwt) of soil were used to prepare soil water extracts (1:10 – w/v). Soil extracts were shaken (Cassel Agitator) for one hour at room temperature, centrifuged (Eppendorf Centrifuge 5403) at 5000 rpm for 20 minutes at 4ºC and the supernatant collected and analyzed colorimetrically (spectrophotometer Tecan Spectra Rainbow A-5082) for nitrate, ammonium and phosphate. Nitrate (NO_3^--N) was determined using a modification of the Cataldo method (Matsumura & Witjaksono 1999), ammonium (NH_4^+-N), using a modified Berthelot reaction (Cruz & Martins-Loução 2000), inorganic N, as the sum of the water extractable NH_4^+-N and NO_3^--N and phosphate (PO_4^{3-}-P), using the Fiske & Subarow method (1925). Nitrate, ammonium, inorganic N and phosphate were expressed as μg N or P per gram of dry soil and mM of N or P in the soil solution (based on soil water content).

The soil bulk samples used for determining organic matter, pH and concentrations of N, phosphorus, potassium and magnesium were dried at 35ºC. Soil pH (Crison micro pH 2002) was measured in a 2.5:1 (v/v) water extract. Organic matter was determined according to ISO norm 10694 by loss on ignition overnight at 600ºC (Nabertherm L3/11/C6). Analysis of total N was done according to ISO standard 13878 by dry combustion using an elemental analyzer (Leco CNS). Extractable phosphorus and potassium were quantified by a modification of the Egner-Riehm method using plasma emission spectrophotometry with an optical detector (ICP-OES), following extraction using ammonium lactate 0.1 M and acetic acid 0.4 M, pH 3.65-3.75. Magnesium was extracted with ammonium acetate 1 M, pH 7 and quantified by atomic absorption spectrophotometry with flame atomization.

Community diversity indices and differences over time

Richness and evenness of the vascular plant communities were calculated (Krebs 1989). The standing plant community was functionally diverse, comprising summer semi deciduous, evergreen sclerophyllous (Correia 1988), ericaceous, leguminous shrubs (Barradas *et al.* 1999), herbaceous and young trees (Table 2.2). The changes in the number of plant species due to the N additions were approached by grouping them into therophyte or perennial (plants that live for more than two years, including those hemicryptophytes that in some cases can be therophytes – Table 2.2), since these two plant groups show distinct temporal responses and dependence on soil nutrient availability (Marschner, Kirkby & Engels 1997). Changes over time (t_0, t_1) were calculated as follows:

$$\text{Parameter Changes (\%)} = \frac{(\text{Parameter}_1 - \text{Parameter}_0)}{(\text{Parameter}_1 + \text{Parameter}_0)/2} \times 100$$

Soil parameters were calculated as mean with variation along the experimental period, i.e., average and standard deviation of the five sampling occasions (spring, summer and autumn 2007 and winter and spring 2008) per plot.

Statistics

Summary statistics of community responses (richness and evenness) were compared for the different treatments. The two-way ANOVA was applied to assess the existence of significant interactions between time and treatment for plant richness and evenness and differences in community parameters per treatment were analyzed by a one-way ANOVA, followed by a Bonferroni test ($p<0.05$), or by a Games-Howell test whenever homogeneity of variances was not confirmed by the Levene's test. Linear correlations between the changes in plant community and soil inorganic N availability were also studied (Pearson's correlations). In all cases, preliminary analyses were performed to ensure there was no violation of the assumptions regarding the tests' application. SPSS software, version 17.0, was used for all tests.

RESULTS

Soil chemical composition

The soil was characteristically low in N and P. Addition of 40 or 80 kg N ha^{-1} yr^{-1} did not significantly increase soil N concentration, which remained at 0.1% for total N and ≈10 μg g^{-1} for inorganic N irrespective of N treatment (Table 2.1). Accordingly, organic matter was also low for all treatments. Potassium and magnesium concentrations were in the range normally found in Portuguese agricultural soils, therefore not limiting, and were similar in all treatments. Soil pH (water) was slightly acidic, but the pH tended to increase with the applied N dose (Table 2.1).

Changes in vascular plant community

In contrast to the soil N concentration (Table 2.1), the standing vascular plant community responded to increased N availability (Table 2.2 and Fig. 2.1). At the beginning of the experiment (Spring 2007), plant communities were similar in all treatments (based on richness and evenness - Fig. 2.1-a, c). However, differences between treatments were observed one year later, in Spring 2008 (Fig. 2.1-b, d).

Table 2.2 – List of the vascular plant species observed in the two consecutive assessments (June 2007 and May 2008, the first and second springs of N fertilization) and their respective changes in cover (2008-2007) according to the N additions (Control, 40A, 40AN and 80AN). Plant species were grouped according to their life form: (i) the first letter represents perennial (P), annual (A) or undetermined (nd); and (ii) the second represents the biological type phanerophyte (P), chamaephyte (C), geophyte (G), hemicryptophyte (H), therophyte (T) and undetermined (nd). + indicates increased cover; 0 indicates no change in cover; - indicated decreased cover; (+) indicates species appearance; and (-) indicates species disappearance (n = 3 experimental plots per treatment). * for *Dittrichia viscosa* refers to statistically significant differences between treatments.

Life form	Family	Species	Control	40A	40AN	80AN
P (P)	*Anacardiaceae*	*Pistacia lentiscus*	-	-	+	-
	Cistaceae	*Cistus crispus*			(-)	+
		Cistus ladanifer	+	+	+	-
		Cistus monspeliensis		+		(+)
		Cistus salvifolius	+	+	+	-
		Halimium halimifolium	+			
	Ericaceae	*Arbutus unedo*				-
		Calluna vulgaris	+	+	+	+
		Erica arborea		+	+	
		Erica scoparia	+	+	+	+
		Erica umbellata				+
	Fabaceae	*Genista triacanthos*	+	+	+	0
		Ulex densus	+	-	+	+
	Fagaceae	*Quercus coccifera*		+	+	0
		Quercus suber			0	
	Lamiaceae	*Lavandula stoechas*	+	-	+	0
		Rosmarinus officinalis		(+)		+
		Salvia sclareoides	(-)			(+)
	Myrtaceae	*Myrtus communis*	+	0	+	-
	Oleaceae	*Olea europaea* var *sylvestris*			+	+
		Phillyrea angustifolia				+
	Pinaceae	*Pinus pinaster*		-		+
	Rhamnaceae	*Rhamnus alaternus*			+	
	Rosaceae	*Rubus ulmifolius*	(-)	(+)	-	
	Thymelaeaceae	*Daphne gnidium*	0	+	-	(+)
P (C)	*Asteraceae*	*Dittrichia viscosa**	-	+	+	+
		Helichrysum stoechas	(-)			-
		Phagnalon saxatile				(-)
	Boraginaceae	*Lithodora prostrata*	0		+	(+)
	Rubiaceae	*Rubia peregrina*				(+)
P (G)	*Asphodelaceae*	*Asphodelus ramosus*	(-)			(+)
	Asteraceae	*Carlina corymbosa*			(+)	
	Hyacinthaceae	*Urginea maritima*		(+)		(-)
	Iridaceae	*Gladiolus illyricus* ssp *reuteri*		(+)	(+)	(+)
		Iris xiphium				(+)

Table 2.2 (continued)

Life form	Family	Species	Control	40A	40AN	80AN
P (G)	*Orobanchaceae*	*Orobanche latisquama*	(+)			
	Ranunculaceae	*Anemone palmata*	(+)			
P (H)	*Apiaceae*	*Daucus carota*			(-)	
	Asteraceae	*Andryala ragusina*	-			
		Cynara sp	(-)			
		Leontodon taraxacoides	-	-	+	0
		Pulicaria odora			(-)	(+)
		Senecio jacobaea	(+)		(-)	
	Campanulaceae	*Campanula rapunculus*		0	(-)	
	Cyperaceae	*Carex flacca*	+	+	+	-
	Fabaceae	*Anthyllis vulneraria*				(+)
	Gentianaceae	*Blackstonia perfoliata*	-	-	(-)	0
		Centaurium erythraea	-	-	(-)	-
	Hypericaceae	*Hypericum* sp	-	-	-	
	Poaceae	*Agrostis* sp	(+)			
		Arrhenatherum album		+		(-)
		Avenula sp	(+)			
		Brachypodium phoenicoides	-	0	-	+
		Dactylis glomerata				(+)
	Rosaceae	*Sanguisorba hybrida*			(-)	(-)
nd (H/T)	*Asteraceae*	*Centaurea melitensis*	(+)			
		Crepis capillaris		(+)		
		Picris echioides		(-)		+
	Fabaceae	*Lotus sp*	(+)	(+)	+	
		Trifolium sp	(-)		(-)	(-)
		Vicia sp		(+)		
A (T)	*Asteraceae*	*Crysanthemum coronarium*		(+)		
		Evax pygmaea				(+)
		Filago minima	-	(-)	(+)	(-)
		Galactites tomentosa	-	(-)	(-)	(-)
		Matricaria recutita	(-)	(-)	(-)	
		Sonchus sp	(-)		(-)	(+)
	Lamiaceae	*Stachys arvensis*	(+)	(+)	(+)	
	Poaceae	*Briza maxima*			(+)	(+)
		Briza minima		(-)		(-)
		Briza minor	(-)		0	(+)
	Primulaceae	*Anagallis arvensis*	(+)	-	+	+
nd	*Apiaceae*	*Apiaceae*	(+)			(+)
	Asteraceae	*Asteraceae*	(-)	(-)	(+)	
	Fabaceae	*Fabaceae*				(-)
	Liliaceae	*Liliaceae*	(+)		(+)	
	Poaceae	*Poaceae*	0		-	0
		Vulpia sp	(-)	(-)		(-)
	Rubiaceae	*Galium* sp			(+)	(+)
		Rubiaceae				(+)

Table 2.2 (continued)

			Control	40A	40AN	80AN
Number of species that:	Increased	+	10	12	19	13
	Decreased	-	10	9	5	8
	Maintained	0	3	3	2	6
	Appeared	(+)	11	9	8	18
	Disappeared	(-)	11	7	12	10

The number of plant species (richness - Fig. 2.1-a, b) decreased in non-fertilized plots (control), did not change in both treatments receiving 40 kg N ha^{-1} yr^{-1} (40A and 40AN), but increased in the 80AN plots. Data show that one year of 80AN was enough to cause a significant increase in the number of plant species in relation to the control ≈ 30%, as well as in relation to the starting point (Spring 2007), ≈16%.

Plant species evenness decreased in control plots (Fig. 2.1-c, d) but decreased even more in the 40A plots. However, it did not change in the plots fertilized with ammonium nitrate, either with 40AN or 80AN.

Changes in the plant community (Fig. 2.1) involved species appearance and disappearance and/or changes in plant species cover (Table 2.2). In control plots, the number of plant species that increased their cover was the same as the ones that decreased it (10) and only three species stayed the same. Eleven 'new' species were detected and another eleven were 'lost'. Fertilization with 40A resulted in twelve species that increased their cover, nine that decreased and three that maintained it. In the second spring after the beginning of the N addition, nine 'new' species and seven 'losses' were observed. Fertilization with 40AN resulted in nineteen species that increased their cover, five that decreased and two that maintained it. In spring 2008, eight 'new' species and twelve 'losses' were observed. The treatment which changed plant species the most was the 80AN with thirteen species that increased their cover, eight that decreased and six that maintained it. After one year of treatment, eighteen 'new' species and ten 'losses' were recorded. The biological types more responsive to the N enrichment were the geophytes, the hemicryptophytes and the therophytes (Table 2.2).

In terms of plant cover, seven groups of plant species could be identified: (i) *Dittrichia viscosa* was the only species which responded significantly to increased N, i.e., its cover decreased in control plots, but increased in fertilized plots (Table 2.2). Similarly, in the second spring of N addition, *Gladiolus illyricus* ssp *reuteri* only appeared in fertilized plots; (ii) cover of *Cistus ladanifer* (the dominant plant species), *C. salvifolius, Genista triacanthos* and *Carex flacca* tended to decrease only in the 80AN; (iii) *Ulex densus, Lavandula stoechas* and *Anagalis arvensis* tended to respond negatively to fertilization with ammonium as the only N source (40A)*;* (iv) in spring 2008, *Orobanche latisquama* (a parasitic plant) and *Anemone palmate* only appeared in the control plots. (v) *Briza maxima* and *Galium* sp were only observed in the second spring of the N addition in plots receiving ammonium nitrate (40AN and 80AN); (vi) *Calluna vulgaris* and *Erica scoparia* increased their cover in all treatments and (vii) *Centaurium erythraea* and *Hypericum* sp decreased their cover irrespective of the treatment.

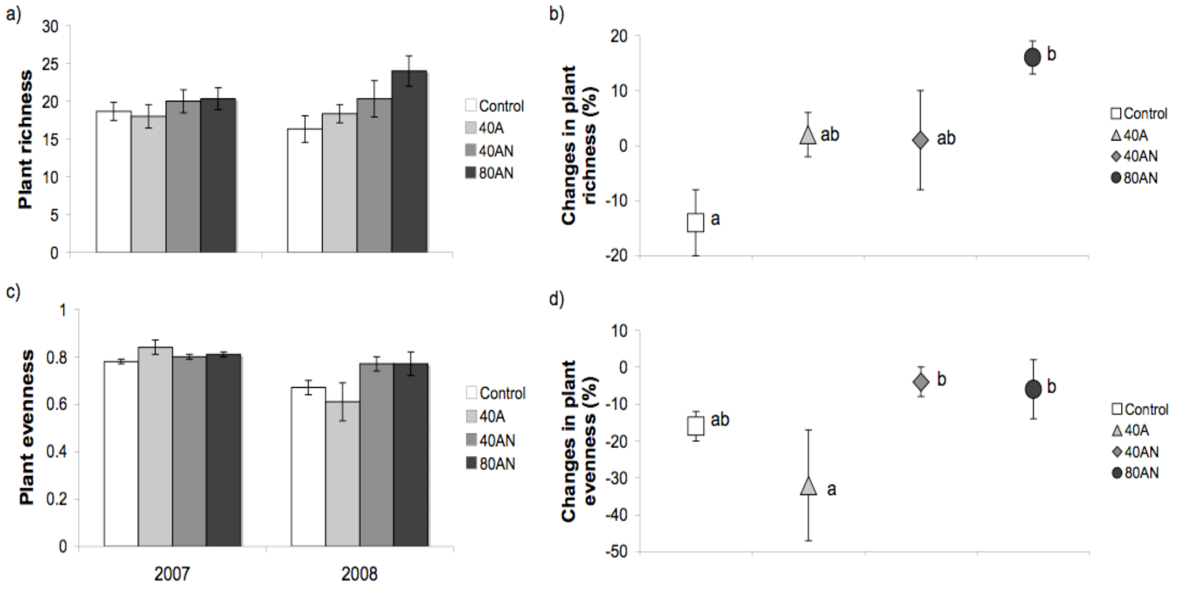

Figure 2.1 – Response of the vascular plant community to distinct N availabilities (Control, 40A, 40AN and 80AN) in terms of plant richness (a-b) and evenness (c-d). Community assessments were performed in the first and second Springs of N fertilization: 2007 and 2008. Changes in each parameter between 2008 and 2007 were positive or negative and were calculated as described in materials and methods. Different letters refer to statistically significant differences between treatments (ANOVA $p<0.05$ followed by a Bonferroni test). There were no significant interactions between treatment and time ($p<0.01$). Values represent the mean (n = 3 experimental plots per treatment) ± SE.

Linking plant community changes with soil N

No relation was found between changes in the whole-plant community and the soil total N pool (Table 2.1 and Fig. 2.1). However, when the distinct N fractions (e.g. inorganic N and ratio of reduced to oxidized N), or the ratio between bioavailable N/P were considered, some correlations became apparent (Table 2.3).

Changes in plant richness were positively correlated with plant richness in 2008 (r = 0.79), the mean of nitrate concentration in the soil solution (r = 0.73), the changes in perennial richness (r = 0.63), the variation of nitrate concentration in the soil solution (r = 0.63) and the mean and variation of soil inorganic N (r = 0.66 and 0.60, respectively); and negatively correlated with the variation (standard deviation) of the ratio between bioavailable N/P (r = -0.75).

Analyzing the changes in richness for the two main plant types (perennials and therophytes – Table 2.2) independently showed that these behaved differently in terms of the number of parameters with significant correlations, and the identity of the variable with which a significant correlation was found (Table 2.3). Changes in perennial richness were positively correlated with the mean of nitrate concentration in the soil solution (r = 0.73), with changes in the plant richness (r = 0.63), and with the mean of inorganic N in the soil, and in the soil solution (r = 0.61 and 0.60, respectively). Changes in therophyte richness were negatively correlated with the variation of soil's ratio of reduced to oxidized inorganic N (r = - 0.58).

Changes in species evenness were positively and strongly correlated with plant evenness in 2008 (r = 0.97). Positive correlations were also obtained with the variation of soil nitrate concentration (r = 0.46) and the mean of inorganic N in the soil, and in the soil solution (r = 0.49 and 0.46, respectively). Changes in species evenness were negatively correlated with plant evenness in 2007 (r = - 0.64 – Table 2.3).

DISCUSSION

Short-term response of a N-limited ecosystem to increased N availability

In contrast to most studies (see Bobbink *et al.* 2010 for worldwide review) our data suggest that increased N availability did not lead to a loss of diversity of vascular plants (Fig. 2.1). On the contrary, increased N availability, created by the addition of 40 or 80 kg N ha^{-1} yr^{-1}, seemed to promote biodiversity. The few other studies reporting increased biodiversity in response to N enrichment (Calvo *et al.* 2005; Allen, Rao & Steers 2009; Pinho *et al.* 2009) were also carried out at sites with low soil N contents. In fact, and according to Bobbink, Hornung & Roelofs (1998), increasing the N availability in extremely nutrient-poor soils (Table 2.1) can increase biodiversity (Fig. 2.1 and Table 2.2). Thus it seems that the initial ecosystem N status is an important factor in predicting the ecosystem's response to N enrichment (Emmett 2007).

Plant species richness and composition (Fig. 2.1 and Table 2.2) were consistent with an early phase of a post-fire succession for a Mediterranean ecosystem (Clemente, Rego & Correia 1996). Immediately after a fire, plant diversity is low (Thompson 2005) since the only plant species are those whose germination is stimulated by high temperatures (e.g. *C. ladanifer*). However, fire is also a disturbance factor which favours the establishment of ruderal plant species resulting in a diversity peak usually observed three years after fire (Thompson 2005), which is then followed by a sharp decline in the next two years corresponding to the decrease of ruderals (Casal 1987; Trabaud 1987). Plant community was assessed on the forth and fifth years after the fire, thus explaining the decrease in plant richness observed in control plots (Fig. 2.1). N enrichment prevented the natural decline in species richness mostly through the appearance and maintenance of geophyte, and therophyte species (Table 2.2).

Table 2.3 – Pearson's correlation coefficients between changes in plant community and soil inorganic N and bioavailable N/P along the time period between the two plant community assessments.

Changes in: Variables:	plant richness	perennial richness	therophyte richness	plant evenness
Plant richness 2007	0.17	-0.11	0.02	0.30
Plant richness 2008	**0.79****	0.40	0.15	0.23
Changes in plant richness	1.00	**0.63***	0.19	0.03
Changes in perennial richness	**0.63***	1.00	-0.23	0.15
Changes in therophyte richness	0.19	-0.23	1.00	0.29
Plant evenness 2007	0.16	-0.01	-0.24	**-0.64***
Plant evenness 2008	0.10	0.20	0.20	**0.97****
Changes in plant evenness	0.03	0.15	0.29	1.00
[NO$_3^-$-N] mean	0.53	0.40	0.22	0.26
[NO$_3^-$-N] sd	0.53	0.29	0.35	**0.46***
[NO$_3^-$-N] in soil solution mean	**0.73****	**0.73****	0.13	0.43
[NO$_3^-$-N] in soil solution sd	**0.63***	0.39	0.49	0.34
[NH$_4^+$-N] mean	0.49	0.51	-0.32	0.31
[NH$_4^+$-N] sd	0.35	0.42	-0.40	0.42
[NH$_4^+$-N] in soil solution mean	0.32	0.44	-0.39	0.37
[NH$_4^+$-N] in soil solution sd	0.11	0.22	0.13	0.13
[inorgN] mean	**0.66***	**0.61***	-0.08	**0.49***
[inorgN] sd	**0.60***	0.33	0.34	0.36
[inorgN] in soil solution mean	0.55	**0.60***	-0.12	**0.46***
[inorgN] in soil solution sd	0.55	0.54	0.03	0.57
[NH$_4^+$-N] / [NO$_3^-$-N] mean	0.50	0.37	-0.26	-0.11
[NH$_4^+$-N] / [NO$_3^-$-N] sd	0.31	0.30	**-.058***	-0.21
[NO$_3^-$-N] / [inorgN] mean	-0.53	-0.31	-0.02	-0.02
[NO$_3^-$-N] / [inorgN] mean	0.22	-0.13	-0.36	-0.30
[inorgN] / [PO$_4^{3-}$-P] mean	-0.45	-0.30	-0.18	0.49
[inorgN] / [PO$_4^{3-}$-P] sd	**-0.75****	-0.46	0.06	0.52

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

The referred decrease in the number and/or cover of ruderal species (*e.g. Dittrichia viscosa* - Table 2.2) is likely to be responsible for the decrease of plant evenness in the control plots (Fig. 2.1-a, b), which is in agreement with the fact that plant communities are naturally uneven (Wilsey & Potvin 2000; Naeem 2009). The addition of ammonium as a single N form (40A) may have been a disturbance factor, resulting in the greatest decline in plant evenness. On the contrary, addition of ammonium and nitrate (40AN and 80AN) may have enabled nitrate-preferring plant species to coexist with those that tolerate ammonium, thus resulting in smaller decreases in plant evenness when compared to the other treatments. Maintenance of high plant evenness in response to fertilization with ammonium nitrate has also been observed by Manning *et al.* (2006).

Assuming N limitation as an important factor of environmental severity, increased N availability, which often promotes the decrease of slow growing conservative species and the settlement of fast growing exploitative species (Emmett 2007), would increase species richness (Grime 1973). The revised Grime's humped-back model (Michalet *et al.* 2006) predicts that the development of the plant community increases the N limitation, so that exploitative species (species with intermediate strategies and competitive ones) would benefit from living under the influence of conservative species (stress-tolerant species). However, as the N limitation is alleviated by the N additions, facilitation would decrease given that exploitative species would be able to exist away from the canopy of the conservative species and therefore the number of species would increase (Fig. 2.1 and Table 2.2). Accordingly, and assuming as conservative species *C. ladanifer, C. salvifolius, G. triacanthos* and *U. densus* (Correia 1988; Clemente 2002), and as exploitative species *D. viscosa* (Karageorgou, Levizou & Manetas 2002), the observed biodiversity increase in Arrábida (Fig. 2.1) may be explained by the coexistence of the remaining conservative with the incoming exploitative. Similar biodiversity changes in response to N availability have been observed for epiphytic lichens (Mitchell *et al.* 2005; Pinho *et al.* 2009). The N-induced biodiversity increases, corresponding to the coexistence of conservative and exploitative species, have probably not been observed in many north temperate ecosystem studies, which typically have higher N availability than our study site. Nevertheless it is important to keep in mind that data refer to very short-term effects so that the biodiversity increase may only be an initial response while the long-term effects may be quite different. In the longer term and according to the humped-back model there might be a loss of more N conservative species as the system becomes more nutrient rich (Michalet *et al.* 2006) and therefore its response may become similar to those in which the majority of studies have been performed.

Linking plant community changes with soil N availability

The variation in the soil bioavailable N/P was the soil parameter that was more correlated with the changes in plant richness (Table 2.3), pointing to the dependence of species on adequate supplies of both N and P. Since most semi-natural ecosystems, and Mediterranean ones in particular (Sardans & Peñuelas 2004), are N and often also P limited (Table 2.1), Fanelli, Lestini & Sauli (2008) also found that the soil's N/P was the best explanatory variable for Mediterranean herbaceous plant communities arranged along a gradient of ruderality.

Nitrate concentrations at the site were comparable to those determined for other sites within the same study area (Cruz *et al.* 2008) and for other Mediterranean ecosystems (Gallardo *et al.* 2006). It is possible that nitrate being the predominant form of inorganic N (Table 2.1) in such a post-fire scenario (Knicker 2007; Meixner *et al.* 2006) accounts for the correlation between soil nitrate and the changes in plant and perennial richness and in plant evenness (Table 2.3). Since plants differ in their tolerance to ammonium (Cruz, Lips & Martins-Loução 2003; Kronzucker *et al.* 2003), the response of the particular system to the variation of the inorganic N present as nitrate is expected to depend on its stage in the ecological succession (Cruz, Lips & Martins-Loução 2003), and on its species composition. Early successional species prefer nitrate, and are relatively ammonium intolerant while late successional are more ammonium tolerant (Kronzucker *et al.* 2003). Thus, soil nitrate concentration may shape plant communities such as the standing plant community which is in an early stage of succession and likely to be dominated by species that prefer nitrate. Similarly to soil nitrate, soil inorganic N was also correlated with the changes in plant and perennial richness and in plant evenness (Table 2.3), which may reflect the strong correlation between the two N fractions (data not shown).

The variation in the ratio of reduced to oxidized inorganic N was the only parameter showing correlation with the changes in therophyte richness (Table 2.3). For a therophyte plant, the transition from seed in the seed bank to seedling or juvenile may depend on the initial seed density, while the transition from juvenile to reproductive adult and from adult plant to seed might depend on the corresponding density in the population (Kluth & Bruelheide 2005). Therefore, changes in the ratio of reduced to oxidized inorganic N may have prevented therophyte plants from using the increased N availability to complete their life cycle (van den Berg *et al.* 2008) in the first and/or second years of N addition.

Understanding biodiversity changes due to increased N availability in a Mediterranean ecosystem

Although *D. viscosa* is quite common within the Mediterranean, it is considered a ruderal plant species due to its abundance in areas that have been modified and altered by anthropic activities (Murciego *et al.* 2007). Therefore the significantly contrasting response of *D. viscosa* in control and fertilized plots (Table 2.2) suggests that this species could be regarded as an indicator of N enrichment in Mediterranean maquis, similarly to *Deschampsia flexuosa* in boreal ecosystems (Bobbink *et al.* 2010). In the following years of this study, other plant species may prove to be consistent indicators of: low N, presence of *Orobanche latisquama* and *Anemone palmata*; N enrichment, presence of *Gladiolus illyricus* ssp. *reuteri;* ammonium enrichment, decrease of *Ulex densus*, *Lavandula stoechas* and *Anagalis arvensis*; nitrate and ammonium enrichment, presence of *Briza maxima* and Galium sp (Table 2.2).

Mediterranean ecosystems are subjected to dry/wet cycles of varying duration and intensity (Jarvis *et al.* 2007). As a result, nutrients (N in particular) may be present in the soil but their concentration in the soil solution can be high (when the soil is drying and/or when there is a nutrient flush) or low (when the soil is water saturated and/or nutrient content is low). Thus, N is only truly available when in solution, making N in the soil solution a more reliable measure of availability in these ecosystems. This may explain the correlation between the changes in plant and in perennial richness with nitrate concentration in the soil solution and not with soil nitrate.

In the present study, both the mean and the variation of N availability along the year were correlated with the changes in plant community (Table 2.3), perhaps due to the characteristics of Mediterranean ecosystems. Plants prefer specific patterns of N availability (Gallardo *et al.* 2006; Cruz *et al.* 2008) which, as in many other biological phenomena, are characterized by a mean and amplitude of variation, with its effects becoming functionally important.

The observed response of *D. viscosa* to N enrichment should be further assessed as a management tool for forecasting, and determining real-time actions to prevent biodiversity changes in response to real N enrichment. Finally, this is a first integrated field study on how European Mediterranean vegetation responds to N enrichment from different forms and doses and highlights the fact that longer-term studies are needed to explore the generality of what has been observed in the present study.

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Chapter 3

Species of arbuscular mycorrhizal fungal spores can indicate increased N availability in Mediterranean-type ecosystems

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Chapter 3

Species of arbuscular mycorrhizal fungal spores can indicate increased N availability in Mediterranean-type ecosystems

ABSTRACT

Mycorrhizal fungi form ecologically important connections between plants and soils, and although nitrogen (N) enrichment has been implicated in the decline of ectomycorrhizal fungal diversity, they are rarely considered in studies investigating the effects of increased N availability on species diversity. This paper describes the effects of N enrichment on the soil fungal community and in particular, on arbuscular mycorrhizal fungal (AMF) spores, in a Mediterranean ecosystem in a Natura 2000 site in southern Portugal (PTCON0010 Arrábida/Espichel). Soil fungal community structure was affected by the addition of 80 kg N ha^{-1} yr^{-1} as NH_4NO_3 within two years. The effects of N addition on AMF diversity (richness and evenness) appear to depend on the form of N, since the addition of 40 kg N ha^{-1} yr^{-1} as ammonium increased AMF spore richness and evenness proportionally more than the addition of 40 kg N ha^{-1} yr^{-1} as ammonium plus nitrate. The composition of AMF species may serve as a sensitive indicator of N enrichment.

INTRODUCTION

Nitrogen (N) availability is increasing globally (Galloway *et al.* 2008), which may cause severe damage to environmental systems at local, regional and global scales, as availability of nutrients is a key factor in determining ecosystem function and stability (Bobbink, Hornung & Roelofs 1998). Sala *et al.* (2000) developed biodiversity change scenarios for terrestrial ecosystems, ranking increased N deposition as the third main driver. Subsequent works inferred that N deposition constitutes a threat to biodiversity (Phoenix *et al.* 2006; Clarisse *et al.* 2009). Although microorganisms comprise much of the Earth's biodiversity and have important roles in ecosystem functioning (Fitter 2005), most studies have focused solely on the threat that increased N availability poses for plant diversity (see Bobbink *et al.* 2010). However, in recent years, there has been a growing awareness amongst plant and soil microbial ecologists of the need to understand the connectivity between plants and soil microbes. Given the importance of fungi in ecosystem processes, such as decomposition and the provision of plant nutrients (through the formation of mycorrhizal symbiosis), it is likely that N availability can potentially interfere with the soil fungal community directly or through effects on biomass/litter composition. Despite the fact that mycorrhizal fungi form ecologically important connections between plants and soils, they are rarely considered in studies investigating the effects of N enrichment on species diversity. Some studies conducted in northern Europe and America, where ectomycorrhizal associations with forest trees are common (see Wallenda & Kottke 1998 for review) have addressed this issue: Lilleskov *et al.* (2002) implicated N enrichment in the decline of ectomycorrhizal fungal diversity. The ecological and functional importance of other types of mycorrhizal fungi varies in accordance with the ecosystem type.

Although arbuscular mycorrhizal fungi (AMF) significantly increase plant access to nutrients, and their effects are more relevant under stress conditions (low nutrient and water availability, namely in Mediterranean ecosystems), few studies have focused on N-driven changes in AMF. Enhanced N availability has been shown to change Mediterranean plant communities (Allen *et al.* 1998; Bonanomi, Caporaso & Allegrezza 2006; Vourlitis, Pasquini & Mustard 2009; Dias *et al.* 2011), which may influence (or be influenced by) the efficiency of the mycorrhizal symbioses in the acquisition of nutrients (namely N and phosphorus) and therefore on the relative competitive capacity of co-existing plant species.

Aims and objectives:

- Study the short-term effects of N enrichment on the soil fungal community (and in particular AMF community) in a Mediterranean ecosystem; and
- Understand the effect of N dose and form on soil fungi and AMF spores.

MATERIALS AND METHODS

The study site is located in Arrábida (Portugal, 38°29’ N, 9º01’ W), with a typical Mediterranean climate: hot and dry summers, and mild and wet winters. The site belongs to the Natura 2000 network (PTCON0010 Arrábida/Espichel). The soils have been classified as Calcic rhodo-chromic luvisoils and calcareous chromic cambiols, according to the FAO system (Cruz *et al.* 2008). The soil is approximately 15 cm deep and has a silt-sand-loam texture (Correia 1988). The vegetation consists of a dense maquis (Eunis habitat type F5.2), established through a secondary succession after a fire event in the summer 2003 (4 years before the beginning of N additions). The applied treatments were: addition of NH_4NO_3, 40 (40AN) and 80 (80AN) kg N ha^{-1} yr^{-1}, or 40 (40A) kg NH_4^+ ha^{-1} yr^{-1}, control plots are not fertilized. Fertilization started in January 2007. N is added in three equal applications throughout the year and each treatment has three replicates (400 m^2 experimental plots).

The effects of N enrichment on soil fungal community and AMF spores were assessed in December 2008. Temperature Gradient Gel Electrophoresis (TGGE) fingerprinting was applied to monitor the impact of the N additions on the soil fungal community structure: total DNA was extracted from the soil samples and universal primers defined for fungal 18S rRNA genes were used for PCR amplification. The resulting amplification products were separated by TGGE. AMF spores were extracted from soil samples by wet sieving following sucrose gradient centrifugation. AMF spores were identified to species from the subcellular structures forming the asexual spores. Differences in community parameters per treatment were analyzed by a one-way ANOVA, followed by a Bonferroni test ($p<0.05$), or by a Games-Howell test whenever homogeneity of variances was not confirmed by the Levene's test. Differences between the two treatments receiving 40 kg N ha^{-1} yr^{-1} (40A and 40AN) were analysed by a t-test ($p<0.05$). When data violated normality, a Kruskal-Wallis test (non-parametric, $p<0.05$), was applied. SPSS software, version 17.0, was used for all tests.

RESULTS AND DISCUSSION

N enrichment changed the soil bacteria (Dias *et al.* 2011) and fungal communities (Fig. 3.1). Based on similarity levels in the dendrogram for the soil fungal community (Fig. 3.1-a), two main clusters could be observed, which included: 80AN plots (75% similarity), and all the remaining treatments including the controls that were very heterogeneous and therefore scattered through the dendrogram. This clustering was corroborated by the soil fungal bands richness (Fig. 3.1-b): 80AN plots showed a significantly higher number of bands than the remaining treatments. Thus, short-term N additions of 80AN to an N poor ecosystem increased the diversity of soil fungi.

In order to evaluate if the changes in the soil fungal community were accompanied by changes in its functionality, the community of AMF spores was also assessed. A total of 16 species of AMF was identified from the genus *Glomus* (12 species)*, Acaulospora* (3 species) and *Paraglomus* (1 species). AMF spores were highly responsive to N enrichment. Two years of N addition was sufficient to induce changes in terms of richness, evenness (Fig. 3.2) and species composition (Table 3.1). Some species were exclusive to the 40A such as *Acaulospoa morrowiae* and *G. mosseae*, while others were detected in all treatments (*e.g. G. coremioides* and *G. fasciculatum)*. The total number of AMF species was within the range of AMF species identified in a similar Californian Mediterranean-type ecosystem (Egerton-Warburton & Allen 2000).

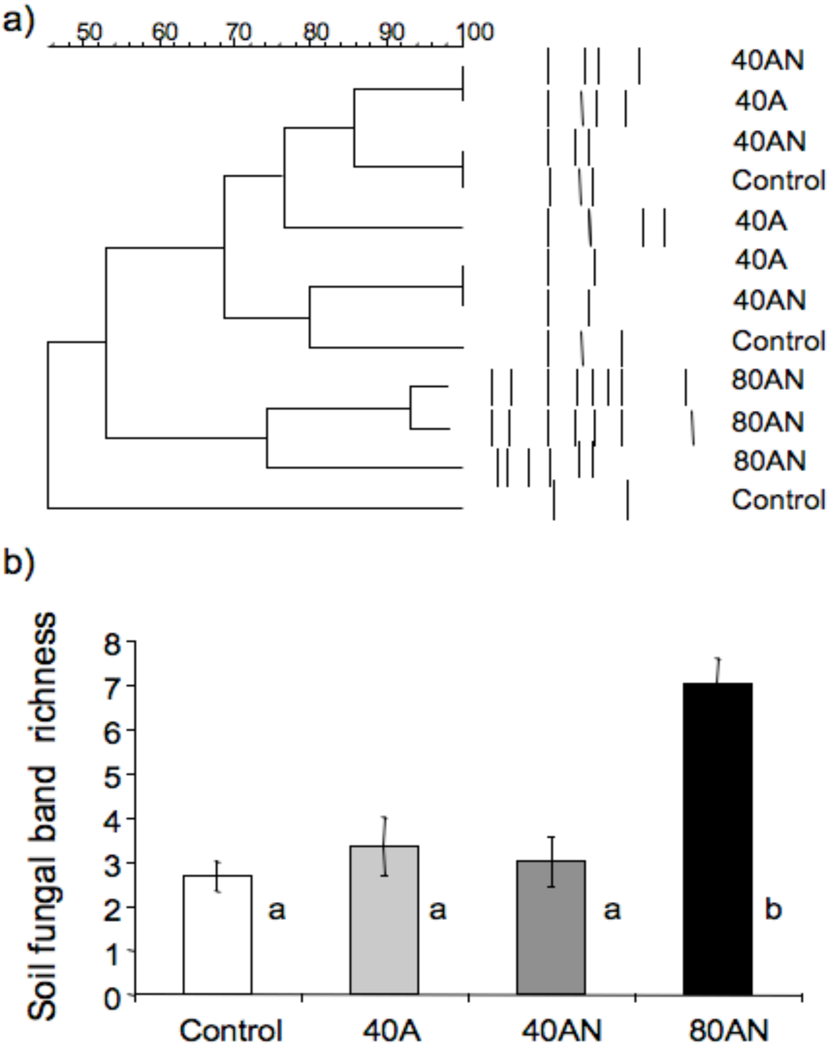

Figure 3.1 - Response of soil fungal community to N additions including ammonium (A) or ammonium with nitrate (AN), in terms of dendrogram (a) and band richness (b) according to treatments (see materials and methods). The soil was sampled in December 2008. Different letters refer to statistically significant differences between treatments (ANOVA $p<0.05$ followed by a Bonferroni test). Values represent the mean (n = 3 experimental plots per treatment) ± SE.

The effects of the N fertilization treatments on AMF spore richness and evenness (Fig. 3.2) were more dependent on the N form than on the N dose. Since the biggest and significant differences in AMF spore richness and evenness were found between the 40A and 40AN treatments (t-test p = 0.046 and 0.05 respectively). Taking into account data from the 80AN, these observations suggest that nitrate influences mycorrhizae more than ammonium, corroborating observations by Atkinson (2009).

N enrichment did not reduce AMF spores richness contrary to other studies (e.g. Egerton-Warburton & Allen 2000; Lilleskov *et al.* 2002; Högberg *et al.* 2003). This opposite response could be due to the low initial N availability in the ecosystem, the short time scale of the N enrichment and/or be related to the observed changes in plant community (Dias *et al.* 2011).

Indeed, Egerton-Warburton, Johnson & Allen (2007) found that the host-plant (C_3 or C_4) was an important determinant of AMF community structure (spores and hyphal community) in grasslands subject to distinct N availabilities.

Table 3.1 - List of the AMF spores species observed in soil samples (n = 3 per experimental plot) collected in December 2008. The species of AMF spores were grouped according to their presence (light grey) or absence (no fill) in soils with distinct N availabilities (Control, 40A, 40AN and 80AN; n = 3 experimental plots per treatment).

AMF spore species	Control	40A	40AN	80AN
Glomus etunicatum	▨			
Glomus sp1	▨			
Acaulospora mellea	▨			
Glomus geosporum-like		▨	▨	▨
Glomus sp4		▨	▨	▨
Acaulospora excavata		▨	▨	▨
Paraglomus occultum		▨	▨	▨
Glomus mosseae		▨		
Glomus sp5		▨		
Glomus sp6		▨		
Acaulospora morrowiae		▨		
Glomus coremioides		▨		▨
Glomus glomerulatum		▨		▨
Glomus fasciculatum	▨	▨	▨	▨
Glomus sp2	▨	▨	▨	▨
Glomus sp3	▨	▨	▨	▨

N enrichment changed the species composition of the AMF spores. Five groups of AMF were identified: those only observed in control plots; those only observed in N fertilized plots; those only observed in the plots receiving 40 kg N ha^{-1} yr^{-1} of ammonium - 40A or 80AN; those observed in all treatments (Table 3.1). Based on the mutually exclusive nature of the first two groups of AMF spores we suggest that species included in these two groups could be used to indicate low and high N availability, respectively. Such shifts in the AMF community in response to N enrichment were also observed by Egerton-Warburton & Allen (2000) in their study of the effects of N enrichment on nine communities of coastal sage scrub, a Mediterranean-type ecosystem dominated by deciduous shrubs.

Changes in the species richness of AMF spores and composition may reflect changes in the AMF communities colonizing plant roots, which could have important consequences for the efficiency of the mycorrhizal symbiosis with respect to nutrient acquisition (namely N and phosphorus), the relative competitive capacity of co-existing plant species and stress tolerance.

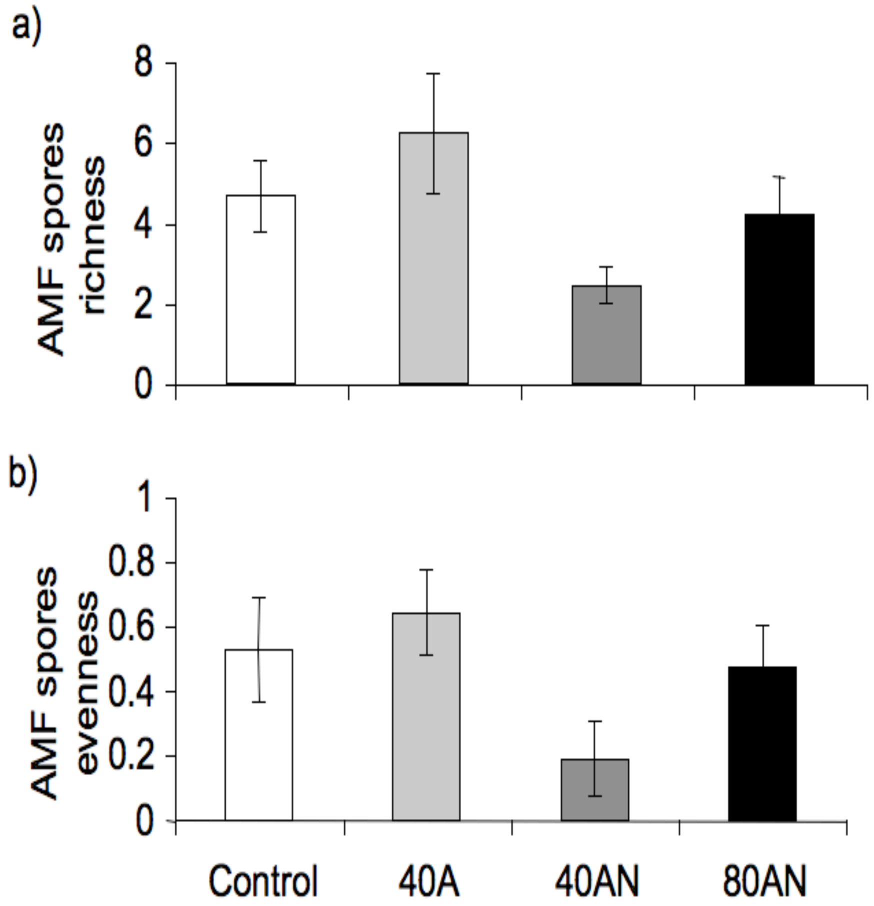

Figure 3.2 – Response of AMF spores richness (a) and evenness (b) to distinct N availabilities. Values represent the mean (n = 9 soil samples per treatment) ± SE.

CONCLUSIONS

- Numerical analysis of TGGE fingerprinting showed that soil fungal community responded to short-term 80AN in terms of richness and structure;
- The effects of N addition on AMF diversity (richness and evenness) appear to depend on the form of the N addition, since 40A had greater AMF richness and evenness than 40AN.
- Changes in soil fungal community were not related to changes in AMF spores.
- The species composition of AMF spores may provide a sensitive indicator of N enrichment, at least in Mediterranean ecosystems.

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Chapter 4

Patterns of nitrate reductase activity vary according to the plant functional group in a Mediterranean maquis

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Chapter 4

Patterns of nitrate reductase activity vary according to the plant functional group in a Mediterranean maquis

ABSTRACT

Background and Aims: Since little is known about how the Mediterranean Basin ecosystems are affected by nitrogen deposition, we aimed to understand the use of nitrogen by distinct plant functional groups (PFG: summer semi-deciduous and evergreen sclerophylls) present in the Mediterranean maquis in order to assess which may be more affected by changes in nitrogen availability.

Methods: The availability of soil inorganic nitrogen, leaf nitrate concentrations and NRA (*in vivo* and *in vitro*) were measured during the year in three plant species from each PFG. The patterns of *in vitro* NRA along the shoot and through the day were also determined.

Results: Although summer semi deciduous species occupied soil patches richer in nitrate, their leaf NRA were significantly lower than that of evergreen sclerophylls species. The pattern of nitrate and ammonium availabilities along the year also distinguished the PFG. Results show that each PFG is composed of a number of physiologically similar species.

Conclusions: Patterns of NRA varied according to the PFG, which may represent distinct specializations of co-occurring species to access nitrogen. Therefore, the NRA can be used as an indicator of the nitrate availability taking into consideration the time of the year, the plant species and its PFG.

Keywords: Mediterranean; Plant functional groups; Nitrate; Ammonium; Pattern of nitrate reductase activity; Nitrogen strategies

INTRODUCTION

Anthropogenic changes in ecosystem functioning threaten human well-being (Sala *et al.* 2000; Cardinale *et al.* 2007). Thus, Rockström *et al.* (2009) identified three main interdependent pressures on global sustainability (changes in the global nitrogen cycle, biodiversity loss, and climate change), with the role of increased nitrogen deposition in biodiversity loss (for review see Bobbink *et al.* 2010) and in alterations of ecosystem functioning (Gruber & Galloway 2008; Rockström *et al.* 2009) being well established. According to Bobbink *et al.* (2010) increased availability of reactive nitrogen is threatening the biodiversity of ecosystems around the world.

Most empirical research linking biodiversity with ecosystem functioning has focused on terrestrial systems with low structural complexity (Maestre & Reynolds 2006; Montès *et al.* 2008). This limits our ability to make confident generalizations on the functional role of biodiversity, and/or to extrapolate the results obtained so far to other communities (Montès *et al.* 2008). Mediterranean-type ecosystems are plant biodiversity hotspots (Phoenix *et al.* 2006) with high structural complexity. The relationship between species richness, functional characteristics and spatio-temporal distribution could be examined in these ecosystems. Moreover, decades of research on these ecosystems have highlighted the importance of plant-plant interactions and environmental heterogeneity (Valladares & Gianoli 2007), both potentially altered by increased nitrogen deposition (Ochoa-Hueso *et al.* 2011). However, the relationship between nitrogen availability and biodiversity has been poorly studied in this type of ecosystem, especially among those located in the Mediterranean Basin (Phoenix *et al.* 2006; Bobbink *et al.* 2010; Ochoa-Hueso *et al.* 2011). Mediterranean ecosystems are thought to be currently experiencing increases in nitrogen deposition (Galloway *et al.* 2004; Phoenix *et al.* 2006) and the greatest proportional biodiversity change (Sala *et al.* 2000; Phoenix *et al.* 2006). Evidence of the link between biodiversity and increased reactive nitrogen availability is starting to emerge: changes in plant (Dias *et al.* 2011) and epiphytic lichen diversity (Pinho *et al.* 2009, 2011). Most semi-natural ecosystems have evolved under nitrogen limitation and Mediterranean ecosystems are no exception (Cowling *et al.* 1996). Thus, plant species are expected to be adapted to the nitrogen source available to them (Chapin, Matson & Mooney 2002; Craine 2009). Given that nitrate is the most common form of reactive nitrogen in the Mediterranean Basin we would expect that the enzyme responsible for processing nitrate, nitrate reductase (NR) will show considerable variability in its activity between plant functional groups.

However, nitrate reductase activity (NRA) is regulated by multiple environmental stimuli (Kaiser *et al.* 2000; Kaiser & Huber 2001). For instance, when plants are deprived of nitrate, NRA falls rapidly (in less than 24h - Kaiser & Spill 1991); if nitrate is added to the soil around plants growing in natural communities, many plants may increase or induce NRA (Kaiser *et al.* 2002; Arslan & Güleryüz 2005). Substrate induction increases the flexibility within the metabolic systems of many plant species. The inducibility of NRA gives flexibility to the metabolic systems of many plant species, constituting an 'economical' response to the wide variation in nitrate supply that occurs under natural conditions (Cruz *et al.* 2003; Sakar *et al.* 2010), especially in Mediterranean ecosystems (Gallardo, Paramá & Covelo 2006; Cruz *et al.* 2008). This, combined with the NR genetic differences between species (Havill, Lee & Stewart 1974; Arslan & Güleryüz 2005; Arslan *et al.* 2009), can contribute to the coexistence of plant species.

The plant species pool of the Mediterranean maquis offers an opportunity to assess the capacity to use nitrate by co-existing species as an indicator of the nitrate status of the ecosystem. But can the NRA of all plant species be used or only that of some? There is growing literature suggesting that focusing on functional traits, rather than species (McGill *et al.* 2006), is more relevant for the assessment of how ecosystems function (Naeem 2009), and a more practical approach for biodiversity hotspots such as Mediterranean ecosystems. The vegetation of Mediterranean ecosystems may be grouped into two plant functional groups (PFG): summer semi-deciduous and evergreen sclerophylls. Each group has been characterized on the basis of its phenology, water relations, carbon exchange properties and abundance during different successional stages (Correia 1988; Werner, Correia & Beyschlag 1999; Werner *et al.* 2001). Our working hypothesis is that the co-existing summer semi-deciduous and evergreen sclerophylls species show distinct patterns of NRA associated with the availability of nitrogen in their ecological niches (Cruz *et al.* 2008) and their phenological and root traits. In order to test our hypothesis, we related leaf *in vivo* and *in vitro* NRA of several plant species belonging to the two PFG with the seasonal availability of water and inorganic nitrogen, to extend the definition of these PFG to their nitrogen use strategies.

MATERIALS AND METHODS

Study site

The study site (38° 27’ 34’’ N, 9° 0’ 20’’ W) is located in Serra da Arrábida in the Arrábida Natural Park, south of Lisbon, Portugal (a Natura 2000 site - PTCON0010 Arrábida/Espichel). Estimated nitrogen deposition is 5.2 kg ha^{-1} yr^{-1} (2.9 kg NO_x + 2.3 kg NH_y - EMEP 2008). The place is situated on a south-facing slope of Jaspe Peak, a calcareous elevation, altitude 270 m. According to the climatic normal (1971-2000) mean annual precipitation was 730 mm; mean maximum temperature, 27.8ºC (August); and mean minimum temperature, 8.1ºC (January). Reported data refer to the nearest climatic station (Setúbal, 15 km distance – Instituto Nacional de Meteorologia e Geofísica). The soil is skeletal (15 cm depth), such that true profiles cannot be discerned. Silt predominates in the soil (57%), while clay and sand contents are 28% and 15% (silt-sand-loam – Correia 1988). Soil pH determined along the experimental period varied between 6.6 and 7.9 for the evergreen sclerophylls species and between 5.6 and 6.8 for the summer semi-deciduous (Cruz *et al.* 2008). The vegetation is a mixed sclerophyll scrub (Eunis class F5.2 – Mediterranean maquis), which developed after a fire eighteen years before this study.

Field sampling

The study site was a homogeneous area (50 m x 50 m) representative of the surrounding vegetation, soil composition, slope, etc. The area was divided into 575 cells of 4 m^2 each. The dominant plant species in each cell was identified and three plant species belonging to the two plant functional groups were selected so that they were abundant and representative of the plant community. The evergreen sclerophylls included *Olea europaea*, *Arbutus unedo* and *Quercus coccifera*; while the chosen summer semi-deciduous species included *Cistus albidus, Cistus salvifolius* and *Rosmarinus officinalis*. Five cells per plant species were identified and further studied. Soil and green leaves were sampled monthly from September 2007 to June (for some parameters until August) 2008. Under the canopy (facing south) of each of the studied plant species, soil water content was measured between 10:00 and 12:00 by time domain reflectrometry (TDR) and soil samples (2 cm diameter and 15 cm depth) were removed, sieved (2mm) and stored at 4ºC until analysis. Soil samples were analysed for inorganic N forms and concentrations. Nitrate and ammonium were extracted from the soil using 2M KCl (1 g soil dry weight to 10 ml of KCl). The concentration of nitrate (in the soil and leaf) was determined by electrophilic substitution of salycilate acid (Matsumura & Witjaksono 1999) while that of ammonium was determined using a modified Berthelot reaction (Cruz & Martins-Loução 2000).

Leaf sampling occurred between 10:00 and 14:00. All the leaves collected were fully-expanded, belonging to the third or fourth pair of leaves (the youngest fully-expanded leaf pair was considered the first), or otherwise indicated, and facing south. Samples were immediately wrapped in aluminium foil and kept in liquid nitrogen until analysis. In the case of NRA determined along the shoot, leaves were collected from the first (youngest) to the seventh (oldest) pair. At the same time, the third and fourth pair of leaves of *Olea europaea* and *Cistus albidus* were collected through the day, every two hours (sunrise was at 6:58 and sunset at 7:32). Sampling along the shoot and along the day only took place at the end of April, when the leaves of all species are active.

Determination of NRA

NRA was determined *in vivo* according to Hageman & Hucklesby (1971), with the modification given by Gebauer, Melzer & Rehder (1984) and *in vitro* according to Kaiser *et al.* (2000). The *in vivo* NRA determination was carried out in two steps. In the first step, whole leaves were cleaned with distilled water and then 100 mg of fresh material was cut into small pieces (~ 0.5 cm). The pieces of leaves were incubated for 90 min at 35ºC in the dark with 5 ml of incubation buffer after vacuum infiltration (5 min) and nitrogen bubbling (10-15 min). The incubation buffer consisted of 100 mM sodium phosphate buffer pH 7.5, 1% iso-propanol and 200 mM KNO_3. The second step consisted of quantifying the nitrite produced, colorimetrically (spectrophotometer Tecan Spectra Rainbow A-5082) at 540 nm by addition to 50 µl of the reaction medium, 125 µl of 5% sulphanilamide in 3N HCl and 125 µl of 0.1% N-naphtyethylendiamine HCl solution. The *in vivo* NRA was expressed as nmol g^{-1} FW h^{-1}.

To determine the *in vitro* NRA, the extraction was performed using an extraction buffer (50 mM Hepes-KOH - pH 7.6 - 0.5% PVP, 0.02% BSA, 0.02% casein, 20 mM DTT, 10 µM FAD, 50 µM leupeptin, 2 mM pefablock, 10 mM $MgCl_2$) in the proportion of 1 g FW of plant material to 2 ml of extraction buffer. Three (out of the five) leaf samples were analysed for NRA. The recovered extracts were centrifuged at 5000 g, for 10 min at 4°C. The resulting crude extracts (650 µl) were desalinated using 5 mL columns filled with Sephadex (G-25-150), centrifuged at 5000 g for 45 seconds, at 4°C. The desalinated extracts were then used to determine the *in vitro* NRA. The activity of NR is modulated by phosphorylation. In the presence of divalent cations phospho-NR forms a catalytically inactive complex by binding to a 14-3-3 protein. If cations are chelated by EDTA, NR becomes fully active (Kaiser *et al.* 2000). Therefore, to determine the potential activity (Pot), one aliquot of extract was pre-incubated for 15 min with a mixture of EDTA/AMP/PO_3^-. The reaction was initiated by addition of the substrates KNO_3 and NADH. The physiological reaction (Phys) was determined under limited concentrations of NADH and NO_3^- and the presence of divalent cations (Mg^{2+}). Both reactions were stopped by addition of 125 µl (10 mM) zinc acetate. The produced nitrite was then quantified as for *in vivo* NRA. The *in vitro* NRA was expressed as µmol g^{-1} FW h^{-1}. The enzyme activation state was determined as the ratio between the potential and the physiological rates. Although the NRA determined in the summer semi-deciduous species did not change, samples from the same plant species grown in the absence of ammonium showed increased NRA (data not shown). Thus, any absence of changes in summer semi-deciduous NRA would not result from inadequacy of the applied protocol.

Statistics

The repeated measures test (General Linear Model) was applied to assess the existence of significant interactions between time (month or hour) and plant species and PFG, for soil and plant parameters. The two-way ANOVA was applied to assess the existence of significant interactions between leaf pair number and PFG. In all cases there were significant interactions between the factors so that differences between PFG were examined for each sampling time and leaf pair number. Summary statistics of soil and plant parameters were compared (two-sided *t*-test, $p<0.05$) for the different sample periods (and leaf pair number), and for PFG. Linear correlations between the soil nitrate and water availability, and plant parameters were also studied (Pearson's correlations). In all cases, preliminary analyses were performed to ensure there was no violation of the assumptions regarding the tests' application. SPSS software, version 19.0, was used for all tests.

RESULTS

The patterns of nitrate and ammonium availabilities along the year in the soil under the canopy of several plant species were quite distinctive of the two PFGs: summer semi-deciduous (*Cistus albidus*, *Cistus salvifolius* and *Rosmarinus officinalis*) and evergreen sclerophylls (*Olea europaea*, *Quercus coccifera* and *Arbutus unedo* - Fig. 4.1). The soil nitrate concentration under the canopy of evergreen sclerophylls species was low throughout the year (< 10 µg g^{-1} - Fig. 4.1-a). Under summer semi-deciduous species the soil ammonium concentration was low and constant along the year (~ 5 µg g^{-1} - Fig. 4.1-b). During the winter and spring months, when water availability was higher, soil nitrate concentrations determined under the canopy of the studied species decreased to minimum levels. In the summer and autumn months, when water availability was lower, the soil patches occupied by the summer semi-deciduous species displayed higher nitrate concentrations than those determined under the canopies of the evergreen sclerophylls. In contrast, the ammonium concentrations determined in the soil patches occupied by summer semi-deciduous species were lower than those determined under the canopy of evergreen sclerophylls. Therefore, plant species belonging to the two PFG were associated with distinct soil nitrogenous environments.

The nitrate concentration in the leaves of summer semi-deciduous species was significantly higher than in evergreen sclerophylls except during the winter months when it reached minimum concentrations in both PFG (Fig. 4.2), thus resembling the temporal pattern of the soil nitrate concentration (Fig. 4.1-a). Correlations between soil nitrate and leaf nitrate concentrations and between the availability of water and leaf nitrate concentration were stronger for summer semi-deciduous species than for evergreen sclerophylls (Table 4.1).

Irrespective of the plant species and the time of the year, leaf *in vivo* NRA was lower than *in vitro* NRA. Also, leaf NRA determined in summer semi-deciduous species was lower than in evergreen sclerophylls (Figs 4.2-b and 4.3). Summer semi-deciduous species displayed low and constant *in vivo* (Fig. 4.2-b) and *in vitro* NRA (potential and physiological – Fig. 4.3-a, b) throughout the year, while evergreen sclerophyllous exhibited differences: lower activities from November to April and higher activities during the rest of the year. Irrespective of the plant species, the patterns of *in vivo* NRA and *in vitro* NRA determined under non-limiting conditions (Potential activity – Fig. 4.3-a) and those determined under limited reducing power conditions (Physiological activity – Fig. 4.3-b) were very similar throughout the year and therefore there were no significant differences in the activation state of the NR (ratio between the physiological and the maximum activities - Fig. 4.3-c). The patterns of the *in vivo* NRA (Table 4.2) and of the *in vitro* NRA determined along the year differed significantly according to the PFG.

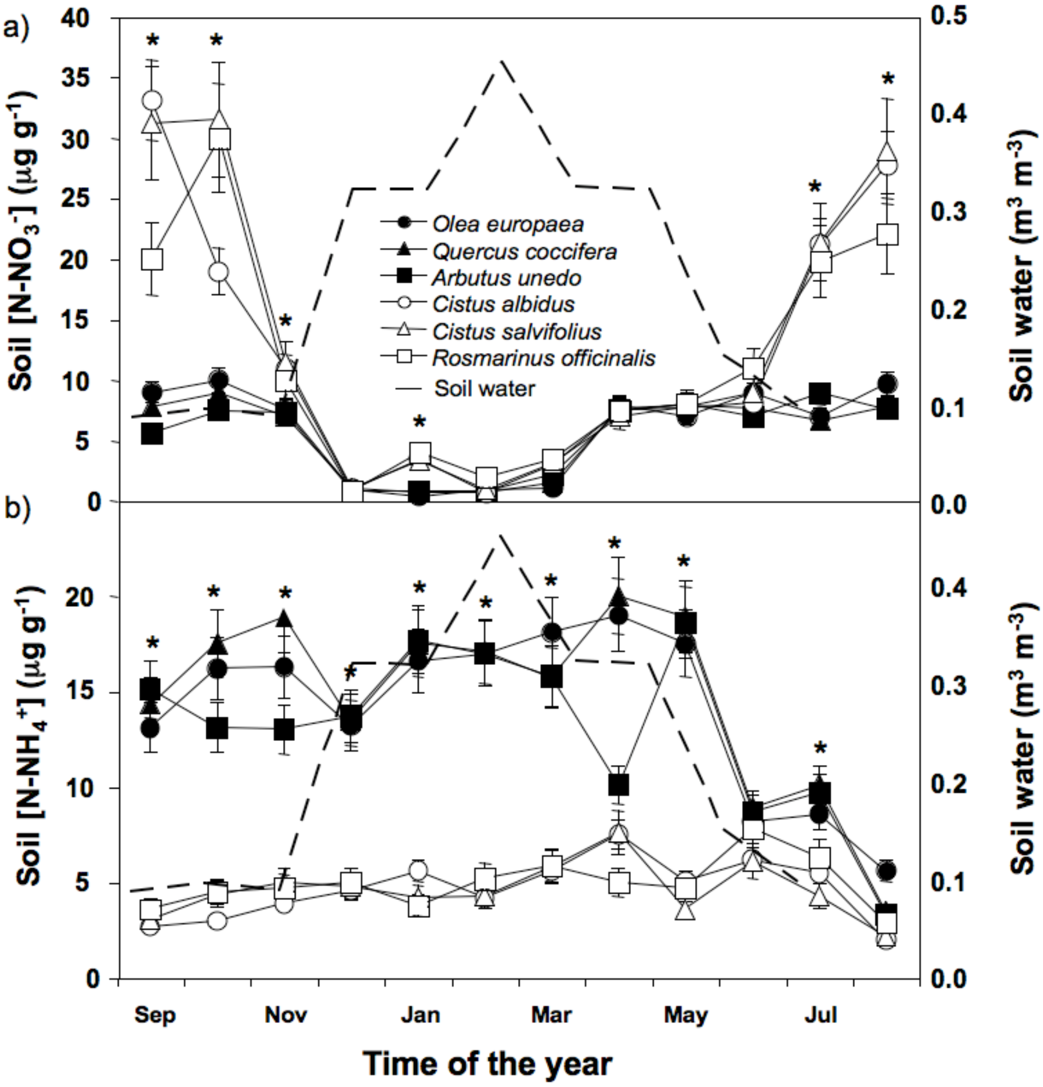

Figure 4.1 – Relation between the plant species belonging to the two PFG (summer semi-deciduous – open symbols; and evergreen sclerophylls – filled symbols) and the concentration of nitrate (a) and ammonium (b) determined along the experimental period under each plant's canopy. There were significant interactions between PFG and time (p<0.05). * refers to statistically significant differences between PFG (t-test p<0.05). Symbols represent the mean (n =3 plants per species) ± SD.

The correlations between the soil nitrate and water availabilities, and plant parameters were assessed (Table 4.1). For the evergreen sclerophylls species, the strongest correlation was between soil nitrate and *in vivo* NRA. Therefore, expressing the *in vivo* NRA as a function of soil nitrate and ammonium concentrations separated the studied plant species according to their PFG and also evidenced two clusters of data points from the evergreen sclerophylls: one cluster comprised the measurements made in winter/spring (closer to zero) and the other, in summer/autumn (higher values of NRA and soil nitrate concentrations - Fig. 4.4). The correlation (Table 4.1) between the *in vivo* NRA of the evergreen sclerophylls species and soil nitrate concentration and the significant effect of soil nitrate concentration on the *in vivo* NRA of the evergreen sclerophylls species (Table 4.2), points to a stimulation of the

NRA by increasing soil nitrate availability. By contrast, and even though the range of soil nitrate concentration under the canopy of summer semi-deciduous species was greater than for the evergreen sclerophylls species, the stimulation of the NRA of the former was smaller (Fig. 4.4-a and Table 4.1). Leaf *in vivo* NRA of summer semi deciduous species was significantly influenced by the interaction between sampling time (month) and the soil nitrate availability (Table 4.2).

Table 4.1 – Pearson's correlation coefficients between soil nitrate and water availability, and plant parameters during the year according to the studied plant species.

PFG	Plant species		Leaf Nitrate	NRA *in vivo*	NRA Potential	NRA Physiological
Evergreen sclerophylls	*O. europaea*	Soil nitrate	**0.45***	**0.98****	**0.72****	**0.42***
		Soil water	**-0.56****	**-0.77****	**-0.50****	**-0.40***
	Q. coccifera	Soil nitrate	**0.48****	**0.95****	**0.56****	**0.46****
		Soil water	**-0.670****	**-0.81****	**-0.75****	**-0.65****
	A. unedo	Soil nitrate	0.13	**0.90****	**0.85****	**0.89****
		Soil water	**-0.50****	**-0.80****	**-0.59****	**-0.53****
Summer semi-deciduous	*C. albidus*	Soil nitrate	**0.49****	**0.56****	0.03	0.29
		Soil water	**-0.79****	**-0.69****	**-0.36***	**-0.64****
	C. salvifolius	Soil nitrate	**0.83****	**0.69****	0.29	0.147
		Soil water	**-0.87****	**-0.86****	-0.08	0.069
	R. officinalis	Soil nitrate	**0.84****	**0.73****	**-0.51****	-0.22
		Soil water	**-0.85****	**-0.88****	**0.63****	**0.38***

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

A negative correlation was observed between the availability of water and nitrate concentration in the leaves and NRA for all the studied plant species. This correlation was stronger for the summer semi-deciduous species than for the evergreen sclerophylls (Table 4.1).

Since the potential for NRA changes with leaf age (Gratani & Bombelli, 2000), we assessed which leaves could potentially contribute to nitrate reduction. Again, and based on the pattern of the *in vitro* NRA along the leaf pairs, the studied plant species were separated according to their PFG (Fig. 4.5). The *in vitro* NRA determined on the youngest and second leaf pairs were very low for all the studied plant species. However, the *in vitro* NRA determined on the third and fourth leaf pairs of the evergreen sclerophylls were approximately twice the NRA measured in the leaves of the summer deciduous species. Of the remaining analysed leaf pairs, the *in vitro* NRA of the summer semi-deciduous species were significantly higher than that of the evergreen sclerophylls.

Although it was not possible to follow the diurnal patterns of *in vitro* NRA in all the studied plant species, it differed between *Cistus albidus* (summer semi-deciduous) and *Olea europaea* (evergreen sclerophylls – Fig. 4.6). At the beginning (6-10 am) and the end (5-7

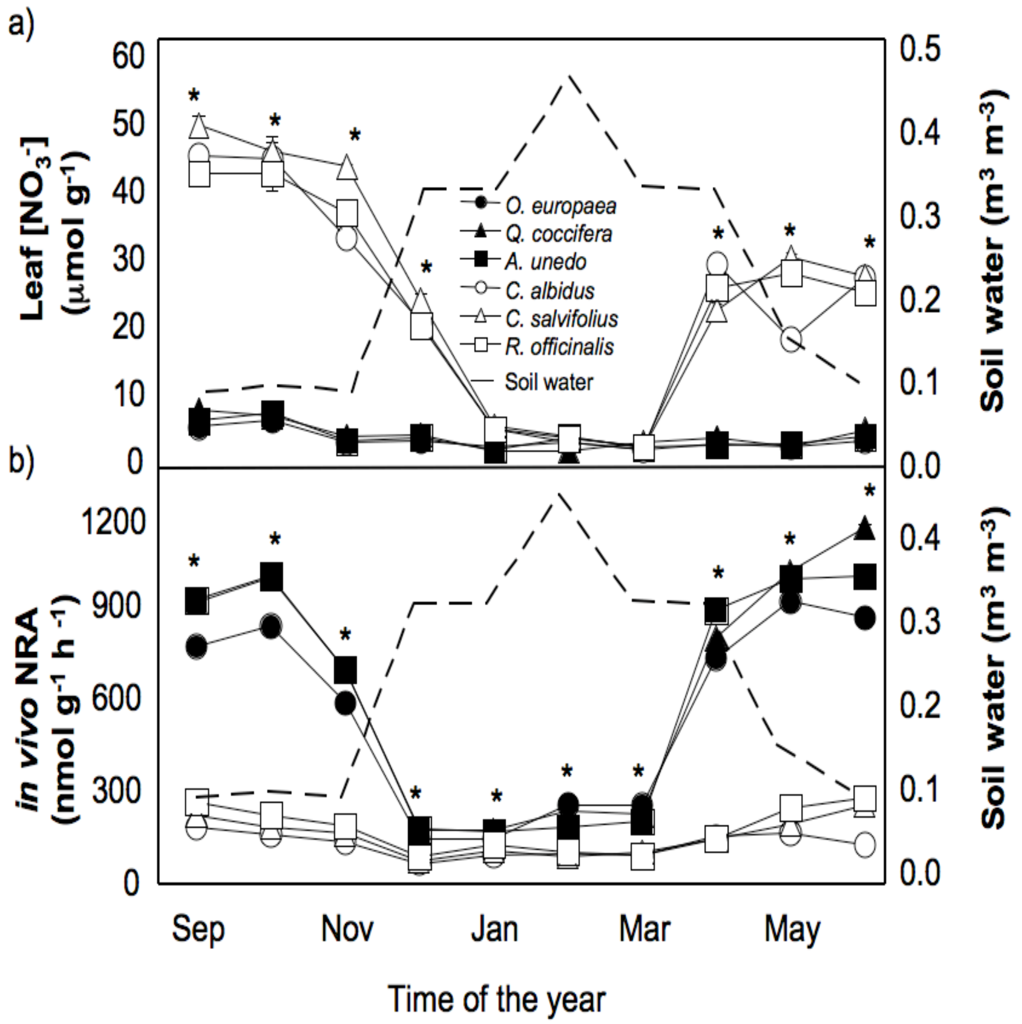

Figure 4.2 –Leaf nitrate concentration (a) and *in vivo* NRA of the studied plant species belonging to the two PFG (summer semi-deciduous – open symbols; and evergreen sclerophylls – filled symbols) along the experimental period. There were significant interactions between PFG and time ($p<0.05$). * refers to statistically significant differences between PFG (t-test $p<0.05$). Symbols represent the mean (n =3 plants per species) ± SD.

pm) of the light period, the *in vitro* NRA of *C. albidus* was significantly higher than that of *O. europaea*. However, during the middle of the light period (11am to 3pm) the *in vitro* NRA of *O. europaea* was significantly higher than that of *C. albidus*.

Based on the analysed parameters, two patterns of nitrate reduction were evident: one shown by *O. europaea*, *Q. coccifera* and *A. unedo*, the three studied evergreen sclerophylls species; and the other by *C. albidus*, *C. salvifolius* and *R. officinalis*, the three summer semi-deciduous species.

DISCUSSION

Soil heterogeneity at the scale of plant species has been described for Mediterranean soils (Gallardo, Paramá & Covelo 2006; Rutigliano *et al.* 2009). Moreover, Cruz *et al.* (2008)

showed that the two main Mediterranean plant functional groups (PFG), summer semi-deciduous and evergreen sclerophylls, significantly affected soil superficial characteristics (e.g. soil pH, organic matter, nitrification potential, etc.) in distinct ways. Accordingly, concentrations of nitrate and ammonium in the soil through the year were in the range found in other Mediterranean ecosystems (Gallardo, Paramá & Covelo 2006; Cruz *et al.* 2008), and clearly reflected the two PFG co-existing at the site: summer semi-deciduous - *Cistus*

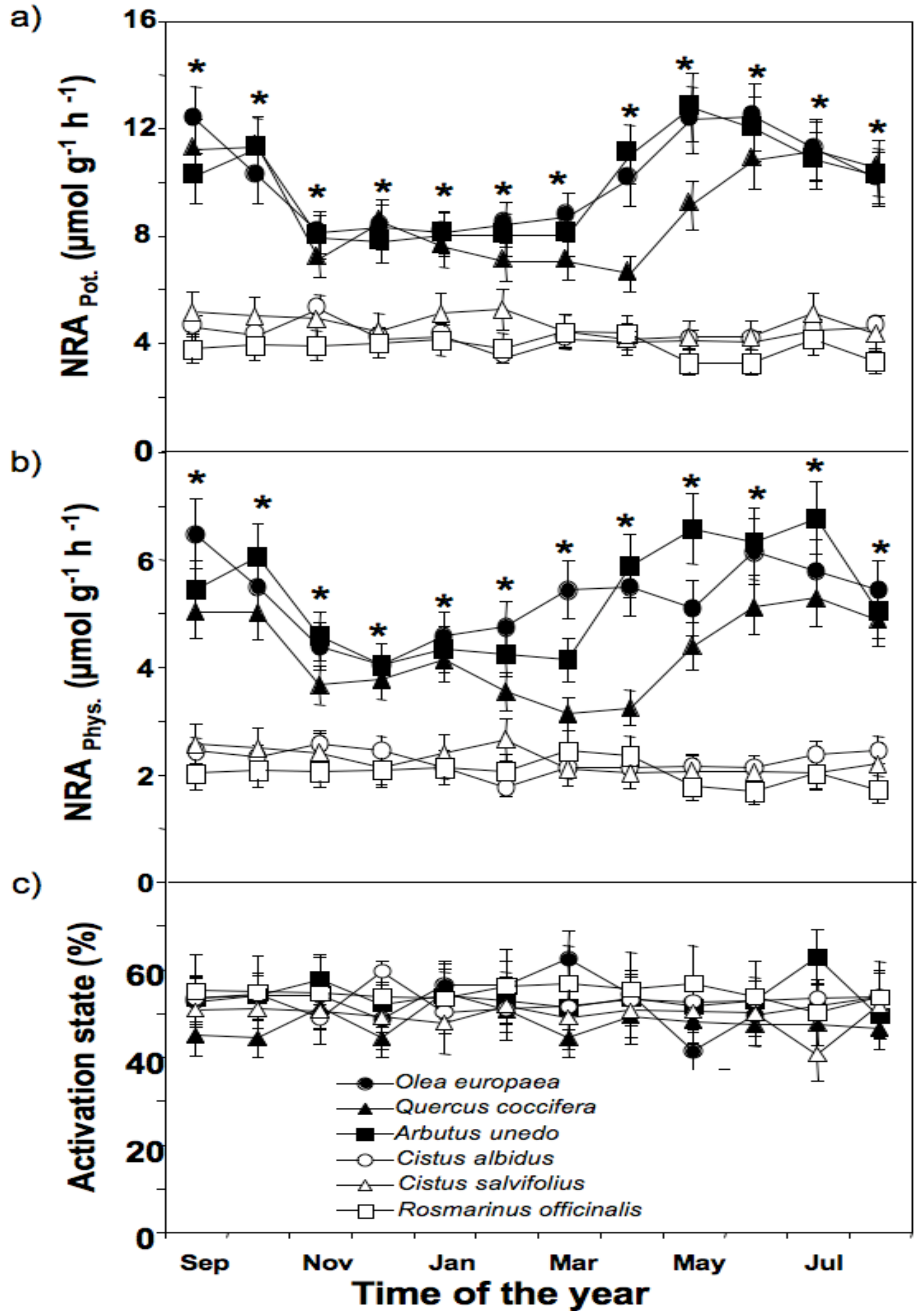

Figure 4.3 – Temporal pattern of $NRA_{Pot.}$ (a), $NRA_{Phys.}$ (b) and the NR activation state (c) of the plant species belonging to the two PFG (summer semi-deciduous – open symbols; and evergreen sclerophylls – filled symbols). There were significant interactions between PFG and time ($p<0.05$). * refers to statistically significant differences between PFG (t-test $p<0.05$). Symbols represent the mean (n =3 plants per species) ± SD.

albidus, *Cistus salvifolius* and *Rosmarinus officinalis*; and evergreen sclerophylls – *Olea europaea*, *Quercus coccifera* and *Arbutus unedo*, i.e., the two main PFG in Mediterranean ecosystems, have distinct soil nitrogenous environments (Fig. 4.1). This may be related to group-specific resource requirements and the use of distinct soil nitrogen pools, decreasing the competition between co-existing groups for limiting nutrients (Kahmen *et al.* 2006).

Table 4.2 – Statistical analyses on leaf *in vivo* NRA for the studied plant species (all and according to their PFG - evergreen sclerophylls and summer semi-deciduous) of the time of sampling (month), soil nitrate concentration and PFG. A repeated-measures ANOVA (p<0.05 are shown in bold type) was conducted for leaf *in vivo* NRA.

Variable	Leaf *in vivo* NRA								
	All studied species			Evergreen scleropylls			Summer semi-deciduous		
	df	F	P	df	F	P	df	F	P
Month	8	30.5	**< 0.001**	8	29.7	**< 0.001**	8	1.4	0.27
Soil [NO_3^-]	75	-	-	45	2.9	**< 0.001**	45	0.6	0.91
Month x Soil [NO_3^-]	38	0.2	0.99	20	0.3	0.99	20	3.0	**0.02**
PFG	1	300.3	**< 0.001**						
PFG x Month	2	1.5	0.40						
PFG x Soil [NO_3^-]	7	0.8	0.68						
PFG x Month x Soil [NO_3^-]	2	2.8	0.08						

The relative abundance of soil nitrate and ammonium depends on the balance between production and consumption. When nitrate is not directly added to the soil (by fertilization or deposition), nitrification is the main process of soil nitrate production. It has been reported that plant species from later successional phases (e.g. evergreen sclerophylls) inhibit soil nitrification (Cruz *et al.* 2008), which could at least partly explain why nitrate concentrations under the canopies of those species were lower than those of ammonium (from November to May - Fig. 4.1-a).

The temporal pattern of nitrate concentration in leaves of both PFG (Fig. 4.2) followed that of the soil (Fig. 4.1-a) especially in the summer semi-deciduous species (Table 4.1), showing that when nitrate was more abundant in the soil (September and October and July and August), it could be detected in the shoot. Thus, leaf nitrate concentration (Fig. 4.2) may be an indicator of soil nitrate availability (Fig. 4.1-a). In general, within this time period and irrespective of the PFG, there was an inverse relation between the concentration of nitrate (in the soil and in the leaves) and the water availability (Table 4.1 and Figs 4.1-a and 4.2-a). This inverse relation may result from nitrate losses (e.g. leaching and/or runoff) or uptake by the biota (soil microbial community and vegetation).

As not many data are available for *in vitro* leaf NRA determined under field conditions, *in vivo* NRA was also determined (Fig. 4.2-b). All the studied plant species displayed *in vivo* leaf NRA (Fig. 4.3-a) comparable with a wide range of Mediterranean geophytes (Arslan & Güleryüz 2005) and shrub species (Arslan *et al.* 2009), but lower than those of plant species characteristic of other calcareous habitats (*Poterium sanguisorba* and *Scabiosa columbaria*– Havill, Lee & Stewart 1974). This may be due to local nutrient availability (Fitter & Hay 2002) and/or the relative growth rates experienced by these plant species in their habitats (Poorter *et al.* 1995; Craine 2009). However, *in vivo* NRA is mainly limited by the availability of nitrate and reducing power (Kaiser *et al.* 2000), so that although they followed the same temporal patterns, *in vitro* NRA (Fig. 4.3) was higher than *in vivo* NRA (Fig. 4.2-a). Given that, in higher plants, NRA is rapidly modulated by environmental conditions (Kaiser & Spill 1991; Kaiser & Huber 2001), NRA is expected to change along the year. This temporal response of NRA to environmental conditions was observed in evergreen sclerophylls but not in summer semi-deciduous species, which indicates that the temporal pattern of NRA also differentiated

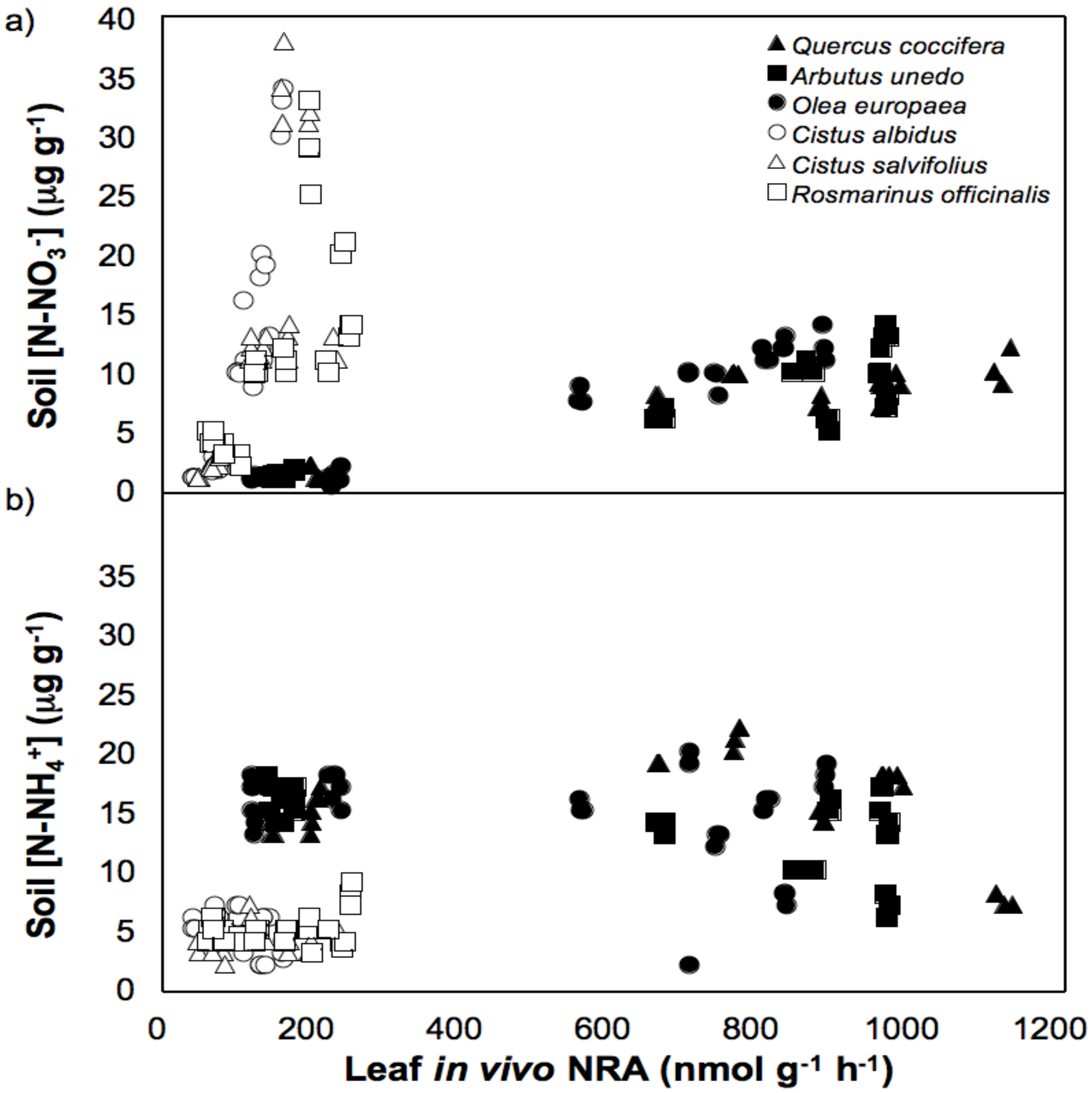

Figure 4.4 – Relation between all the determinations of leaf *in vivo* NRA and the respective concentrations of soil nitrate (a) and ammonium (b) along the experimental time and according to the plant species (summer semi-deciduous – open symbols; and evergreen sclerophylls – filled symbols; see Table 4.1 for correlations). Symbols represent the individual values (n = 3 measurements x 9 sampling times per species).

between summer semi-deciduous and evergreen sclerophyllous species (Table 4.2 - Karavatas & Manetas 1999).

Since NR is a substrate-inducible enzyme (Kaiser *et al.* 2000; Kaiser & Huber 2001; Arslan & Güleryüz 2005), the NRA of a plant has been assumed to reflect the long-term nitrate supply to the plant so that in ecological studies NRA can indicate nitrate availability (Lee & Stewart 1978; Arslan & Güleryüz 2005; Sakar *et al.* 2010). This was the case for the evergreen sclerophylls species, but not for the summer semi-deciduous species (Tables 4.1 and 4.2). Surprisingly, even though summer semi-deciduous species occupied nitrate-richer

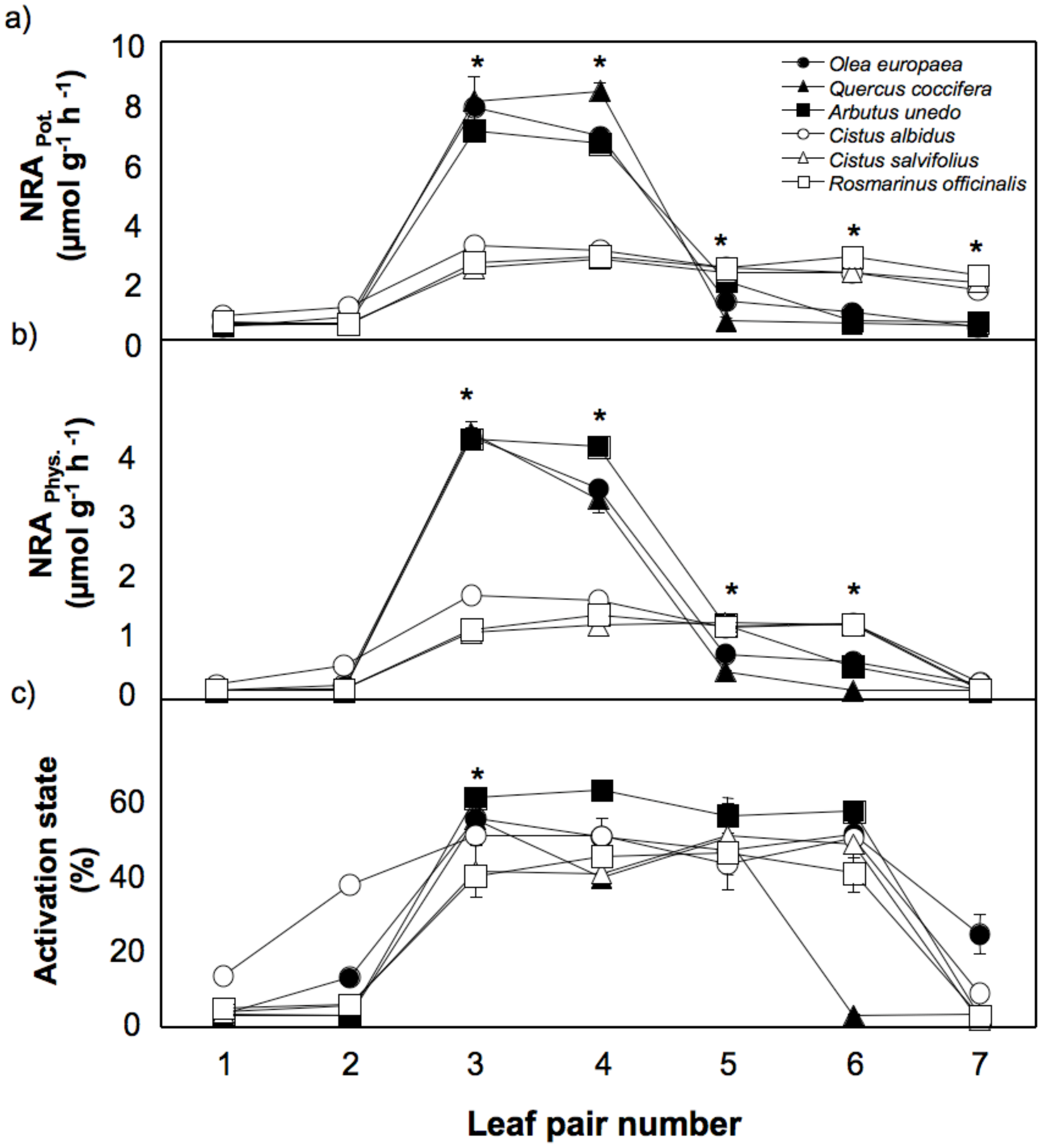

Figure 4.5 – Comparison between the *in vitro* $NRA_{Pot.}$ (a), $NRA_{Phys.}$ (b) and the NR activation state (c) along the branches of the plant species belonging to the two PFG (summer semi-deciduous – open symbols; and evergreen sclerophylls – filled symbols). There were significant interactions between PFG and time ($p<0.05$). * refers to statistically significant differences between PFG (t-test $p<0.05$). Symbols represent the mean (n =3 plants per species) ± SD.

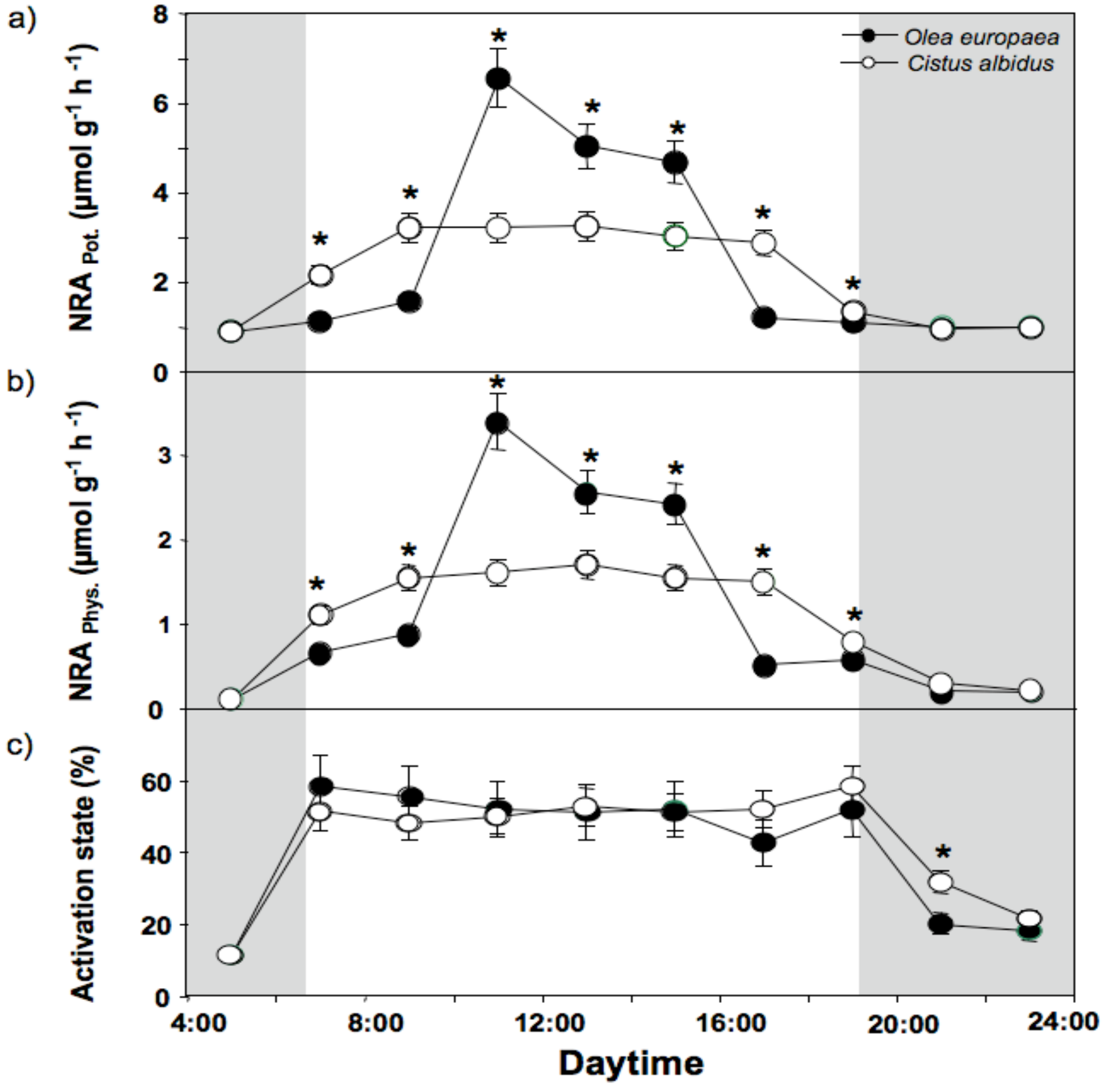

Figure 4.6 – Diurnal pattern of the *in vitro* $NRA_{Pot.}$ (a), $NRA_{Phys.}$ (b) and the NR activation state (c) according to the plant species: *Cistus albidus* (summer semi-deciduous) and *Olea europaea* (evergreen sclerophylls). Sunrise occurred at 7am and sunset at 7pm. There were significant interactions between PFG and time ($p<0.05$). * refers to statistically significant differences between PFG (t-test $p<0.05$). Symbols represent the mean (n =3 plants per species) ± SD.

soil patches and had more nitrate in the leaves than the evergreen sclerophylls (Fig. 4.1-a), *in vivo* and *in vitro* NRA determined in the former was lower than in the latter (Figs 4.2-b and 4.3). In fact, the NRA of summer semi-deciduous species responded less to increases in nitrate availability than the evergreen sclerophylls (Tables 4.1 and 4.2 and Figs 4.1-a and 4.4). However, as NR can facilitate the transduction of many environmental stimuli into metabolic activity (Kaiser & Spill 1991; Kaiser & Huber 2001; Kaiser *et al.* 2002), it is possible that besides the stimulation of NRA by nitrate (Figs 4.1-a and 4.2-a), there was, in the summer semi-deciduous species, a predominant inhibitory signal. The fact that nitrate accumulated in the leaves of summer semi-deciduous species (Fig. 4.2-a) and that ammonium concentrations in the soil under the canopy of these species were below 8 µg g^{-1} (Figs 4.1-b and 4.4-b) suggest that ammonium can be an inhibitory signal. Ammonium can affect the metabolism of nitrate because most steps in plants' nitrate assimilatory pathway are nitrate-inducible but ammonium, or its metabolic products, can inhibit the reduction of nitrate (Orebamjo & Stewart 1975; Oaks, Aslam & Boesel 1977; Emmett 2007) through inhibition of NR synthesis. This hypothesis should be further assessed as a potential mechanism contributing to the natural replacement of summer semi-deciduous species by evergreen sclerophylls during plant succession (Werner, Correia & Beyschlag 1999; Werner *et al.* 2001). Non-exclusively, the two PFG also differ in rooting depths; summer semi-deciduous tend to have a superficial root system while evergreen sclerophylls have a more complex root system with both superficial and deeper roots (Correia 1988; Canadell *et al.* 1996). Although a small fraction of root biomass might be found at depths below 1 m, the functional significance of those roots is important for ecosystem water and carbon fluxes and nutrient cycling (Canadell *et al.* 1996). Deep roots improve water uptake and increase the probability of survival in Mediterranean communities (Lloret, Casanovas & Peñuelas 1999). Also, in the Brazilian Cerrado, deep roots have also been shown to access nitrate that had been leached (Canadell *et al.* 1996). Thus, the deeper root system of evergreen sclerophylls may enable them 'escape' the summer drought. On the contrary and given that higher nitrate availability coincided with lower water availability (Fig. 4.1-a), summer semi-deciduous may not have been able to reduce the nitrate due to their shallow root system.

The pattern of NRA along the twig was different for the two PFG (Fig. 4.5), which may be related to their phenology, i.e., the life span of the summer semi-deciduous leaves is less than one year while that of evergreen sclerophylls is 1 to 2 years (Correia 1988; Oliveira & Peñuelas 2004). This corresponds to a substantial carbon cost in nitrate assimilation so that NRA along the twig needs to be linked to the metabolic activity of the leaves, in particular the carbon balance (Foyer *et al.* 1998) and availability of reducing power. The greater importance of the third and fourth pairs of leaves relative to the others in evergreen sclerophylls species may result from a combination of factors and is in agreement with the slow relative growth rate of these species (Chapin, Matson & Mooney 2002). Summer semi-deciduous species displayed lower NRA but the relative contribution of each pair of leaves to the nitrate reduction was more uniform, suggesting a more homogeneous contribution of all the leaves to the plant metabolism, which is characteristic of plants from the initial stages of succession and with higher relative growth rates.

The NRA of summer semi-deciduous and evergreen sclerophylls species also differed throughout the day (Fig. 4.6). The diurnal pattern of NRA may result from the complex mechanisms regulating the activity of the NR and its interactions with other enzymes and metabolites such as soluble sugars, amino acids, malate and nitrate concentrations in the roots and in the xylem and phloem (Matt *et al.* 2001b). The differences in the patterns of diurnal activities will have drastic consequences on the partitioning of the newly assimilated carbon (Matt *et al.* 2001a; Cruz *et al.* 2003).

CONCLUSIONS

Altogether, the experimental data are in agreement with other preliminary studies made in the same area (Cruz *et al.* 2008), which together suggest that each of the PFG is composed of a number of physiologically similar plant species, as suggested by Kummerow (1973). Therefore, and given that phenology and rooting depth of the plant species appeared to have influenced NRA, the use of NRA as an indicator of the nitrate availability has to take into consideration the time of the year, the plant species and its PFG.

In the study area, evergreen sclerophylls and summer semi-deciduous co-exist. However, in later phases of succession, evergreen sclerophylls tend to dominate to the detriment of the summer semi-deciduous species. This may be accompanied by a higher occupation of the soil space by roots of evergreen sclerophylls, with the concomitant decrease of nitrate and increase of ammonium concentrations in the soil (Chapin, Matson & Mooney 2002). It is possible that in earlier phases of the ecological succession, when summer semi-deciduous species dominate, their leaf NRA could be a more robust indicator of the nitrate supply (Lee & Stewart 1978; Arslan & Güleryüz 2005; Sakar *et al.* 2010).

Based on the estimates of increased nitrogen deposition for the Mediterranean Basin (Galloway *et al.* 2004; Phoenix *et al.* 2006), it is possible that the ecological nitrogen niche occupied by summer semi-deciduous species will become narrower, with the concomitant lengthening of that of evergreen sclerophylls. However, more research is needed to understand how the Mediterranean maquis will respond to increased nitrogen deposition, namely to different forms and doses.

Finally, the observed differences between plant species belonging to the two PFG may be the consequence of evolutionary trade-offs, and represent specializations of the endemic species to increase their chances of getting access to nitrogen, so that different and complementary nitrogen strategies can contribute to the benefits of PFG diversity on ecosystem functioning (Kahmen *et al.* 2006).

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Chapter 5

The strength of the biotic compartment in retaining nitrogen additions prevents nitrogen losses from a Mediterranean maquis

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The strength of the biotic compartment in retaining nitrogen additions prevents nitrogen losses from a Mediterranean maquis

ABSTRACT

Nitrogen (N) is one of the nutrients most limiting to ecosystem productivity. However, N availability is increasing globally, which may affect ecosystem functions and stability. To understand the role of each ecosystem compartment in the cycling of increased N, we studied the initial response of a nutrient-poor ecosystem, a Mediterranean maquis, to increased N deposition. N availability (dose and form) was modified by three N additions over the year (middle autumn/winter, spring and summer). Soil inorganic N pools (nitrate in particular) strongly reflected the N additions in autumn, almost matching the total N added over the three additions. *Cistus ladanifer*, the dominant plant species, responded to the increased N (cover and N concentration in leaves and litter). Given that leaf shedding occurs in the summer, the importance of this N pool returning to the soil through litter decomposition on the total soil inorganic N in autumn was investigated. Data suggest that living plants and litter have a crucial role in preventing N losses from Mediterranean maquis. This is the first integrated field study on how European Mediterranean ecosystems retain increased N of different forms and doses, however longer-term studies are needed to explore the generality of this study's observations.

Keywords: Mediterranean; Temporal patterns; Soil inorganic N; Litter; N cycling; *Cistus ladanifer*

INTRODUCTION

Temporal patterns of inorganic nitrogen (N) turnover and plant growth can influence important aspects of plant community and ecosystem dynamics; e.g. N losses, plant productivity, community composition, changes in N and/or phosphorus (P) limitation, decomposition rates and N niche complementarity (Augustine & McNaughton 2004). Anthropogenic activities have led to global N enrichment (Galloway *et al.* 2008), threatening ecosystems at local, regional and global scales (Cassman *et al.* 2003), since demand has become decoupled from N availability. Nutrient availability is a key determinant of ecosystem function and stability (Bobbink, Hornung & Roelofs 1998; Phoenix *et al.* 2006). When N availability exceeds an ecosystem's N retention capacity, a shift from a closed internal N cycle to an open and leaky cycle occurs, with the excess N being leached and/or emitted from the ecosystem (De Schrijver *et al.* 2008). Most research on the effects of N enrichment on ecosystems has focused on temperate synchronous systems (availability of resources coincides with plant growth) from northern Europe and America (Bobbink *et al.* 2010; Phoenix *et al.* 2006). There are some documented studies on asynchronous temperate ecosystems (e.g. in alpine tundra there are large increases in the inorganic N pool during snowmelt - Brooks, Williams & Schmidt 1998 - and the highest rates of N mineralization occur during late summer or early fall, after plant senescence - Jaeger *et al.* 1999). However, these are exceptions and not the most common situation (Augustine & McNaughton 2004). Therefore not much is known about asynchronous ecosystems (availability of distinct resources and plant growth do not occur at the same time) such as those in Mediterranean regions (Jackson *et al.* 1988). Mediterranean-type ecosystems occur worldwide and are a product of the Mediterranean climate: hot dry summers, and mild wet winters (Rivas-Martínez, Penas & Díaz 2004). The few studies of the N retention capacity of Mediterranean-type ecosystems have been made in California (e.g. Holub & Lajtha 2004), while, as far as we are aware, none have been made of the Mediterranean Basin.

We hypothesize that the mild temperatures and less limiting water availabilities, which result in intense biological activity during the Mediterranean spring, coincide with periods of low soil inorganic N pools. During this period, most additional N is likely to be taken up by plants and microorganisms, and retained within the ecosystem's biotic compartment. However, during the dry summer, a significant proportion of the N will return to the soil through leaf shedding. In autumn, when water no longer limits biological activity, N returns to the inorganic form through decomposition processes. As a result, soil inorganic N pools are expected to be the highest in autumn. For this reason we studied soil inorganic N over time, plant community and the N stored in plants and litter in a Mediterranean maquis in response to N additions. Our objective was to evaluate the short-term N retention capacity of a Mediterranean maquis (i.e., a type of Mediterranean habitat that comprises closed vegetation, usually with 100% cover, mainly shrubs with few annuals and some geophytes, trees are nearly always present, some of which may be in shrub form) and understand which ecosystem compartments are involved in the cycling of the increased N in such an asynchronous ecosystem. Specifically, we examined relationships between the N-driven response of N concentration in the soil, leaves and litter through two consecutive springs (2007 and 2008).

MATERIALS AND METHODS

Study site

The study site (38° 29’ N - 9° 01’ W) is in Serra da Arrábida in the Arrábida Natural Park, south of Lisbon, Portugal (a Natura 2000 site - PTCON0010 Arrábida/Espichel). It is located in a sub-humid thermomediterranean bioclimatic domain (Rivas-Martínez, Penas & Díaz 2004). According to records (1971-2000 - Instituto Nacional de Meteorologia e Geofísica), mean annual precipitation is 730 mm; mean maximum temperature, 27.8°C (August); and mean minimum temperature, 8.1°C (January). Estimated background N deposition is 5.2 kg ha^{-1} yr^{-1} (2.9 kg NO_x + 2.3 kg NH_y - http://webdab.emep.int/Unified_Model_Results/AN/).

The study site is located on a southeast-facing slope (5%) at 130 m a.s.l. that is protected from public access and has not been affected by human activity in recent years. Soil is skeletal (15 cm depth) so that true profiles cannot be discerned and soil density is 1.3 g cm^{-3}. Silt predominates in the soil (50%), while sand and clay contents are 32% and 18%, respectively (silt-sand-loam texture). The vegetation consists of a dense maquis (Eunis class F5.2 – Mediterranean maquis), which developed after a fire event (summer 2003) four years before the first N addition. The vegetation is dominated by summer semi-deciduous species which exhibit leaf dimorphism, shedding a large fraction of their leaves and twigs in the summer, and their development is coupled to water availability in the upper soil layers (Correia & Catarino 1994; Cruz *et al.* 2008). At the moment, the dominant plant species is a *Cistaceae*, *Cistus ladanifer* L. (Dias *et al.* 2011). Other abundant plant species include *Erica scoparia* L. (*Ericaceae*), *Calluna vulgaris* (L.) Hull (*Ericaceae*), *Genista triacanthos* Brot. (*Fabaceae*), *Ulex densus* Welw. ex Webb (*Fabaceae*), *Dittrichia viscosa* L. (*Asteraceae*), and *Myrtus communis* L (*Myrtaceae*). Herbaceous species comprise ≈ 10% of the total plant cover, of which many are annual plants.

Experimental design and fertilization schedule

The criteria for choosing the N doses and forms used in the experiment were: a) N doses lower than the N deposition reported for other areas in Mediterranean-type ecosystems (145 kg N ha^{-1} yr^{-1} - Fenn *et al.* 2003; Meixner & Fenn 2004) but high enough to establish ‘worst case’ scenarios of N enrichment in this type of habitat; and b) mimic the most likely N pollution scenarios in the experimental area, i.e., combined inputs from urban/industrial sites and agricultural (addition of nitrate and ammonium) and predominantly agricultural sources (addition of ammonium). Therefore, N availability was modified by the addition of 40 and 80 kg N ha^{-1} yr^{-1} in the form of N-NH_4NO_3 (designated 40AN and 80AN) and 40 kg N ha^{-1} yr^{-1} as a 1:1 mixture of N-NH_4Cl and N-$(NH_4)_2SO_4$ (designated 40A). Control plots were not fertilized. Beginning in January 2007, the dry N salts were homogenously added, by hand, in three equal applications over a year: middle autumn/winter, spring and summer. The N granules dissolved rapidly (1-7 days depending on the N addition period). N additions were scheduled so that no precipitation was predicted for at least one week after the N addition. Data reported in the present paper is based on four N additions that took place in January, April and August 2007; and January 2008, always following the corresponding soil sampling. Each treatment was replicated three times, 3 plots each of 400 m^2. In order to restrict boundary effects and dilution processes, all measurements, analyses and sample collection were performed within an internal 100 m^2 square. To prevent N ‘contamination’ through runoff from N-plots, the experimental plots were distributed in three rows along the 5% slope, with the controls being located in the top row.

Soil and plant sampling and plant assessment

Soil was sampled from the four corners and the centre of the internal 100 m^2 square of each plot. Soil samples (2 cm diameter and 15 cm depth) were removed, sieved (2mm) and stored at 4ºC until analysis. Sampling took place in May, August and October 2007; and February and April 2008, corresponding to the distinct seasons. Individual soil samples (five per plot) were used to determine soil pH, moisture and concentrations of nitrate ($N\text{-}NO_3^-$), ammonium ($N\text{-}NH_4^+$) and inorganic N. Bulk soil samples (equal mixtures of the five soil samples from each experimental plot) collected in May 2007 and April 2008 were used for soil characterization (Table 5.1, see below for methods).

The vegetation at the study site was assessed within one 5x5m square per plot (within the internal 100 m^2) during spring (June 2007, May 2008). *C. ladanifer* plant cover was calculated from the total projected crown area (calculated from two perpendicular diameters, assuming elliptical shape).

C. ladanifer’s leaves were sampled in the spring. Samples consisted of twigs containing approximately 4-5 pairs of fully expanded leaves. One twig per plant was sampled from ten random *C. ladanifer* plants within the internal 100 m^2 square. The leaves from the ten twigs were bulked to form one composite sample per plot. Litter was sampled near to each of the five soil sampling points in each plot. Litter sampling took place in the summer. Only leaf litter from *C. ladanifer* was analyzed.

Chemical analysis

From each individual soil sample (five per plot and per sampling time), 10 g fresh weight (fwt) of soil were used to gravimetrically determine soil water content (Kern EG300 3M). Five g (fwt) of soil were used to prepare soil water extracts (1:10 – w/v). Soil extracts were shaken (Cassel Agitator, 600 rpm) for one hour at room temperature, centrifuged (Eppendorf Centrifuge 5403) at 5000 g for 20 minutes at 4ºC. The supernatant was collected and analyzed colorimetrically (spectrophotometer Tecan Spectra Rainbow A-5082) for nitrate

(Matsumura & Witjaksono 1999), ammonium (Cruz & Martins-Loução 2000), and for soil pH (Crison micro pH 2002). Soil inorganic N was the sum of the water extracted N-NH_4^+ and N-NO_3^-, and was expressed as µg N per g of dry soil. The bulk soil samples used for determining organic matter and concentrations of total N and extractable P, potassium (K) and magnesium (Mg) were dried at 35ºC until constant dry weight (dwt). Organic matter was determined according to ISO standard 10694 by loss on ignition overnight at 600ºC (Nabertherm L3/11/C6). Analysis of total N was carried out according to ISO standard 13878 by dry combustion using an elemental analyzer (Leco CNS). Extractable phosphorus and potassium were quantified by a modification of the Egner-Riehm method using plasma emission spectrophotometer with an optical detector (ICP-OES), following extraction using ammonium lactate 0.1 M and acetic acid 0.4 M, pH 3.65-3.75. Mg was extracted with ammonium acetate 1 M, pH 7 and quantified by atomic absorption spectrophotometry with flame atomization. Leaf and leaf litter samples were dried at 60ºC, ground (MM 2000) and analyzed for total N and carbon (C - the same procedure as for soil samples).

Calculations

Changes in the cover of *Cistus ladanifer* over time (t_0, t_1) were calculated according to Sheppard *et al.* (2008) as follows:

$$\text{Changes over time (\%)} = \frac{(\text{Parameter}_1 - \text{Parameter}_0)}{(\text{Parameter}_1 + \text{Parameter}_0)/2} \times 100$$

Changes in the treatments (m_n) in relation to the control (M_c) were calculated as follows:

$$\text{Changes in relation to the control } (\mu g\ g^{-1}) = m_n - M_C$$

Where 'm' corresponds to each individual value (e.g. concentration of N as soil inorganic N and litter N); 'M_C' corresponds to the mean value of the control for each parameter; and 'n' corresponds to the distinct experimental plots.

Transformation of the applied N doses into soil inorganic N concentrations (µg N g^{-1} dwt) was based on the following soil characteristics: 15 cm depth and 1.3 g cm^{-3} of density, resulting in ~2000 t of soil per ha. Therefore, the addition of 40 and 80 kg N per ha corresponded to doses of 20 and 40 µg N g^{-1}, respectively. The transformation of the applied N doses into soil inorganic N concentrations will be referred to as "total N added", while the concentration of soil inorganic N in relation to the control determined in autumn will be referred to as "measured N".

The possible contributions to the soil inorganic N concentration determined in autumn were identified as: inorganic N present in the soil in the previous season – [N] summer; the subsequent N addition – [N] addition; and the extra N present in the litter (in relation to the control) which was shed during the summer – [N] litter. The [N] addition and the [N] litter were also transformed into soil inorganic N in the soil (µg g^{-1} soil dwt). The effects of the N additions on the three contributors were then compared to the control. The changes of litter N concentration in relation to the control were estimated assuming an annual litter production of 2.3 t ha^{-1} in a Mediterranean maquis (Schultz 2002). Then we calculated the sum of the soil inorganic N determined in the summer, the summer N application and the N contained in the litter (‘calculated N’).

Statistics

Summary statistics of soil and plant parameters were compared for the different treatments. The repeated measures test (General Linear Model) was applied to assess the existence of significant interactions between time and treatment for soil and plant parameters and differences per treatment in leaf and litter parameters were analyzed by a one-way ANOVA. In both cases, these were followed by a Bonferroni test ($p<0.05$ for all comparisons except for the changes in *C. ladanifer* cover $p<0.1$), or by a Games-Howell test whenever homogeneity of variances was not confirmed by a Levene’s test. Differences between ‘total N added’ and ‘measured N’ and between ‘measured N’ and the ‘calculated N’ were analyzed by the t-student test ($p<0.01$). In all cases, preliminary analyses were performed to ensure that there was no violation of the assumptions regarding the tests’ application. SPSS software, version 19.0, was used for all tests.

RESULTS

Soil responses to N additions

In the first spring after the beginning of the experiment (2007), concentrations of N, P, K and Mg in the soil were similar irrespective of the treatment (Table 5.1); the soil was very poor in N and P. Soil analyses in the first and second springs after the beginning of the N additions showed that total N concentrations were not related to the applied N doses (0, 40 or 80 kg N ha^{-1} yr^{-1}) since they were similar for all treatments. In contrast, soil P and Mg had decreased significantly by the second spring irrespective of the treatment. Although not significantly different, the decrease in K concentrations was most pronounced in 40A plots. The K and Mg concentrations were still within the range normally found in agricultural soils, therefore not expected to be limiting. Soil organic matter in springs 2007 and 2008 were similar, while soil pH (in water) decreased in the second spring.

Soil total N, inorganic N, NO_3^- and NH_4^+ concentrations in spring 2007 and 2008 were not related to the treatments. On the first sampling occasion (May 2007), the N-plots had only received 1/3 of the annual dose: 40A and 40AN ≈ 13 kg N ha^{-1} and 80AN ≈ 27 kg N ha^{-1}, but by spring 2008, the fertilized plots had received 4/3 of the annual dose: 40A and 40AN ≈ 53 kg N ha^{-1} and 80AN ≈ 107 kg N ha^{-1}.

Table 5.1 – Soil surface (0-15 cm) properties (N, phosphorus - P, potassium - K, magnesium - Mg - and organic matter concentrations - OM - and pH) at the first (May 2007) and second (April 2008) springs after the beginning of the experiment according to the distinct N additions (Control, 40A, 40AN and 80AN).

Soil properties		N (mg g^{-1})	P* (µg g^{-1})	K (µg g^{-1})	Mg* (µg g^{-1})	OM (mg g^{-1})	pH* (H_2O)
Control	2007	0.9 ± 0.2	8.0 ± 3.1	115 ± 22	126 ± 19	57 ± 1	5.8 ± 0.2
	2008	1.0 ± 0.1	5.0 ± 1.2	98 ± 21	77 ± 13	58 ± 0	5.5 ± 0.1
40A	2007	1.0 ± 0.2	5.3 ± 1.3	178 ± 29	135 ± 11	60 ± 0	6.5 ±0.3
	2008	1.1 ± 0.1	3.3 ± 0.3	118 ± 30	95 ± 7	57 ± 1	5.4 ± 0.2
40AN	2007	1.0 ± 0.1	7.0 ± 1.5	133 ± 3	117 ± 9	64 ± 0	6.3 ± 0
	2008	1.3 ± 0	4.0 ± 0.6	132 ± 22	89 ± 4	60 ± 0	5.6 ± 0.1
80AN	2007	1.2 ± 0.3	7.3 ± 2.8	151 ± 37	137 ± 18	70 ± 1	6.7 ± 0.3
	2008	1.2 ± 0.2	4.7 ± 1.2	141 ± 25	88 ± 1	69 ± 1	5.9 ± 0.2

* refers to statistically significant differences between the two years; There were no significant interactions between treatment and time ($p<0.01$). Values represent the mean (n = 3 experimental plots per treatment) ± SE.

However, when the concentrations of soil inorganic N and NO_3^- were determined in the summer, autumn and winter between the two springs, it could be seen that in autumn (late October 2007) they reflected the N added to the system (Fig. 5.1). NO_3^- was the predominant form of inorganic N in the soil, except in summer (August 2007 - Fig. 5.1). The temporal pattern of soil total inorganic N (Fig. 5.1-b) therefore resembled that of NO_3^- (Fig. 5.1-c), reflecting the N additions in autumn when the three annual N additions had already been applied. In autumn, soils from fertilized plots had more inorganic N than the controls (40A < 40AN < 80AN plots), corresponding to 11, 22 and 32 µg of N g^{-1} of soil dwt more than the control (see materials and methods). After transforming the applied N doses (40 and 80 kg N ha^{-1} yr^{-1}) into soil inorganic N concentrations (see materials and methods - µg N g^{-1} soil dwt), the comparison between the 'total N added' and the 'measured N' showed significant differences for the 40A plots but not the 40AN and 80AN plots. Therefore, the N added to 40AN and 80AN plots appears to have been retained by the system, becoming detectable in soil total inorganic N measured in autumn. In 40A plots the total soil inorganic N measured in autumn was significantly lower than the total N added (Table 5.2).

Cistus ladanifer responses to N additions

At the beginning of the experiment (Spring 2007), *C. ladanifer* plant cover was similar in all treatments (Fig. 5.2-a). However, differences between treatments were observed one year later, in Spring 2008 (Fig. 5.2-a, b). The 80AN treatment caused a decrease in the *C. ladanifer* (Dias *et al.* 2011) plant cover in relation to the control (Fig. 5.2-b).

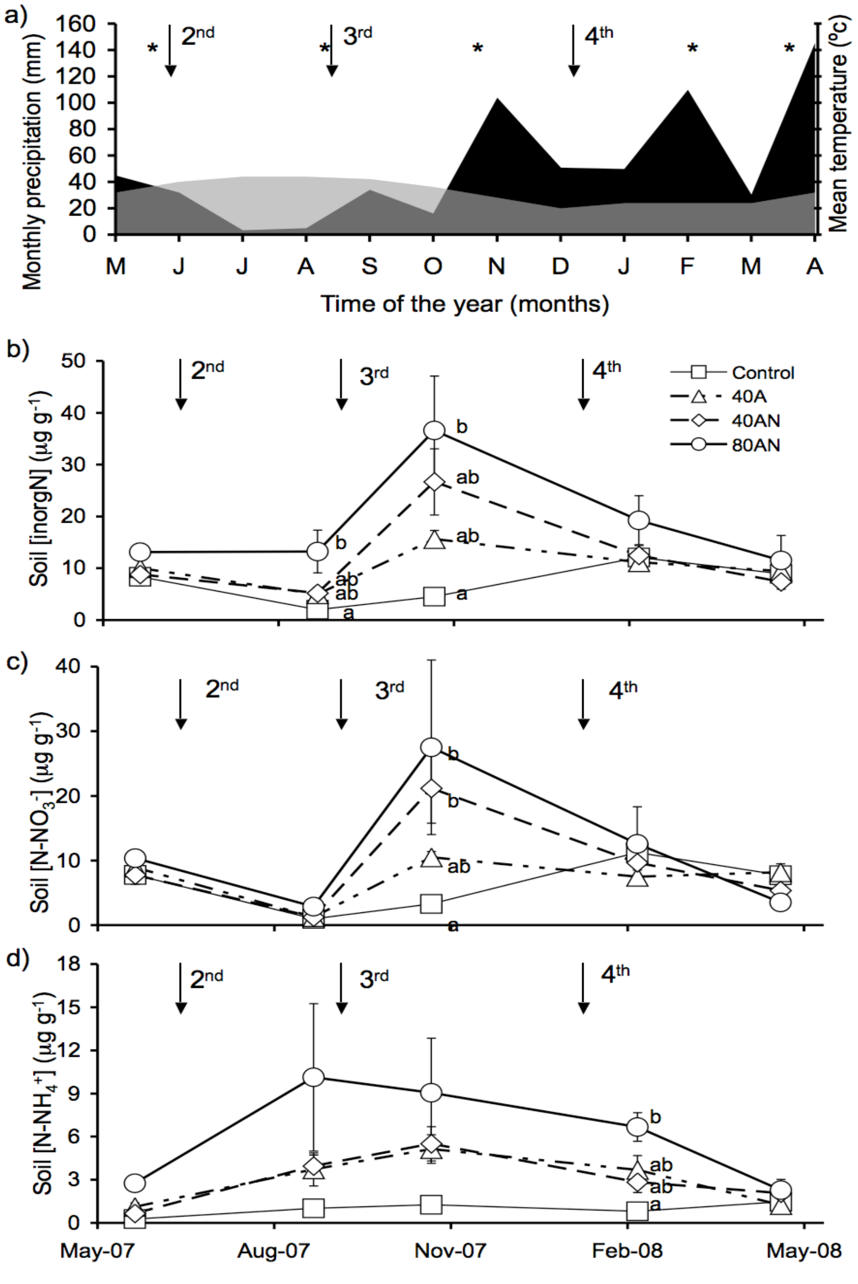

Figure 5.1 – Mean monthly temperature (grey), total monthly precipitation (black) and time of N additions (arrows) and soil sampling (asterisks) from May 2007 to May 2008 (a). Soil inorganic N (b), nitrate (c) and ammonium (d) concentrations according to the distinct N additions (Control, 40A, 40AN, and 80AN). Different letters refer to statistically significant differences between the between treatments (ANOVA $p<0.05$ followed by a Bonferroni test). There were significant interactions between treatment and time ($p<0.01$). Values represent the mean (n = 3 experimental plots per treatment) ± SE.

The N concentration of *C. ladanifer* leaves was also affected by the N form, since only the additions of ammonium nitrate (40AN and 80AN) led to a significant increase in relation to the control (Fig. 5.3-b). In contrast, the N concentration of *C. ladanifer* leaf litter responded to the N dose, with only the litter from the 80AN plots having significantly higher N concentrations than the control. The C/N ratio of *C. ladanifer* leaves also depended significantly on the N form applied (Fig. 5.3-c).

Table 5.2 –Total N added to the soil and the soil inorganic N concentrations in relation to the control determined in autumn (measured N) according to the N additions (40A, 40AN and 80AN).

N addition	Total N added ($\mu g\ g^{-1}$)	Measured N ($\mu g\ g^{-1}$)	
40A	20	11 ± 2	*
40AN	20	22 ± 6	ns
80AN	40	32 ± 10	ns

* refers to significant differences between total N added and measured N (t-student $p<0.01$). Values represent the mean (n = 3 experimental plots per treatment) ± SE.

Components of soil inorganic N concentration in autumn

To understand why soil inorganic N concentration was highest in autumn, the possible components of this N pool were assessed: inorganic N present in the soil in the previous season ([N] summer), the subsequent N addition ([N] addition) and the N present in the litter which was shed during the summer ([N] litter- see materials and methods). The N contained in *C. ladanifer* leaf litter produced in 40A had 2.9 mg more N per gram of litter than the control (data not shown), which corresponded to an addition of 3.5 $\mu g\ N\ g^{-1}$ soil (Fig. 5.4). Similarly, litter produced in 40AN and 80AN corresponded to the addition of 3.1 and 6.4 $\mu g\ N\ g^{-1}$ soil. When the three possible components of the autumn soil inorganic N concentration were combined as 'calculated N', there were no significant differences between this and the 'measured N'.

DISCUSSION

Short-term response of a Mediterranean soil to N enrichment

Soil fertility of the Mediterranean Basin is considered to be moderate to high within Mediterranean-type ecosystems (Cowling *et al.* 1996). However, soils at the experimental site (Table 5.1) had lower N concentration than those reported in other studies in the Mediterranean Basin (e.g. > 0.16%, Gallardo *et al.* 2000; > 0.4%, Ferran, Delitti & Vallejo 2005; > 0.2%, Sardans *et al.* 2008; > 0.2%, Rutigliano *et al.* 2009). The level of extractable P was low in comparison with other Mediterranean Basin soils (> 12 µg g^{-1}, Dumontet *et al.* 1996; > 7 µg g^{-1}, Carreira, Lajtha & Niell 1997; > 7 µg g^{-1}, Ferran, Delitti & Vallejo 2005; > 30 µg g^{-1}, Saura-Mas & Lloret 2009) but comparable with those from Australian and South African Mediterranean-type ecosystems (Milewski 1983; Mitchell, Brown & Jongens-Roberts 1984; Hobbs, Richardson & Davis 1995). Soil organic matter values were within the range observed in other Mediterranean Basin soils (Peñuelas, Filella & Terradas 1999; Gallardo *et al.* 2000; Cruz *et al.* 2008) but so low that these soils are at risk of desertification due to soil erosion (López-Bermúdez & García-Gómez 2006). Soil acidification (Table 5.1) is among the most commonly reported effects of N enrichment and may result from cation loss, aluminium release and nitrification. Although soils at the experimental site were slightly acidic, nitrification occurred since NO_3^- was the predominant N source present in the soil, even in the treatments where N was added as NH_4^+ (Fig. 5.1). However, soil acidification tends to be less intense in soils of the Mediterranean Basin than in other ecosystems, due to their high cation exchange capacity and alkaline sands blown in from North Africa (Ochoa-Hueso *et al.* 2011).

Many studies of soil inorganic N availability have sampled soils in spring, when water and temperature do not limit biological activities (Fig. 5.1-a), and conditions are optimal for plant growth, leading to the conclusion that values obtained could be used as indicators of the system's N availability. However, comparison of soil inorganic N concentrations determined in the two consecutive springs (2007 and 2008) showed no differences between treatments or between years (Fig. 5.1-b) thus constituting a baseline of inorganic N availability for the ecosystem. This may imply that the N added to the system was either lost (NO_3^- leaching, runoff, NH_4^+ volatilization, denitrification) or incorporated in the biota (microbial community and vegetation). If most of the added N had been lost from the system, the peak of soil inorganic N observed in autumn (Fig. 5.1-b) should only reflect (if at all) the previous N addition (summer 2007). However, the observed increase in soil inorganic N in autumn was related not only to the previous N addition, but also with the 'total N added' at that sampling time (Table 5.2). The mild temperatures and the adequate water availability (Fig. 5.1-a) may have stimulated the decomposition of the large litter input derived from the leaf shedding of summer semi deciduous plants (Correia 1988) and the death of annual plants in the summer. In fact, N mineralization has been shown to peak in early autumn in Mediterranean maquis soils (Rutigliano *et al.* 2009; Simões, Madeira & Gazarini 2009). Thus, the reduced biological activity in the summer that allowed inorganic N to accumulate, combined with the summer N fertilization and the high autumn N mineralization (Rutigliano *et al.* 2009), all appear to contribute to the peak of soil inorganic N in autumn (Fig. 5.4). The inorganic N concentrations detected in the soil in autumn suggest that the ecosystem was more efficient in retaining N-NH_4NO_3 than solely N-NH_4^+. This shows that although undetectable as soil inorganic N along the year, most of the added N was used and conserved in the system as would be expected in a nutrient limited ecosystem (Craine 2009). These results clearly show that early autumn is the best time of the year to measure soil inorganic N as an indicator of the N fluxes between the biotic and abiotic compartments. The N incorporation into the biotic compartments is in agreement with the fast responses of the ecosystem structure and function to increased N availability (Dias *et al.* 2011). If a large fraction of the N added to the system is moving through the biotic and abiotic compartments of the ecosystem, then large impacts on ecosystem processes can be expected (e.g. Emmett 2007; Bobbink *et al.* 2010).

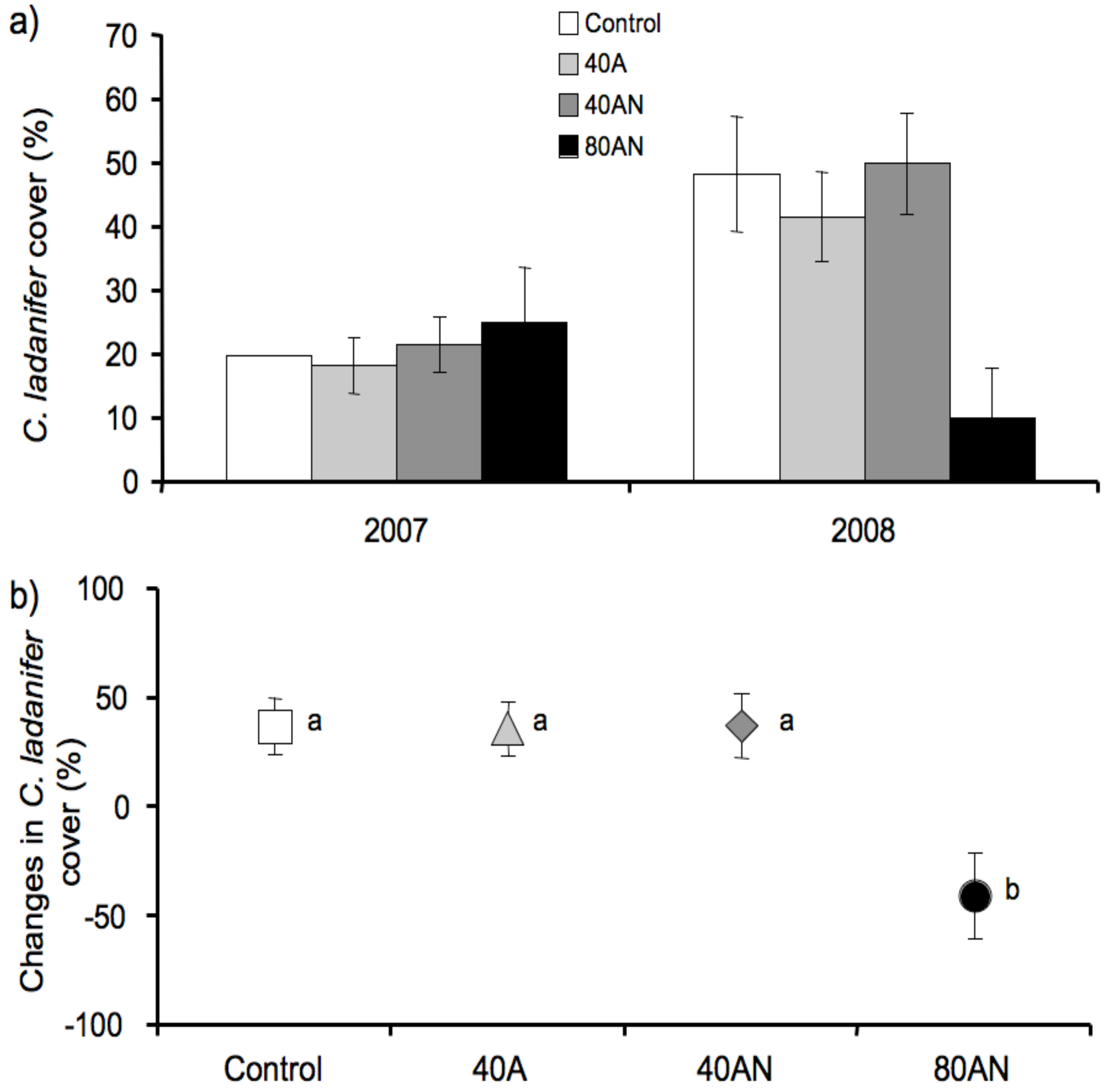

Figure 5.2 – *C. ladanifer* plant cover (a) and its response to the distinct N additions (Control, 40A, 40AN, and 80AN). Plant cover (%) was assessed on the first and second springs after the beginning of the N fertilizations, which allowed calculating its changes over time (see material and methods). Different letters refers to statistically significant differences between the two springs (ANOVA $p<0.1$ followed by a Bonferroni test). There were no significant interactions between treatment and time ($p<0.01$). Values represent the mean (n = 3 experimental plots per treatment) ± SE.

Short-term response of the plant community to N additions

The cover of *C. ladanifer* (a slow growing conservative species - Dias *et al.* 2011) increased under all treatments except 80AN (Fig. 5.2). In nutrient limited ecosystems, N additions tend to induce changes in the plant community promoting a decrease in the plant cover of the slow growing species and an increase in that of the fast growing species.

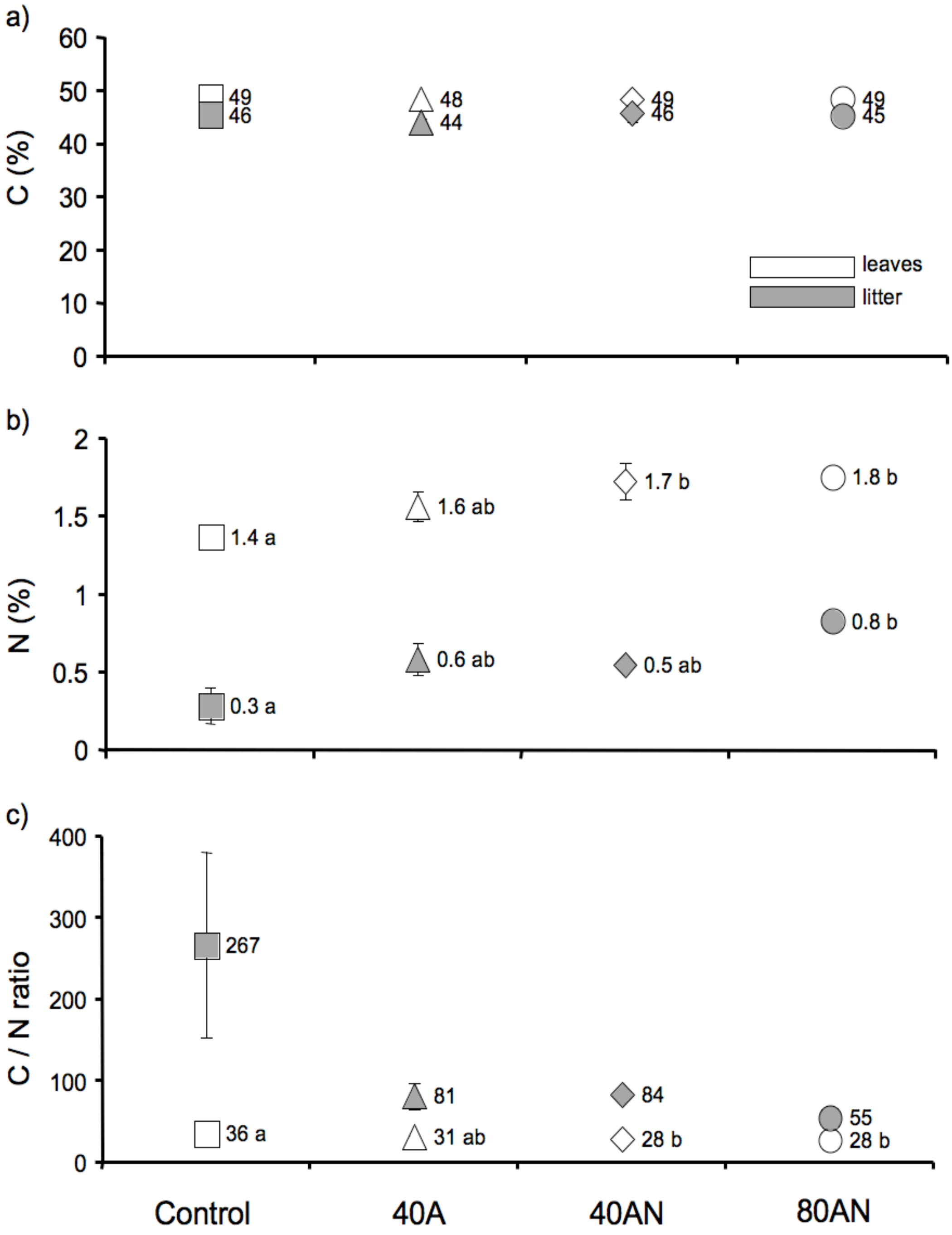

Figure 5.3 – C and N concentrations and the C/N ratio of leaves and leaf litter of *C. ladanifer* in response to the distinct N additions (Control, 40A, 40AN and 80AN). Different letters refers to statistically significant differences between treatments (ANOVA *p*<0.05 followed by a Bonferroni test). Symbols and values represent the mean (n = 3 experimental plots per treatment) ± SE.

Under conditions of nutrient limitation, the efficiency of a plant using limiting nutrients depends not only on the uptake efficiency but also on the retention time (Emmett 2007). Accordingly, the N-driven response of the N concentration in *C. ladanifer*'s leaves points to this species' efficient N uptake (Fig. 5.3-b) and was in agreement with the use of plant tissue N concentration as a N accumulation indicator (Sutton *et al.* 2005). However, if plants were only N limited, the extra N would have been used to produce biomass so that the N content would have increased but not the N concentration. Thus, increased leaf N concentration together with decreased soil P concentration (Table 5.1) may indicate N and P co-limitation (Bishop *et al.* 2010). On the other hand, plants characteristic of nutrient-poor ecosystems have efficient ways of reabsorbing nutrients from old leaves (Craine 2009), depriving the litter of N and giving rise to high C/N ratios in the litter (litter from control plots – Fig. 5.3-c).
As the N limitation was alleviated by the N additions, the N resorption efficiency from the old leaves may have decreased (Kobe, Lepczyk & Iyer 2005), resulting in increased N concentration in the litter (Fig. 5.3-b), i.e., improved litter quality (Witkowski 1989; Vourlitis, Pasquini & Mustard 2009). The overall N-driven response of leaf and litter N concentrations and the C/N ratio was similar to that observed in two other Mediterranean-type ecosystems with different plant communities: one dominated by summer semi deciduous (coastal sage scrub), the other by evergreen sclerophylls (chaparral - Vourlitis, Pasquini & Zorba 2007; Vourlitis & Pasquini 2009). Therefore, litter N concentration should be a good indicator of the N availability in Mediterranean-type ecosystems.

N cycling through biotic and abiotic compartments

Combined analysis of the soil inorganic N concentrations (Fig. 5.1) and the changes in the N concentration (and C/N ratio) of *C. ladanifer* leaves and litter (Fig. 5.3) suggest that under these Mediterranean conditions, the N cycling through biotic and abiotic compartments allowed soil inorganic N to reflect cumulative N additions in autumn (Fig. 5.1-b). This suggests that the N added to the system is rapidly taken up by the biota and it is only after leaf shedding in summer and litter decomposition in autumn that the added N is detectable in the soil. The fact that there were no differences between the soil inorganic N in 40AN and 80AN plots and the respective total N added (Table 5.2) can only be explained by efficient internal cycling of the previously added N within the ecosystem. If the soil sampling had occurred in late autumn it is likely that the peak in soil inorganic N availability would be missed, since the N concentration in *C. ladanifer* litter increases through the autumn and winter (Simões, Madeira & Gazarini 2009). The retention of N by litter has also been shown in other Mediterranean communities (e.g. Fioretto *et al.* 2005; Holub & Lajtha 2004), and could help explain the sharp decline in soil inorganic N availability in winter (Fig. 5.1-b).

An inverse relationship between litter C/N ratio and decomposition rate (Bosatta & Staaf 1982; Taylor, Parkinson & Parsons 1989) has also been established for Mediterranean plant species (Rutigliano *et al.* 2009), so that the N-driven lower litter C/N ratio (Fig. 5.3-c) may have stimulated decomposition. Also, the decomposition of higher quality litter has been shown to result in a rapid (~2 months) increase of inorganic N (Sirulnik *et al.* 2007). Litter decomposition, together with the soil inorganic N concentration in the summer and the following N addition contributed to the observed autumn inorganic N (Fig. 5.4), explaining the inorganic N concentrations measured in autumn. Litter decomposition may be an important component of the soil inorganic N measured in autumn. Data suggest that living plants and litter have a crucial role in preventing N losses from Mediterranean maquis.

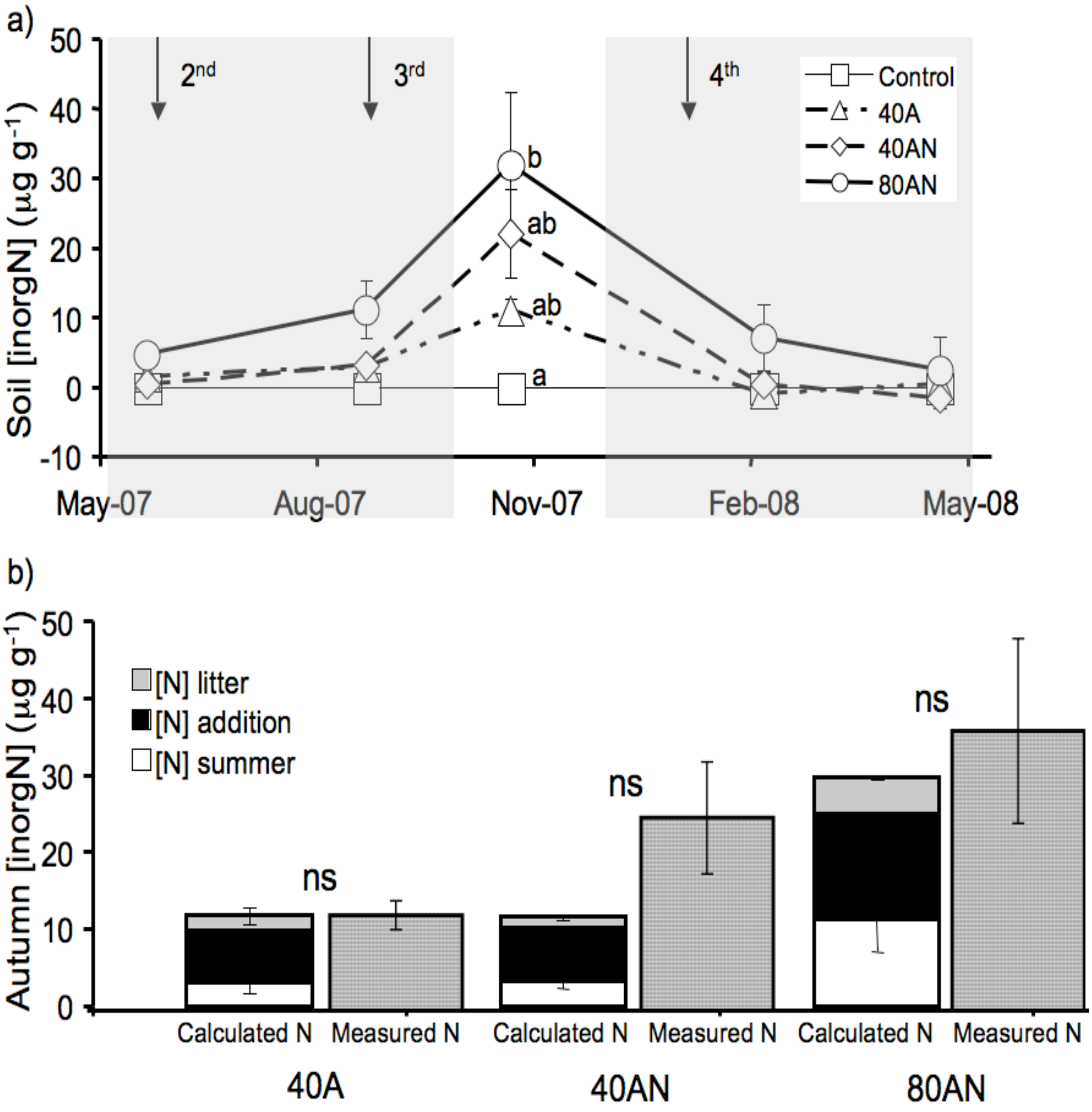

Figure 5.4 – Soil inorganic N concentration in relation to the control along the experimental period in response to distinct N additions (a – see material and methods) and comparison between the ‘calculated N’ (stacked columns – see material and methods) and the ‘measured N’ (dashed columns) concentrations of soil inorganic N in autumn and according to the N additions (40A, 40AN and 80AN – b). There were no significant differences between ‘calculated N’ and ‘measured N’ (t-student $p<0.001$). Values represent the mean (n = 3 experimental plots per treatment) ± SE.

CONCLUSIONS

The results of this study suggest that most of the added inorganic N was retained in this N-limited Mediterranean maquis, affecting its structure and function. Also, the form of the added N influenced the overall N retention during the study period: NH_4^+ additions resulted in lower N recovery from the soil than the additions of NH_4NO_3. The added N was retained in the biotic compartment during the growth season, then returned to the soil after the dry period through litter decomposition (autumn). The data highlight the sensitivity of Mediterranean Basin ecosystems, which constitute an important worldwide biodiversity hotspot, to N deposition. Thus, the present N-manipulation study points to the role of N availability as a driving force for biodiversity changes, especially in Natura 2000 sites such as this manipulation site. However, caution should be used when extrapolating data to other Mediterranean-type ecosystems, which may differ in soil fertility, and even to other Mediterranean habitats that differ in plant community. Finally, this is the first integrated field study of how European Mediterranean ecosystems retain N enrichment of different forms and doses, however longer-term studies are needed to explore the generality of these observations.

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Chapter 6

N-driven changes in plant community affect leaf litter traits and delay organic matter decomposition in a Mediterranean maquis

This chapter was submitted to:
Dias, T., Oakley, S., Alarcón-Gutiérrez, E., Ziarelli, F., Trindade, H., Martins-Loução, M.A., Sheppard, L., Ostle, N. & Cruz C. (2012) N-driven changes in plant community affect leaf litter traits and delay organic matter decomposition in a Mediterranean maquis**.** *Plos One*, submitted.

N-driven changes in plant community affect leaf litter traits and delay organic matter decomposition in a Mediterranean maquis

ABSTRACT

Background: Organic matter (OM) decomposition is controlled by climate, litter quality and soil microorganisms. Nitrogen (N) availability also influences decomposition and nutrient-cycling dynamics. However, reported N effects on decomposition are controversial and most studies have only addressed decomposition of individual plant species grown under high N availability.

Methodology/Principal Findings: We accounted for the integrated effects of N enrichment on litter decomposability taking into consideration not just individual species but N-driven changes in plant community. We collected one type of soil and four types of leaf litter (from four N addition treatments) from a N-manipulation field experiment for a laboratory controlled decomposition study. Distinct leaf litter traits were quantified (N and lignin concentration and C/N and lignin/N ratios) and correlated with OM decomposition, soil microbial PLFAs and CO_2 and N_2O emissions. The leaf litter consisted mostly of leaves from summer semi-deciduous shrubs. However, the high N treatment (80 kg N ha^{-1} yr^{-1}) had twice the amount of evergreen sclerophyll leaf litter and both higher lignin and N concentrations giving lower C/N and lignin/N ratios than the control. As a result, OM decomposition in the microcosms containing litter with both higher N and lignin content was delayed in relation to the 40 kg N ha^{-1} yr^{-1} treatments. Similarly, the microcosms containing litter from the higher N dose showed lower values of soil microbial PLFAs, CO_2 and N_2O net fluxes than those from the 40 kg N ha^{-1} yr^{-1} treatments.

Conclusions/Significance: Our results suggest that at the ecosystem level, N-driven changes in plant community affected leaf litter traits (e.g. increased litter lignin and N content and decreased lignin/N ratio), which were powerful determinants of litter decomposition rates. Results demonstrate that increasing N availability in this nutrient poor Mediterranean maquis will select plants with litter traits that delay decomposition and potentially increase soil OM accumulation.

Keywords: Evergreen sclerophylls; Summer semi-deciduous; Lignin content; Nitrogen content; Microcosm; Plant functional groups; Soil microbial community

INTRODUCTION

The balance between vegetation inputs and organic matter (OM) decomposition determines the size of the soil OM pools (Baer *et al.* 2010) and controls nutrient cycling in terrestrial ecosystems (Knorr, Frey & Curtis 2005). In its turn, the rate of litter decomposition is controlled by climate, litter quality (Fioretto *et al.* 1998, 2001; Alarcón-Gutiérrez *et al.* 2008; Austin & Ballaré 2010) and soil microorganisms (Fioretto *et al.* 2001). However, nitrogen (N) availability can also influence decomposition and nutrient-cycling dynamics (Knorr, Frey & Curtis 2005; Liu *et al.* 2010) with potential consequences for OM decomposition and accumulation.

Increased N availability can change plant community litter traits directly and/or indirectly. N enrichment directly increases litter N concentration and decreases C/N ratio (Vourlitis, Pasquini & Mustard 2009; Dias *et al.* 2012) making litter more readily decomposable. Indirectly, N enrichment is a powerful driver of plant diversity changes (Sala *et al.* 2000), with alterations in plant traits (e.g. species-specific N and lignin concentrations) strongly influencing litter inputs and decomposition rates (Cornwell *et al.* 2008).

Knorr, Frey & Curtis (2005) conducted a meta-analysis of empirical studies to examine the effects of N enrichment on litter decomposition concluding that N enrichment may increase, decrease or have no effect on litter decomposition depending on fertilization rate, site-specific ambient N-deposition level, and litter quality. However, none of the analysed studies had in consideration the effect of increased N availability on the changes on local plant biodiversity and consequently on the changes of leaf litter traits.

In this work we aimed at assessing the effects of increased N availability on leaf litter decomposability at the individual species and plant community levels. In order to highlight the effects of each component, the study was performed in a N-poor Mediterranean ecosystem very responsive to N availability (Dias *et al.* 2011a, 2012) and with marked differences in the leaf traits of the main plant functional groups (summer semi-deciduous and evergreen sclerophyllous - Correia & Catarino 1994).

In particular, in the semi-natural Mediterranean Basin ecosystems, where N deposition is predicted to increase three fold by 2050 (Galloway *et al.* 2004; Phoenix *et al.* 2006), the dynamics of litter decomposition need to be understood in order to inform management of these biodiversity hotspots (Phoenix *et al.* 2006). The few studies made on the effects of increased N on decomposition of Mediterranean litter (Sirulnik *et al.* 2007; Alarcón-Gutiérrez *et al.* 2008; Kazakou *et al.* 2009) suggest that N enrichment may enable Mediterranean-type ecosystems to retain more soil OM and act as a C sink or source (Ochoa-Hueso *et al.* 2011).

In our approach we used soil (one type) and litter (from four N addition treatments - Control, 40 kg NH_4^+-N ha^{-1} yr^{-1} and 40 and 80 kg NH_4NO_3-N ha^{-1} yr^{-1}) from an ongoing N-manipulation field study in a Mediterranean Basin maquis (Portugal) where increased N concentration (and decreased C/N ratio) of the dominant plant species (Dias *et al*. 2012) and changes in plant community (Dias *et al*. 2011a) had already been observed. Our working hypothesis is that N-driven plant community changes that cause an increase in litter lignin concentration may result in suppressed litter decomposition and consequently, in soil OM accumulation.

MATERIALS AND METHODS

N-manipulation field study and experimental design

The study site (38º 29' N - 9º 01' W) is located in Serra da Arrábida in the Arrábida Natural Park, Portugal (a Natura 2000 site - PTCON0010 Arrábida/Espichel) within a sub-humid thermomediterranean bioclimatic zone (Rivas-Martínez, Penas & Díaz 2004). Estimated background N deposition is 5.2 kg ha^{-1} yr^{-1} (2.9 kg NO_x + 2.3 kg NH_y - http://webdab.emep.int/Unified_Model_Results/AN/).

The N-addition field experiment study is sited on a southeast-facing slope (5%) at 130 m a.s.l. Soil is skeletal (15 cm depth) with a bulk density of 1.3 g cm^{-3}. Silt dominates (50%), while sand and clay contents are 32% and 18%, respectively (i.e. a silt-sand-loam texture). The vegetation consists of a dense maquis (Eunis class F5.2 – Mediterranean maquis), developed after a fire event (summer 2003) four years before the first experimental N addition. The vegetation is dominated by summer semi-deciduous species, exhibiting leaf dimorphism, shedding an important fraction of leaves and twigs in the summer (Cruz *et al*. 2008). These species are common in open and disturbed stands, but through succession they are progressively eliminated under the canopies of evergreen sclerophylls characteristic of the late successional stages (Correia & Catarino 1994). Sclerophyll leaves are long-lived, consistent, hard and coriaceous (Mooney *et al*. 1983). At the time of sampling for this study, the plant community was dominated by *Cistus ladanifer* L.; other abundant plant species include *Erica scoparia* L., *Calluna vulgaris* (L.) Hull, *Genista triacanthos* Brot., *Ulex densus* Welw. ex Webb, *Dittrichia viscosa* L., *Quercus coccifera* L., *Pistacea lentiscus* L. and *Myrtus communis* L. Herbaceous species comprise ≈ 10% of the total plant cover (Dias *et al*. 2011a).

The field N-addition experiment commenced in 2007 with the addition of 40 and 80 kg N ha^{-1} yr^{-1} in the form of NH_4NO_3 (40AN and 80AN, respectively) and 40 kg N ha^{-1} yr^{-1} as a 1:1 mixture of NH_4Cl and $(NH_4)_2SO_4$ (40A). Control plots have been maintained without N-addition. Beginning in January 2007, the dry fertiliser was spread evenly, by hand, in three equal applications in winter, spring and summer. Each treatment was replicated with 3 plots each of 400 m^2. In order to restrict boundary effects and dilution processes, all measurements, analyses and sample collection were performed within an internal 100 m^2

square. To prevent N ‘contamination’ through runoff from the N-plots, the experimental plots were distributed in three rows along the 5% slope, with the controls being located in the top row (for further details please see Dias *et al*. 2011a, 2012).

Soil and leaf litter sampling

Soil and leaf litter were sampled in August 2009, prior to the summer N addition, and after two and half years of N addition treatments (addition of 40A and 40AN corresponded to a cumulative N addition of ~ 107 kg N while addition of 80AN corresponded to ~ 213 kg N). Leaf litter was collected from each experimental plot (no N addition - L-C; 40A - L-40A; 40AN - L-40AN; and 80AN - L-80AN). Intact leaf litter from the uppermost layer (0–2 cm above the soil surface) was hand collected from four points per experimental plot, bulked (4 samples from each plot x 3 plots per treatment) air-dried (35ºC for two weeks) and analyzed for its plant group composition (Fig. 6.1) and total C, N, P, potassium, calcium, magnesium, sulphur, sodium and manganese (Table 6.2). Three sub samples of litter per treatment were stored at -20ºC for lignin analysis (Table 6.1 and Supplementary data Table 6.1). Soil was collected (50 cm diameter and 15 cm depth) from all control plots, sieved (2mm), bulked (4 samples from each plot x 3 control plots) and air-dried (35ºC for six weeks).

Litter decomposition microcosm experimental design

Each microcosm consisted of 500 g of dry soil from the control field plots (no N addition) mixed with 6 g dwt of roughly ground litter (pestle and mortar, 1mm), as described by Madritch & Hunter (2003). The microcosm (combinations of soil + different litters) treatments were: 1) soil only (M-nL); 2) soil + litter from Control plots (M-C); 3) soil + litter from the field addition of 40 kg NH_4^+-N ha^{-1} yr^{-1} (M-40A); 4) soil + litter from the field addition of 40 kg NH_4NO_3-N ha^{-1} yr^{-1} (M-40AN); and 5) soil + litter from the field addition of 80 kg NH_4NO_3-N ha^{-1} yr^{-1} (M-80AN). The microcosms treatments were analyzed for pH (H_2O), OM, total C and N, extractable P, potassium and magnesium (Table 6.3) and incubated under controlled conditions with a photoperiod of 8h day: 16 h night at 25ºC and 40-60% of water filled pore space (determined gravimetrically) for three months (Madritch & Hunter 2003). Each microcosm treatment was replicated 3 times and microcosms were randomly distributed in the incubator.

Chemical analysis

Frozen (-20ºC) bulk litter (collected from the N-manipulation field experiment) and soil microcosms samples were analyzed for their concentration in lignin and aromaticity (Table 6.1 and Supplementary data Table 6.1) by Cross-Polarization Magic-Angle Spinning ^{13}C Nuclear Magnetic Resonance (^{13}C CPMAS NMR), using a Bruker Avance III 400 MHz spectrometer and a commercial two-channel, 4-mm Bruker probe head. Dried samples were ground (particle size 0.3 mm) and analyzed as described by Alarcón-Gutiérrez *et al*. (2009). Based on previous studies (see Alarcón-Gutiérrez *et al*. 2009) it was possible to characterize the signals of the ^{13}C CPMAS NMR spectra of the distinct litter types. By the deconvolution software DmFIT, the aromaticity index (AI) was calculated as AI = 100 [A (110-160 ppm) / A (0-160 ppm)] (Lorenz *et al*. 2006), while lignin concentration was estimated using the following equation established by Haw, Maciel & Schroeder (1984): % lignin = (100%)(183/9.92) *I*´lig/(183/9.92) *I*´lig + (162/6) *I*´carb).

Dried (35ºC) litter samples were analyzed for total C, N, P, potassium (K), calcium (Ca), magnesium (Mg), sulphur (S), sodium (Na) and manganese (Mn) as described by Fioretto *et al*. (2001) and Fioretto, Papa & Fuggi (2003). At the beginning of the experiment, soil microcosms, i.e., soil plus litter, were analyzed for pH (H_2O), OM and concentrations of total C, N, extractable P (ammonium lactate 0.1 M), K (and acetic acid 0.4 M, pH 3.65–3.75.) and Mg (ammonium acetate 1 M, pH 7) were determined as described by Dias *et al*. (2011a, 2012). At the end of the experiment microcosms were analyzed for pH (H_2O), total C and N concentration, nitrate (NO_3^--N - Matsumura & Witjaksono 1999), ammonium (NH_4^+-N - Cruz & Martins-Loução 2000) and phosphate (PO_4^{3-}-P - Fiske & Subarow 1925) as described in Dias *et al*. (2011a, 2012). Soil inorganic N (inorgN) was the sum of the water extracted NH_4^+ and NO_3^-. NO_3^-, NH_4^+, inorgN and PO_4^{3-} were expressed as µg N or P per gram of dry soil.

Soil microbial PLFA extraction and quantification

Soil was taken from the microcosms at the end of the incubation and freeze-dried at -20°C. Soil microbial composition was determined on 1 g of freeze-dried soil, using phospholipid fatty acid (PLFA) analysis with the Bligh & Dyer method (1959), adapted by White *et al*. (1979) and described in Treonis *et al*. (2004). The lipid extract was fractioned on silicic acid columns, into different polarity classes by sequential elution with chloroform, acetone and methanol. A C19:0 internal standard was added before the methylation step and used for calculating retention times and quantification. Samples were analyzed using a gas chromatogram (Agilent 5890GC) equipped with a flame ionisation detector and capillary column (Varian CP Sil 5 CB) using the following oven conditions: initial 50°C for 5 minutes, followed by a ramping of 10°C/minute up to 270°C, then 3°C/minute up to 320°C and held at 320°C for 10 minutes. Fatty acids were identified by retention time by comparison with previously identified samples and by GC-MS on an Agilent 6890GC connected to an Agilent 5973 Mass Selective Detector. Total PLFA concentration was calculated using all identified PLFA's.

CO_2 and N_2O fluxes

At the end of the experiment (3 months), 100 g FW samples of soil at 60% of water filled pore space from each microcosm (three per treatment) were placed into sealed perspex columns (20 cm height x 5 cm diameter) in a growth chamber, in the dark at 25ºC as described by Sánchez-Martín *et al.* (2008) in order to measure CO_2 and N_2O fluxes. CO_2 and N_2O concentrations were measured daily for three days, directly through a sampling port fitted into the air outlet tap, using a trace gas analyzer (TGA - 1412 Photoacoustic Field Gas-Monitor; Innova AirTech Instruments, Ballerup, Denmark) as described by Fangueiro *et al.* (2008). The CO_2 and N_2O fluxes were calculated as the difference between the concentrations in the airflow before closing the columns and 30 min after (Sánchez-Martín *et al.* 2008).

Calculations and statistical analysis

The variation in the concentration of soil OM (ΔOM - Fig. 6.2-b) and of total C (ΔC – Fig. 6.3) and N (ΔN) were calculated as the percentage of variation in the concentration of the given variable during the experiment, in relation to its initial concentration:

$$\Delta \text{ parameter } (\%) = \frac{(\%\text{ Parameter}_{\text{end}} - \%\text{ Parameter}_{\text{beginning}})}{\%\text{ Parameter}_{\text{beginning}}} \times 100$$

Summary statistics of litter, microcosms and gases parameters were compared for the different treatments. A two-way ANOVA was used to assess the existence of significant interactions between time and treatment for soil OM, total C and N, pH and gas fluxes. Differences per treatment in litter and microcosms parameters were analyzed by a one-way ANOVA, followed by a Bonferroni test ($p<0.05$), or by a Games-Howell test whenever homogeneity of variances was not confirmed by the Levene’s test. Linear correlations between the indicators of soil OM decomposition (ΔOM and ΔC and CO_2 and N_2O fluxes) and factors influencing soil OM decomposition were examined using Pearson’s correlations. In all cases, preliminary analyses were performed using SPSS software, version 19.0.

RESULTS

Ecological N-driven trade-offs in litter traits

Leaf litter produced by the standing plant community was mainly composed of summer semi-deciduous leaves (mostly *Cistus ladanifer* and *Cistus salvifolius*) with a small contribution from evergreen sclerophylls (e.g. *Myrtus communis*, *Quercus coccifera*, *Pistacea lentiscus* - Fig. 6.1). However, leaf litter produced under the higher N dose (L-80AN) changed the composition and proportion of the two plant functional groups and introduced a new one, graminoids. Summer semi-deciduous species decreased by approximately 30 %, evergreen sclerophylls doubled and grasses (e.g. *Brachypodium phoenicoides*, *Gastridium ventricosum*, etc – data not shown) 'appeared' (~10%). The leaf litter produced by the 40A and 40AN field N addition treatments was similar to that produced by the Control (L-C).

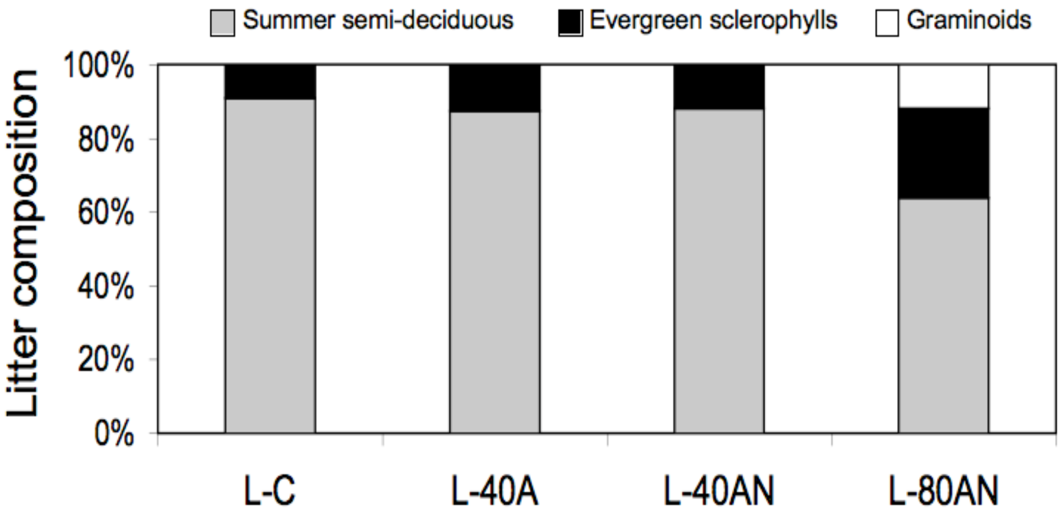

Figure 6.1 - Effect of the field N additions (no N addition, 40A, 40AN and 80AN) on the respective litters' (L-C, L-40A, L-40AN and L-80AN) composition. Litters were collected at the study site in summer 2009 (two and half years of N additions). Stacked bars represent the percentage of the three plant groups (summer semi-deciduous, evergreen sclerophylls and graminoids) to the bulk sample used in the decomposition experiment.

Comparatively to the other treatments, the intensity of the *O*-alkyl-C region (45-110 ppm - polysaccharides) decreased in L-80AN litter (Supplementary data Table 6.1) indicating a reduction of these molecules; other important regions where intensity increased in the L-80AN were the aromatic, phenolic and carboxyl regions. Changes in the litter lignin concentration, aromaticity and the humification index (alkyl C to *O*-alkyl C ratio – Table 6.1), revealed differences in the quality of L-80AN compared with L-C, L-40A and L-40AN. Given that the microcosms' C concentrations were well below the detection limit of the ^{13}C CPMAS NMR procedure (data not shown), it was not possible to monitor the chemical changes that occurred during the decomposition experiment.

Irrespective of the N additions, the litter produced by the standing maquis vegetation had low concentrations of N and P (Table 6.2). However, two and half years of N additions (40A, 40AN and 80AN) did affect litter traits: L-80AN had a higher N concentration than the Control (L-C), and the ammonium nitrate treatments (L-40AN and L-80AN) presented a lower C/N ratio than the Control (L-C). The remaining analysed nutrients (as well as some of their ratios) were not significantly affected by the N treatments. At the beginning of the decomposition experiment, the microcosms had similar total N concentrations although their C/N ratios differed: M-nL had the lowest C/N ratio while M-C, M-40A and M-40AN had significantly higher C/N ratios and M-80AN had an intermediate C/N ratio (Table 6.3).

Table 6.1 – Ratios and the concentration of lignin and aromaticity of the different types of litter (L-C, L-40A, L-40AN and L-80AN) derived from the ^{13}C CPMAS NMR spectra (Supplementary data Table 6.1). Litters were collected at the study site in August 2009 (two and half years of N additions). Different letters refer to statistically significant differences between treatments (ANOVA *p*<0.05 followed by a Bonferroni test) and are shown in bold. Values represent the mean (n = 3) ± SE.

Ratios	L-C	L-40A	L-40AN	L-80AN
Alkyl C / *O*-alkyl C	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0
Alkyl C / Carboxyl C	3.6 ± 0.3	3.2 ± 0.3	3.3 ± 0.3	3.3 ± 0.3
O-alkyl C /Aromatic C	**4.8 ± 0.3 ^{a}**	**4.2 ± 0.3 ^{ab}**	**4.6 ± 0.3 ^{a}**	**3.6 ± 0.3 ^{b}**
Concentration (%)				
Lignin	**25.7 ± 0.9 ^{a}**	**27.6 ± 0.9 ^{ab}**	**26.1 ± 1.3 ^{a}**	**31.1 ± 1.7 ^{b}**
Aromaticity	**20.3 ± 0.7 ^{a}**	**21.6 ± 0.7 ^{ab}**	**20.6 ± 0.9 ^{a}**	**22.0 ± 1.1 ^{b}**

Consequences of the altered litter traits on organic matter decomposition

At the end of the microcosm experiment, the concentrations of OM and total C and N in the microcosms were remeasured to determine the ΔOM (a measure of soil OM decomposition - Fig. 6.2), ΔC (Fig. 6.3) and ΔN (data not shown).

Table 6.2 – Effect of the distinct N additions (no N addition, 40A, 40AN and 80AN) on the respective litters' (L-C, L-40A, L-40AN and L-80AN) nutrients concentration. Litters were collected at the study site in August 2009. Different letters refer to statistically significant differences between treatments (ANOVA *p*<0.05 followed by a Bonferroni test) and are shown in bold. Values represent the mean (n = 3 experimental plots per treatment) ± SE.

	L-C	L-40A	L-40AN	L-80AN
C (mg g^{-1})	453.7 ± 8.9	454.5 ± 3.6	455.3 ± 10.3	443.7 ± 7.0
N (mg g^{-1})	**5.7 ± 0.3 ^{a}**	**7.0 ± 0.6 ^{a}**	**8.0 ± 0.6 ^{ab}**	**10 ± 0.6 ^{b}**
P (mg g^{-1})	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
K (mg g^{-1})	1.0 ± 0.1	1.2 ± 0.2	1.4 ± 0.1	1.3 ± 0.1
Ca (mg g^{-1})	10.8 ± 1.6	12.8 ± 1.2	12.4 ± 1.0	13.6 ± 1.1
Mg (mg g^{-1})	1.1 ± 0.1	0.9 ± 0.1	1.3 ± 0.1	1.1 ± 0.1
S (mg g^{-1})	0.8 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1
Na (μg g^{-1})	236.0 ± 5.0	289.0 ± 55.6	253.7 ± 20.2	366.3 ± 88.5
Mn (μg g^{-1})	411.0 ± 33.1	314.7 ± 78.0	418.7 ± 64.8	336.3 ± 67.9
C/N ratio	**85.2 ± 2.8 ^{a}**	**66.6 ± 6.5 ^{ab}**	**58.8 ± 5.5 ^{b}**	**44.3 ± 2.5 ^{b}**
N/P ratio	23.6 ± 2.8	30.4 ± 3.2	26.3 ± 2.6	39.1 ± 5.7

ΔOM and ΔC followed a similar pattern and therefore were strongly and positively correlated (Table 6.4). M-40A and M-40AN lost ~ 20% of its initial OM and C concentrations, thus resulting in the highest ΔOM, i.e., the highest decomposition rate (Fig. 6.2-b). M-80AN resulted in intermediate ΔOM (~12% of initial OM concentration) and of total C while M-nL and M-C showed the lowest ΔOM (<5% of initial OM concentration), i.e., the lowest decomposition rate. There was a significant correlation between litter's lignin/N ratio and microcosms' ΔC when excluding the data points from the microcosms incubated with litter produced solely with the addition of ammonium (M-40A - Fig. 6.3). There was no significant ΔN during the experiment (data not shown).

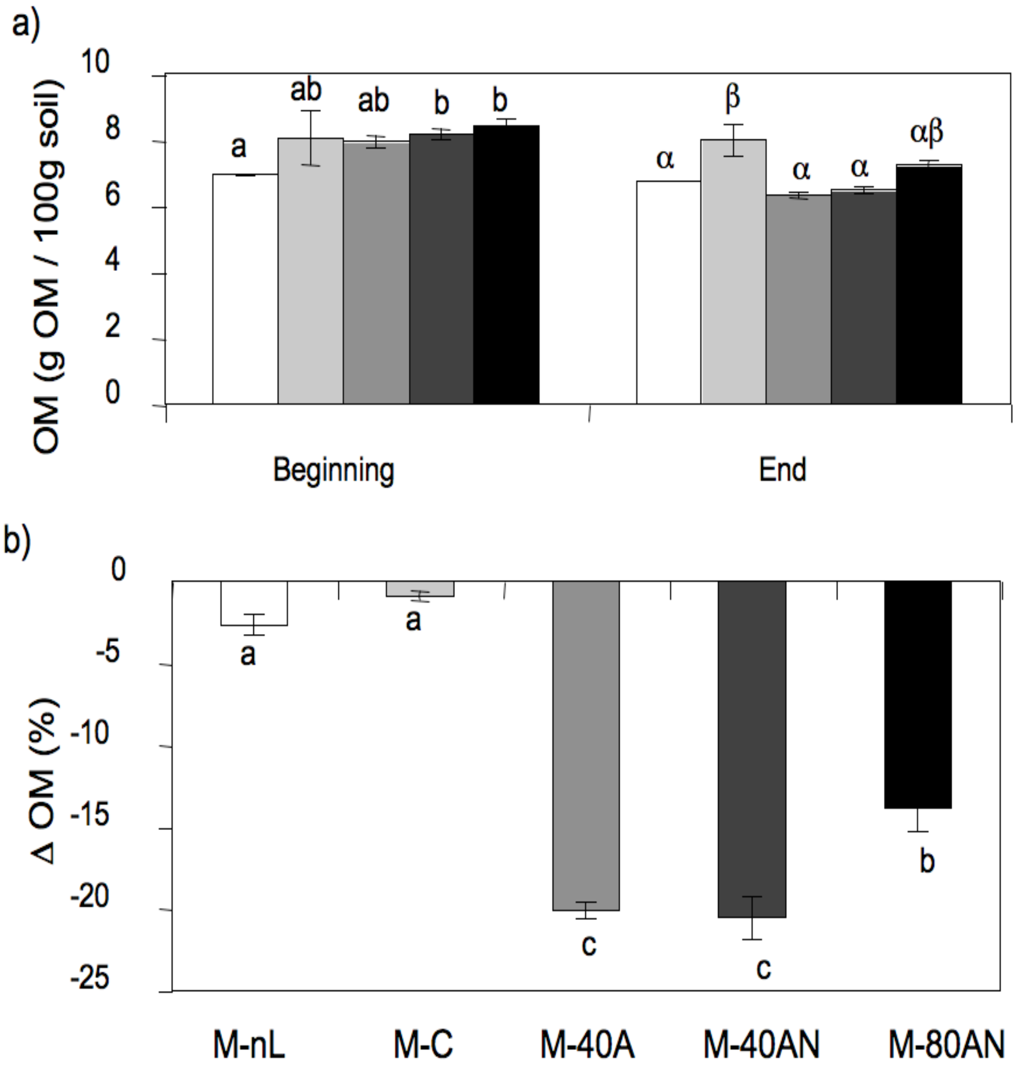

Figure 6.2 – Concentration of OM determined in the microcosms (M-nL, M-C, M-40A, M-40AN and M-80AN) at the beginning and at the end of the experiment [a - there were significant interactions between time and treatments (p<0.05)]; and the corresponding ΔOM (%) in relation to the beginning of the incubation (b). Different letters refer to significant differences between treatments (ANOVA p<0.05 followed by a Bonferroni test). Bars represent the mean (n = 3 microcosms per treatment) ± SE.

Addition of different litters significantly altered the soil microbial community structure (Fig. 6.4 and Supplementary data Table 6.2) and activity of the soil (Fig 6.6). In general, the analyzed

PLFAs were more abundant in M-40A and/or M-40AN, followed by M-C, M-nL and were depressed by the litter from the high N treatment (M-80AN). The exceptions were C16:1 (n-7), C17:1 (n-8), C18:1 (n-5) and C19:1 for which there were no significant differences (Supplementary data Table 6.2). Given that the majority of the analyzed PLFAs followed a similar pattern, it was not possible to identify a biomarker for changes in litter traits. Moreover, total microbial PLFAs followed the same pattern as individual PLFAs (Fig. 6.4-a) with bacteria dominating the microcosms' microbial communities. The pattern of total bacterial PLFAs (Fig. 6.4-b) and of total fungal PLFAs (Fig. 6.4-c) was similar to that of total PLFAs (Fig. 6.4-a). The availability of N per unit of soil PLFAs also distinguished between M-80AN and the other treatments (Fig. 6.5). Microbial community structure (total PLFAs and total bacterial and fungal PLFAs) was strongly correlated ($r \geq 0.7$) with CO_2 and N_2O fluxes between microcosms' soil and atmosphere (Table 6.4).

Table 6.3 – Characterization of the distinct soil microcosms (M-nL, M-C, M-40A, M-40AN and M-80AN) at the beginning of the experiment; pH (H_2O); OM, organic matter; C, total carbon; total N, nitrogen; P, extractable phosphorus; K, extractable potassium; Mg, extractable magnesium; C/N ratio; N/P ratio; and C/P ratio. Different letters refer to statistically significant differences between treatments (ANOVA *p*<0.05 followed by a Bonferroni test) and are shown in bold. Values represent the mean (n = 3 microcosms per treatment) ± SE.

	M-nL	M-C	M-40A	M-40AN	M-80AN
pH (H_2O)	**5.5 ± 0.0** ^{a}	**5.3 ± 0.0** ^{b}	**5.1 ± 0.0** ^{c}	**5.1 ± 0.0** ^{c}	**5.2 ± 0.0** ^{bc}
OM (%)	**6.9 ± 0.0** ^{a}	**8.1 ± 0.5** ^{ab}	**7.9 ± 0.1** ^{ab}	**8.2 ± 0.0** ^{b}	**8.4 ± 0.2** ^{b}
C (mg g^{-1})	**20.4 ± 1.5** ^{a}	**25.6 ± 1.4** ^{b}	**25.6 ± 1.5** ^{b}	**25.6 ± 1.6** ^{b}	**25.4 ± 1.6** ^{b}
N (mg g^{-1})	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
P (μg g^{-1})	8.3 ± 1.5	11.0 ± 1.4	11.0 ± 1.8	11.8 ± 1.4	11.4 ± 1.1
K (μg g^{-1})	142.3 ± 10.3	152.5 ± 10.7	155.3 ± 11.4	156.9 ± 11.0	156.5 ± 10.4
Mg (μg g^{-1})	111.7 ± 10.9	123.8 ± 11.5	121.4 ± 11.7	126.2 ± 9.7	123.0 ± 9.4
C/N ratio	**21.0 ± 0.6** ^{a}	**25.0 ± 0.7** ^{b}	**24.6 ± 0.5** ^{b}	**24.3 ± 0.7** ^{b}	**23.6 ± 0.5** ^{ab}
N/P ratio	120.9 ± 12.7	94.8 ± 6.7	97.8 ± 9.6	90.7 ± 6.4	95.3 ± 3.8
C/P ratio	2537 ± 247	2375 ± 200	2402 ± 237	2200 ± 127	2246 ± 74

The functional changes in soil microbial activity due to altered litter traits (Fig. 6.1 and Tables 6.1 and 6.2) were assessed by measuring CO_2 and N_2O fluxes between soil and atmosphere (Fig. 6.6). Given that there were no significant differences in the gas fluxes measured (see material and methods), each microcosm was presented as an average of three measurements. Soil respiration was significantly higher in M-40A and M-40AN while N_2O fluxes increased (less potential to act as N_2O sink). Fluxes of these two gases were strongly correlated (r = 0.89). CO_2 and N_2O fluxes were strongly correlated with ΔOM, ΔC, microcosm initial C concentration and the availability of nitrate and inorganic N, available N/P and microbial community structure (PLFAs - Table 6.4).

In general, OM decomposition (e.g. ∆OM and ∆C) was not correlated with litter traits (e.g. litter's C/N ratio) or initial microcosm characteristics (e.g. microcosm initial P concentration). However, there were significant correlations with the following litter traits: N, P and lignin concentrations, and its ratios (N/P and lignin/N – Table 6.4). The strongest correlations were with CO_2 and N_2O fluxes and initial total C concentration and final concentration of NO_3^-, total inorganic N, phosphate and the ratio between inorganic N and phosphate (available N/P).

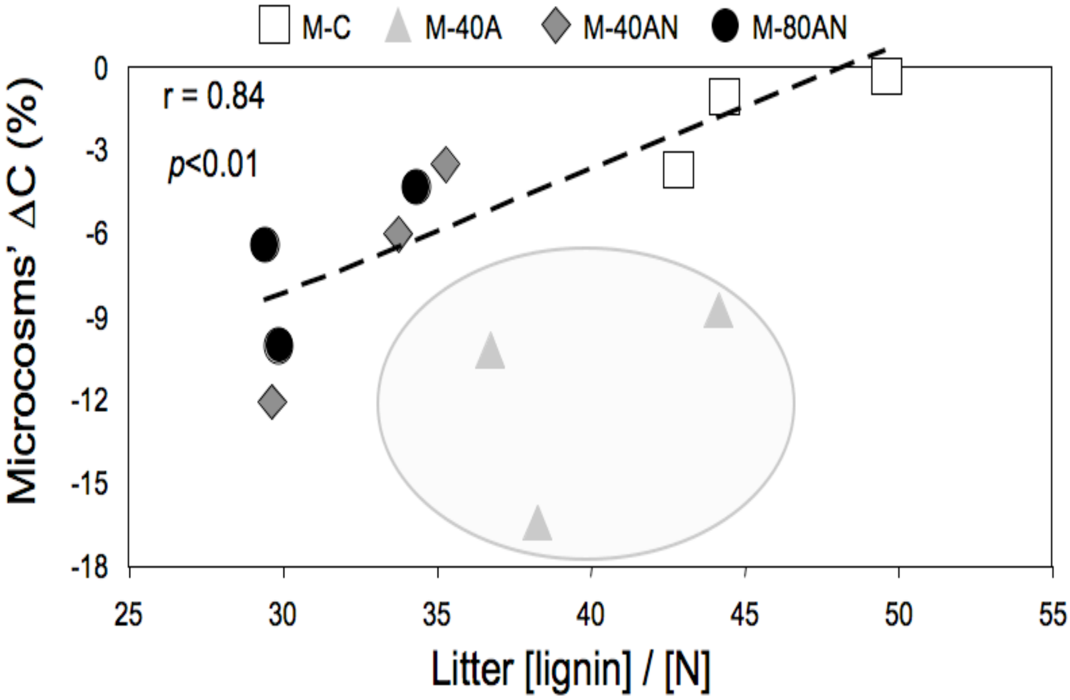

Figure 6.3 - Relation between litter's lignin/N ratio and the respective microcosms' ∆C. Symbols represent the individual values (n = 3 microcosms per treatment). Excluding the data points from the M-40A (triangles), there was a significant linear correlation between the two variables (n = 9 data points).

DISCUSSION

Ecological N-driven trade-offs in litter traits

The dominance of leaf litter from summer semi-deciduous and evergreen sclerophylls species is consistent with the trait-based functional grouping of the Mediterranean vegetation into these two groups (Correia & Catarino 1994; Cruz *et al.* 2008; Dias *et al*. 2011b). The increased proportion of evergreen sclerophylls in the leaf litter produced in the field under the higher N dose (L-80AN - Fig. 6.1) resulted mainly from a decrease in the abundance of summer semi-deciduous (Dias *et al*. 2012) and alterations in other plant species composition and abundance (Dias *et al.* 2011a), which is likely to influence decomposition (Cornwell *et al.* 2008).

Table 6.4 - Pearson's correlations between the indicators of OM decomposition (ΔOM, ΔC and CO_2 and N_2O fluxes) and the considered factors influencing OM decomposition. * Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed).

	ΔOM	ΔC	CO_2 flux	N_2O flux
ΔOM	1.00	0.87**	-0.67**	-0.87**
ΔC	0.87**	1.00	-0.76**	-0.88**
ΔN	0.22 ns	0.22 ns	0.06 ns	-0.02 ns
Litter [N]	-0.63*	-0.46 ns	0.40 ns	0.59*
Litter C/N ratio	-0.23 ns	-0.31 ns	0.61*	0.49 ns
Litter [lignin]	-0.54*	-0.45 ns	0.49 ns	0.61*
Litter [lignin]/[N]	0.59*	0.27 ns	0.05 ns	-0.30 ns
Soil C/N ratio	-0.43 ns	-0.67**	0.68**	0.64*
$\Delta [H^+]$	0.80**	0.79**	-0.76**	-0.78**
CO_2 flux	-0.67**	-0.76**	1.00	0.89**
N_2O flux	-0.87**	-0.88**	0.89**	1.00
$[NO_3^-]$	0.58*	0.77**	-0.85**	-0.79**
$[NH_4^+]$	0.46 ns	0.53*	-0.73**	-0.68**
[inorgN]	0.59*	0.77**	-0.86**	-0.81**
$[PO_4^{3-}]$	-0.70**	-0.43 ns	0.10 ns	0.38 ns
Available N/P	0.81**	0.86**	-0.78**	-0.86**
Litter [P]	-0.55*	-0.44 ns	0.61*	0.67*
Litter [Mn]	-0.38 ns	-0.30 ns	0.55*	0.50 ns
Litter C/P ratio	-0.41 ns	-0.44 ns	0.49	0.52*
Litter N/P ratio	-0.58*	-0.46 ns	0.32 ns	0.51 ns
Soil [C]	-0.79**	-0.76**	0.59*	0.74**
Soil [N]	-0.58*	-0.29 ns	0.16 ns	0.33 ns
Soil [P]	-0.29 ns	-0.05 ns	0.32 ns	0.30 ns
Total PLFAs	-0.37 ns	-0.57*	0.85**	0.62*
Fungal PLFAs	-0.52 ns	0.66**	0.86**	0.70**
Bacterial PLFAs	-0.36 ns	-0.55 ns	0.84**	0.62*

Irrespective of the type of ecosystem, increased N availability changes biodiversity (Sala *et al.* 2000; Bobbink *et al.* 2010) by altering the competitive interactions between species (Bobbink *et al.* 2010). It is clear that in our experimental site the addition of 80 kg NH_4NO_3-N ha^{-1} yr^{-1} favoured the proportion of litter from evergreen sclerophylls and graminoids in detriment of summer semi-deciduous plants (Fig. 6.1). Since evergreen sclerophylls are more conservative in respect to nutrients and more water use efficient (Correia & Catarino 1994; Canadell *et al*. 1996; Dias *et al*. 2011b), they may have advantage over the summer semi-deciduous under conditions (such as increased N availability) that decrease P and/or water availability to individual plants (Craine 2009).

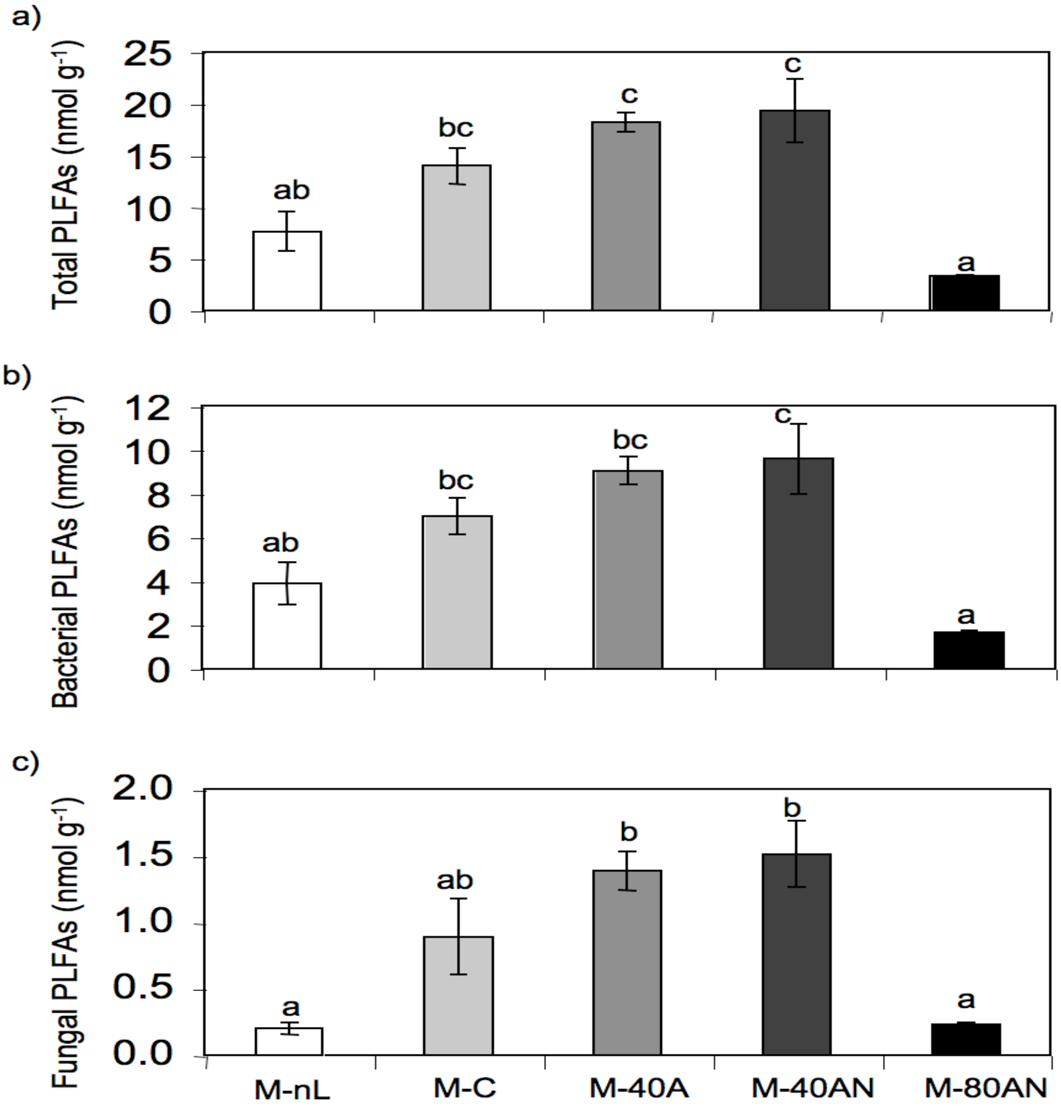

Figure 6.4 – Microcosms' total (a), bacterial (b) and fungal (c) PLFAs concentrations determined at the end of the experiment and according to the distinct treatments (M-nL, M-C, M-40A, M-40AN, and M-80AN). Different letters refer to statistically significant differences between treatments (ANOVA $p<0.05$ followed by a Bonferroni test). Bars represent the mean (n = 3 microcosms) ± SE.

Summer semi-deciduous and evergreen sclerophylls also differ in their strategy to use N; summer semi-deciduous appear to use it for biomass production (data not shown) while evergreen sclerophylls invest comparatively more N in the synthesis of structural compounds (e.g. lignin – Table 6.1) which might increase stress tolerance (e.g. water stress, herbivory, etc – Craine 2009). Due to the higher lignin concentration of evergreen sclerophyll leaves (in relation to summer semi-deciduous - Fioretto *et al.* 2005), the increased proportion of evergreen sclerophylls in the leaf litter produced under the higher N dose led to higher lignin concentrations in the leaf litter (L-80AN - Table 6.1). Therefore when increased N availability changes the balance between plants, groups of plants or litter, that have distinct leaf traits, such as happened in the 80AN treatment, effects are expected on decomposition.

However, N additions (field treatments) also resulted in increased litter N concentration and lower C/N ratios (Table 6.2), which can improve litter decomposability (Vourlitis, Pasquini & Mustard 2009; Dias *et al*. 2012). Thus, assessing the effects of increased N availability on decomposition must take account of the trade-offs between on the one hand higher lignin and on the other, higher N concentrations.

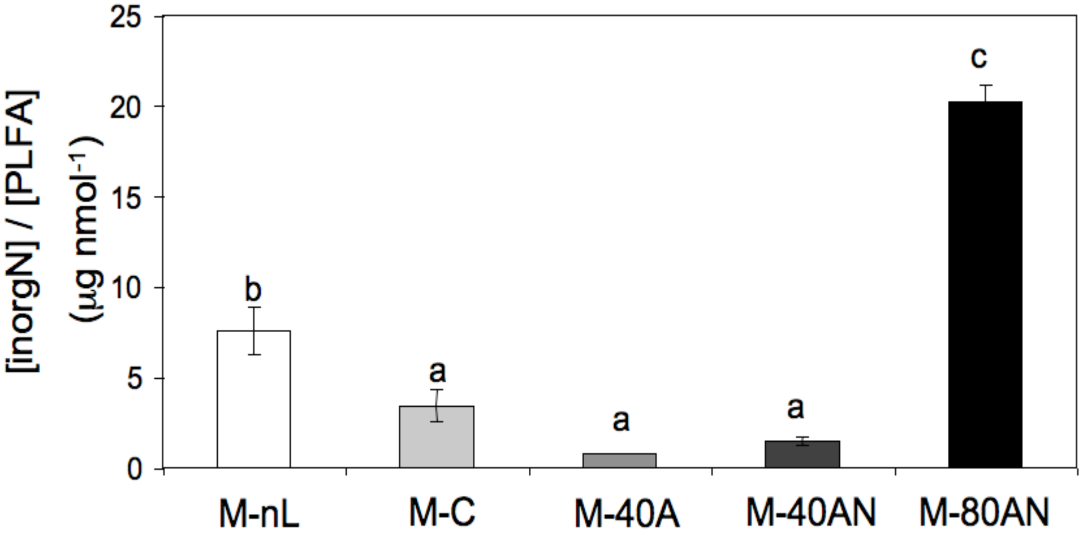

Figure 6.5 – Microcosms' availability of inorganic N per total microbial PLFAs at the end of the experiment and according to the distinct treatments (M-nL, M-C, M-40A, M-40AN, and M-80AN). Different letters refer to statistically significant differences between treatments (ANOVA $p<0.05$ followed by a Bonferroni test). Bars represent the mean (n = 3 microcosms) ± SE.

Consequences of altered litter traits on organic matter decomposition

Soil C/N ratio is regarded as a determinant parameter in OM decomposition (Davidson & Janssens 2006). As a general rule, net mineralization occurs at C/N ratio < 20 (Craine 2009). The fact that soil microcosms presented C/N ratios in the range 21 - 25 (Table 6.3) may imply that within that range, changes in the C/N ratio are not likely to interfere with decomposition. In fact, the lower loss of OM observed in the M-80AN may reflect the higher aromaticity and lignin concentration of the litter mixture in comparison with the other N addition treatments (M-40A and M-40AN - Fig. 6.2 and Table 6.1). Lignin is known to be relatively resistant to microbial decomposition (Austin & Ballaré 2010) and breaks-down to produce aromatic phenolic compounds that are known to suppress hydrolytic enzyme activity crucial to decomposition (Freeman, Ostle & Kang 2001). The effect of this suppression has overcome the stimulatory effect of leaf litter's increased N concentration and may explain why OM decomposition in M-80AN was lower than in M-40A and M-40AN (Fig. 6.2-b).

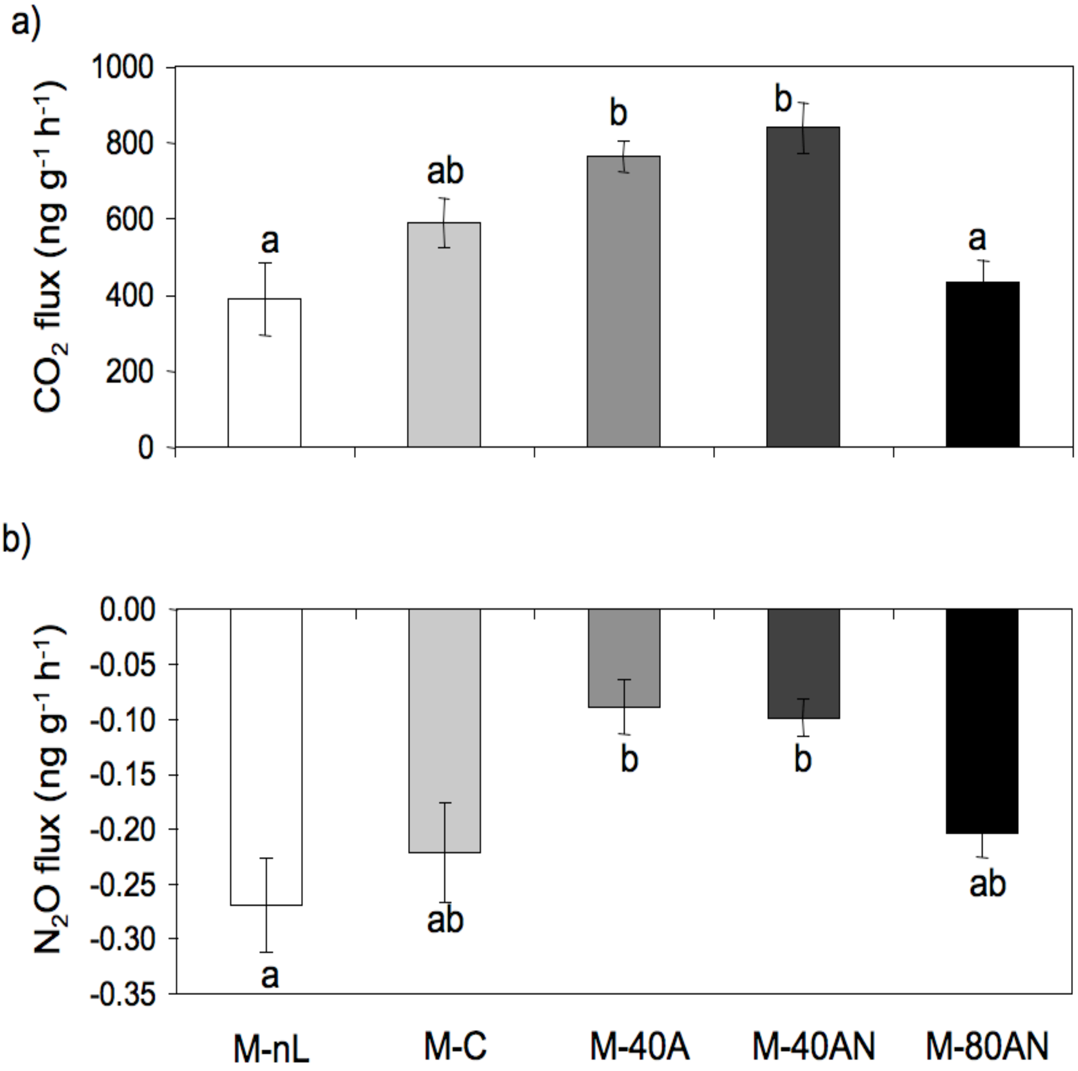

Figure 6.6 - CO_2 (a) and N_2O (b) net fluxes between soil and the atmosphere at the end of the experiment and according to the treatments (M-nL, M-C, M-40A, M-40AN and M-80AN - see materials and methods). Different letters refer to statistically significant differences between treatments (ANOVA $p<0.05$ followed by a Bonferroni test). Bars represent the mean (n = 3 microcosms) ± SE.

In addition, litter's lignin/N ratio is an estimate of litter decay dynamics (Aber & Melillo 1982; Aber, Melillo & McClaugherty 1990; Knorr, Frey & Curtis 2005), so that when considering data from the microcosms incubated with litter produced without N addition (M-C) and with the addition of both nitrate and ammonium (M-40AN and M-80AN), there was a significant correlation between litter's lignin/N ratio and microcosms' ΔC (Fig. 6.3). Which may be related with the importance of the N sources, namely of nitrate, in the regulation of secondary metabolism, such as the phenylpropanoid metabolism responsible for the synthesis of lignin (Fritz *et al*. 2006).

Our results show that at the community/ecosystem level the positive effect of higher litter N concentration on decomposition was offset by a negative effect of increased litter lignin content and soil N availability (Fig. 6.5), that have reduced soil microbial biomass (Fig. 6.4) and activity (Fig. 6.6). The very low soil microbial biomass in M-80AN (Fig. 6.4) reflects changes in leaf litter composition (Fig. 6.1 and Tables 6.1 and 6.2), which is evidence of the influence of plant community richness and composition (including species and community litter traits) on soil microbial community structure and therefore on OM decomposition (e.g. De Deyn, Cornelissen & Bardgett 2008; Fornara, Tilman & Hobbie 2009).

The microbial activity based on the net CO_2 and N_2O fluxes from the microcosms was within the range reported for field-studies in the Mediterranean Basin (Rosenkranz *et al*. 2006). It has been shown that Mediterranean forest soils are mostly weak N_2O emitters (<10µg N m^{-2} h^{-1}) or even temporal sinks for atmospheric N_2O (Fenn, Poth & Johnson 1996; Bernal *et al*. 2003; Butterbach-Bahl & Kiese 2005), which has been linked to their very low N availability (Rosenkranz *et al*. 2006). The pattern of net CO_2 and N_2O fluxes (very small in the M-80AN and highest for M-40A and M-40AN – Fig. 6.6) appear to reflect differences in OM decomposition (Fig. 6.2) and the size of the soil microbial communities (Fig. 6.4 and Supplementary data Table 6.2). However, the correlation between total PLFAs and N_2O fluxes was lower than that with CO_2 fluxes (Table 6.4), which reflects the fact that only a specific group of microorganisms (i.e., nitrifiers consume N_2O in nitrifier denitrification - Chapuis-Lardy *et al*. 2007) was responsible for the observed negative fluxes (Fig. 6.6).

CONCLUSIONS

As far as we are aware, this is the first study of the integrated effects of N enrichment on litter decomposability based on N-driven community responses in litter traits. Data shows that N additions reduced the abundance of summer semi-deciduous species (Dias *et al*. 2012) and benefited evergreen sclerophylls and graminoids (Fig. 6.1). This change in plant community composition has resulted in a change in the quality of litter inputs that has reduced the size of the microbial biomass (Fig. 6.4) and suppressed soil OM decomposition rates (Fig. 6.2), despite high N levels. Although it is important to also quantify *in situ* leaf litter decomposition (e.g. litter bags), which would also account for the N-driven changes in the soil microbial community, at the end of the N-manipulation field experiment, soil OM concentration was higher in the 80AN plots (Dias *et al.* submitted). Thus, the accumulation of OM in the field confirms the slower decomposition rates observed in the laboratory. In addition, under natural conditions, OM accumulates much more in the soil patches under the influence of evergreen sclerophylls (~16%) than under that of summer semi-deciduous (~10% - Cruz *et al.* 2008). Therefore, taking into account the estimates of increased N deposition for semi-natural Mediterranean Basin ecosystems, it is possible that the ecological niche occupied by summer semi-deciduous species will narrow (Dias *et al*. 2011b) while that of evergreen sclerophylls widens, which may lead to lower soil decomposition rates (Fig. 6.2) and an overall accumulation of OM. These results demonstrate how anthropogenic N deposition, and how the N forms (Fig. 6.3) can be a powerful determinant of ecosystem function. Specifically, results confirm the importance of the interaction between plant community change and plant-soil interactions in nutrient-poor ecosystems, such as this Mediterranean maquis and highlight the important role of N availability (concentration and source) in controlling ecosystem nutrient cycles.

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SUPPLEMENTARY DATA

Table 6.1 – Relative intensities (%) of the different types of litter (L-C, L-40A, L-40AN and L-80AN) derived from the ^{13}C CPMAS NMR spectra. Different letters refer to statistically significant differences between treatments (ANOVA *p*<0.05 followed by a Bonferroni test) and are shown in bold. Values represent the mean (n = 3) ± SE.

Region of spectra (ppm)	L-C	L-40A	L-40AN	L-80AN
Alkyl C (0-45)	18.1 ± 0.6	17.7 ± 0.6	17.8 ± 1.3	18.3 ± 0.8
Methoxyl C (45-60)	2.2 ± 0.2	2.5 ± 0.0	2.4 ± 0.2	2.4 ± 0.2
O-alkyl C (45-110)	**55.4 ± 0.2 ^{a}**	**53.8 ± 0.6 ^{a}**	**54.9 ± 0.7 ^{a}**	**50.6 ± 1.1 ^{b}**
Aromatic C (110-160)	**11.7 ± 0.6 ^{a}**	**13.0 ± 0.8 ^{ab}**	**12.0 ± 0.7 ^{ab}**	**14.3 ± 1.1 ^{b}**
Phenolic C (140-160)	**7.6 ± 0.1 ^{a}**	**7.5 ± 0.2 ^{a}**	**7.5 ± 0.2 ^{a}**	**8.3 ± 0.2 ^{b}**
Carboxyl C (160-190)	**5.0 ± 0.2 ^{a}**	**5.6 ± 0.3 ^{ab}**	**5.5 ± 0.2 ^{a}**	**6.3 ± 0.4 ^{b}**

Table 6.2 – Microcosms' microbial PLFAs concentration determined at the end of the experiment and according to the distinct treatments (M-nL, M-C, M-40A, M-40AN, and M-80AN). Different letters refer to statistically significant differences between the treatments (ANOVA *p*<0.05 followed by a Bonferroni test). Values represent the mean (n = 3 microcosms per treatment) ± SE.

[PLFA] (nmol g^{-1})	M-nL	M-C	M-40A	M-40AN	M-80AN
C14:0	0.12±0.04 ^{ab}	0.16±0.03 ^{ab}	0.20±0.01 ^{ab}	0.24±0.06 ^{b}	0.04±0.00 ^{a}
C15:0i	0.52±0.16 ^{ab}	0.80±0.13 ^{ab}	1.05±0.04 ^{b}	1.08±0.22 ^{b}	0.22±0.01 ^{a}
C15:0a	0.29±0.09 ^{ab}	0.46±0.05 ^{ab}	0.54±0.05 ^{b}	0.57±0.11 ^{b}	0.11±0.01 ^{a}
C15.0	0.10±0.03 ^{ab}	0.18±0.03 ^{b}	0.17±0.00 ^{ab}	0.20±0.04 ^{b}	0.04±0.00 ^{a}
C16:0i	0.47±0.13 ^{ab}	0.71±0.07 ^{b}	0.84±0.02 ^{b}	0.94±0.16 ^{b}	0.17±0.01 ^{a}
C16:1 (n-7)	0.04±0.04	0.04±0.04	0.08±0.04	0.10±0.06	0.02±0.00
C16:1	0.34±0.06 ^{ab}	0.76±0.09 ^{bc}	0.99±0.08 ^{c}	1.07±0.17 ^{c}	0.17±0.01 ^{a}
C16:1 (n-5)	0.11±0.03 ^{ab}	0.21±0.02 ^{abc}	0.26±0.02 ^{bc}	0.30±0.05 ^{c}	0.05±0.00 ^{a}
C16:0	1.27±0.29 ^{ab}	2.39±0.25 ^{bc}	3.04±0.16 ^{c}	3.04±0.45 ^{c}	0.52±0.02 ^{a}
C17:1 (n-8)	0.11±0.04	0.06±0.06	0.09±0.05	0.19±0.02	0.04±0.00
7Me-C17:0	0.07±0.03 ^{ab}	0.13±0.01 ^{abc}	0.20±0.01 ^{c}	0.18±0.04 ^{bc}	0.04±0.00 ^{a}
brC17:0	0.49±0.20 ^{ab}	0.70±0.02 ^{b}	0.87±0.07 ^{b}	0.88±0.15 ^{b}	0.15±0.01 ^{a}
iC17:0	0.19±0.05 ^{ab}	0.30±0.03 ^{b}	0.33±0.01 ^{b}	0.37±0.06 ^{b}	0.07±0.01 ^{a}
aC17:0	0.21±0.05 ^{ab}	0.38±0.04 ^{bc}	0.47±0.02 ^{c}	0.49±0.08 ^{c}	0.09±0.01 ^{a}
7,cy-C17:0	0.21±0.02 ^{ab}	0.43±0.05 ^{bc}	0.49±0.04 ^{bc}	0.58±0.09 ^{c}	0.09±0.00 ^{a}
brC18:0	0.26±0.06 ^{ab}	0.42±0.04 ^{b}	0.41±0.04 ^{b}	0.41±0.04 ^{b}	0.07±0.00 ^{a}
C18:2 (n-6)	0.20±0.05 ^{a}	0.89±0.28 ^{ab}	1.39±0.15 ^{b}	1.51±0.25 ^{b}	0.24±0.01 ^{a}
C18:1 (n-9)	0.86±0.19 ^{ab}	1.57±0.28 ^{abc}	2.08±0.05 ^{bc}	2.36±0.37 ^{c}	0.39±0.01 ^{a}
C18:1 (n-7)	0.54±0.11 ^{ab}	1.49±0.37 ^{ab}	2.23±0.49 ^{b}	2.07±0.32 ^{ab}	0.30±0.01 ^{a}
C18:1 (n-5)	0.18±0.03	0.11±0.11	0.22±0.11	0.38±0.04	0.06±0.00
C18:0	0.32±0.06 ^{ab}	0.65±0.06 ^{c}	0.63±0.02 ^{bc}	0.73±0.09 ^{c}	0.13±0.01 ^{a}
C19:1	0.07±0.01	0.06±0.06	0.27±0.06	0.23±0.03	0.04±0.00
7,8cy-C19:0	0.69±0.16 ^{ab}	1.10±0.08 ^{bc}	1.37±0.01 ^{c}	1.44±0.22 ^{c}	0.27±0.01 ^{a}

Chapter 7

Enhanced N availability increases plant diversity in a Mediterranean ecosystem: evidence from a medium-term field manipulation experiment

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Chapter 7

Enhanced N availability increases plant diversity in a Mediterranean ecosystem: evidence from a medium-term field manipulation experiment

ABSTRACT

Enhanced nitrogen (N) availability is one of the main causes of biodiversity loss. However, in nutrient-limited ecosystems, short-term enhanced N availability can promote diversity. Mediterranean Basin ecosystems are nutrient-limited biodiversity hotspots, but no information is available on their long- or medium-term responses to enhanced N availability. Since 2007, we have been manipulating the form and dose of available N in a Mediterranean Basin maquis in southwestern Europe, with low ambient N deposition (5.2 kg N ha^{-1} yr^{-1}) and low soil N content (0.1%). N availability was modified by the addition of 40 kg N ha^{-1} yr^{-1} as a 1:1 NH_4Cl to $(NH_4)_2SO_4$ mixture, and 40 and 80 kg N ha^{-1} yr^{-1} as NH_4NO_3. Control plots were not fertilized. Over the following 4.5 years the effects on soil properties and plant diversity were assessed. Enhanced N availability resulted in an increased number of plant species that was more strongly related to the cumulative ammonium than with the cumulative nitrate addition over the experiment. Exposure to 40 kg NH_4^+-N ha^{-1} y^{-1} (either solely or with nitrate) led to a significant increase in the number of plant species, but did not increase productivity while the exposure to 40 kg NH_4NO_3-N ha^{-1} y^{-1} increased biomass production but did not promote plant diversity. The N-benefited species were small short-lived plants, typical of disturbed and/or eutrophied sites while N-affected were perennial shrubs characteristic of sites with very low nutrient availability. Soil inorganic N pools (NO_3^- and NH_4^+) and organic matter increased under the higher N dose. Data suggest that agriculture, the main source of enhanced NH_4^+-N, may affect the structure of the maquis in the surrounding areas and promote soil erosion as a consequence of decreased permanent plant cover. While industrial and urban activities, responsible for enhanced NO_3^--N availability may promote biomass accumulation and therefore increase the fire risk. This information is of significant importance for land use management in biodiverse and fragmented ecosystems such as the Mediterranean ones, especially in Natura 2000 sites.

Keywords: Mediterranean ecosystems; Nitrogen effects; Species richness; Species evenness; Plant cover; Soil erosion; Productivity

INTRODUCTION

Enhanced nitrogen (N) availability has been acknowledged as one of the main causes of biodiversity loss (Sala *et al.* 2000; Phoenix *et al.* 2006; Clarisse *et al.* 2009; Bobbink *et al.* 2010; De Schrijver *et al.* 2011). Although most global biodiversity is contained within natural and semi-natural ecosystems (Bobbink, Hornung & Roelofs 1998), most of our knowledge of the effects of increased N on such ecosystems comes only from northern Europe and America (Bobbink *et al.* 2010). Mediterranean-type ecosystems sit in the 'neglected ecosystems list' (Bobbink *et al.* 2010; Dias *et al.* 2011a; Ochoa-Hueso *et al.* 2011) despite being a global conservation priority (Myers *et al.* 2000; Olson & Dinerstein 2002; Klausmeyer & Shaw 2009) that rivals with tropical rainforests (Cowling *et al.* 1996; Dallman 1998; Klausmeyer & Shaw 2009). However, except for the Mediterranean California (Fenn *et al.* 2010; Fenn, Allen & Geiser 2011; Ochoa-Hueso *et al.* 2011), not much is known about the effects of increased N in Mediterranean Basin ecosystems, where N deposition is estimated to increase three fold by 2050 (Galloway *et al.* 2004; Phoenix *et al.* 2006).

The most distinctive features of Mediterranean-type ecosystems, in terms of controlling the response to increased N, are: climate (highly seasonal, with warm and dry summers, which contrast with cool and rainy winters); soils (low nutrients and organic matter and high bases such as carbonates); dominance of dry N deposition; and asynchrony between N availability and biological activity (Ochoa-Hueso *et al.* 2011). These distinguish Mediterranean-type ecosystems from the well-studied temperate ones, so that lessons learned from them concerning N impacts may not be applicable. Even within Mediterranean-type ecosystems, differences in soil fertility (Cowling *et al.* 1996) and phosphorus (P) availability in particular (Ochoa-Hueso *et al.* 2011) can compromise extrapolations from Californian ecosystems to those in the Mediterranean Basin (Ochoa-Hueso *et al.* 2011). Therefore, in 2007 a N-manipulation (doses and forms) field experiment was established in a severely nutrient-limited Mediterranean maquis. Within one year, changes in plant community were observed and in contrast to many other studies, but in agreement with what was expected for poor nutrient availability, plant diversity increased (chapter 2, Dias *et al.* 2011a). This observation raised key questions:

(i) Was this effect transient? For how long (and/or under how much more N) will plant diversity increase? This is especially relevant since most European ecosystems have already reached a threshold, from where diversity has declined in response to N deposition (De Schrijver *et al.* 2011).

(ii) Are there consistent plant responders to increased N in Mediterranean maquis? *Dittrichia viscosa* responded significantly to increased N (chapter 2, Dias *et al.* 2011a) but along a post-fire succession changes in plant cover and species composition occur naturally (Thompson 2005), which may confound the identification of consistent plant responders to enhanced N availability.

(iii) Are the N-driven changes in diversity linked with functional alterations, namely plant productivity? Experiments that directly manipulate species diversity often report a positive effect of diversity on productivity, whereas observations of natural communities reveal more complex relationships, and nutrient additions generally result in increased productivity and decreased diversity (Jiang, Wan & Li 2009).

(iv) Does the form of N matter? Since the co-existing plant species occupy distinct N ecological niches, with NH_4^+ availability as a determinant (Cruz *et al.* 2008; chapter 4, Dias *et al.* 2011b), the plant's response to enhanced N availability may vary according to the N form.

To address these questions, we maintained our measurements over further 4.5 years, focusing on the N-driven changes, in soil surface characteristics and plant community.

MATERIALS AND METHODS

Study site

The study site (38° 29' N - 9° 01' W) is located in Serra da Arrábida in the Arrábida Natural Park, Portugal (a Natura 2000 site - PTCON0010 Arrábida/Espichel). It is within the sub-humid thermomediterranean bioclimatic domain (Rivas-Martínez, Penas & Díaz 2004). According to records (1971-2000 - Instituto Nacional de Meteorologia e Geofísica), mean annual precipitation is 730 mm; mean maximum temperature, 27.8ºC (August); and mean minimum temperature, 8.1ºC (January). Over the experimental period (2007-2011), mean annual precipitation was 870 mm; mean maximum temperature, 21.6ºC; and mean minimum temperature, 12.9ºC. Total monthly precipitation and mean monthly temperature over the experimental period (from 2007 to 2011) are shown in Figure 7.1.

The study site is located on a southeast-facing slope (5%) at 130 m a.s.l. that is protected from public access and has not been managed in recent decades. The soils of Serra da Arrábida are classified as calcic rhodo-chromic luvisols and calcareous chromic cambisols (FAO System - Specht *et al.* 1988). The soil is skeletal (topsoil layer of approximately 15 cm) with a bulk density of 1.3 g cm^{-3}. Silt predominates (50%), while sand and clay contents make up 32% and 18%, respectively (silt-sand-loam texture).

The vegetation consists of a dense maquis which is a type of Mediterranean habitat that comprises closed vegetation, usually with 100% cover, mainly shrubs with few annuals and some geophytes, trees are nearly always present, some of which may be in shrub form (Eunis class F5.2 – Mediterranean maquis). The standing community developed after a fire event (summer 2003) four years before the first N addition and included as the dominant plant species, a *Cistaceae*, *Cistus ladanifer* L. (chapter 2, Dias *et al.* 2011a). Other abundant plant species included *Erica scoparia* L. (*Ericaceae*), *Calluna vulgaris* (L.) Hull (*Ericaceae*), *Genista triacanthos* Brot. (*Fabaceae*), *Ulex densus* Welw. ex Webb (*Fabaceae*), *Dittrichia viscosa* L. (*Asteraceae*), and *Myrtus communis* L (*Myrtaceae*). Herbaceous species comprised ≈ 10% of the total plant cover, of which many were annual plants (chapter 2, Dias *et al.* 2011a).

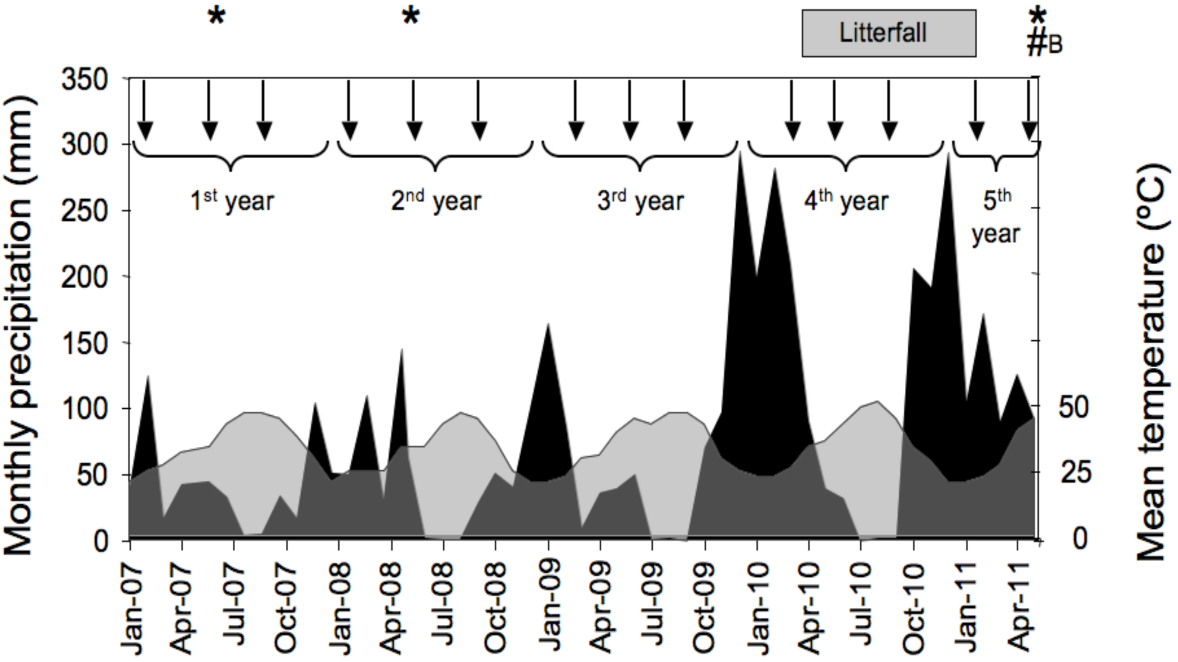

Figure 7.1 - Mean monthly temperature (light grey) and total monthly precipitation (black). Arrows represent the time of N additions along the period of the N treatments (January 2007 to June 2011). Asterisk refers to the time plant community assessments (2007, 2008 and 2011), litterfall was collected between April 2010 to December 2010, and "#B" refers to the time of aboveground biomass sampling (June 2011).

Experimental design and fertilization schedule

Background N deposition is estimated to be 5.2 kg ha^{-1} yr^{-1} (2.9 kg NO_x + 2.3 kg NH_y - based on the model used by the European Monitoring and Evaluation Programme - http://webdab.emep.int/Unified_Model_Results/AN/). The chosen N doses were high enough to establish 'worst case' scenarios of N enrichment in this type of habitat, but lower than the N deposition reported for highly N polluted areas in Mediterranean-type ecosystems (145 kg N ha^{-1} yr^{-1} - Fenn *et al.* 2003; Meixner & Fenn 2004). The N forms mimicked the most likely N pollution scenarios within the Mediterranean Basin (e.g. predominantly agricultural sources, and combined inputs from urban/industrial sites and agricultural). Control plots were not N-fertilized, while N availability was modified by the addition of 40 kg N ha^{-1} yr^{-1} as a 1:1 mixture of NH_4Cl and $(NH_4)_2SO_4$ (designated 40A) and 40 and 80 kg N ha^{-1} yr^{-1} in the form of NH_4NO_3 (designated as 40AN and 80AN respectively). Therefore 40A and 40AN provide the same N dose while 40A and 80AN provide the same NH_4^+ dose. To prevent N 'contamination' through runoff from N-plots, the experimental plots were distributed in three rows along the 5% slope, with the controls being located in the top row.

Beginning in January 2007, the dry N salts were homogenously added, by hand, sprinkled over the surface, in three equal applications over the year: middle autumn/winter, spring and summer (Fig. 7.1). Each treatment was replicated three times (3 plots of 400 m^2 each). To restrict boundary effects and dilution processes, all measurements, analyses and sample collection were performed within an internal 100 m^2 square.

Effects of N addition on superficial soil properties

Soil was sampled from the four corners and the centre of the internal 100 m^2 square of each plot. Soil samples (2 cm diameter and 15 cm depth) were removed, sieved (2mm) and stored at 4ºC until analysis. Sampling took place in May 2007 and 2011 corresponding to the first and fifth springs of the N additions. Individual soil samples (five per plot) were analyzed for nitrate (NO_3^--N - Matsumura & Witjaksono 1999), ammonium (NH_4^+-N - Cruz & Martins-Loução 2000), pH (H_2O) and organic matter as described in Dias *et al.* (2011a, chapter 2). Soil inorganic N (inorgN) was the sum of the water extracted NH_4^+ and NO_3^-. NO_3^-, NH_4^+ and inorgN were expressed as µg N per gram of dry soil. Bulk soil samples (equal mixtures of the five soil samples from each experimental plot) were dried (35ºC) and used for quantifying soil total N and C and the C/N ratio by dry combustion using an elemental analyzer (EuroVector, Italy).

Effects of the N additions on plant cover and diversity

The vascular plant community composition was assessed in June 2007, May 2008 – chapter 2, Dias *et al.* 2011a – and June 2011. The vegetation at the study site was sampled within one 5x5m square per experimental plot (within the internal 100 m^2). Percentage of vascular plant species cover (herbaceous included), and of bare soil were recorded. Each species' cover was calculated from the total projected crown area (calculated from two perpendicular diameters, assuming elliptical shape). Plant species observed in the three community assessments were associated in Table 7.2 according to their biological types (Raunkier 1934) described in Flora Digital de Portugal (http://www.jb.utad.pt/pt/herbario/cons_reg.asp). The subdivisions of the Raunkier system are based on the location of the plant's growth-point (bud) during seasons with adverse conditions (cold seasons, dry seasons): phanerophytes (P) - usually woody perennials that project their buds on stems more than 25 cm above soil level; chamaephytes (C) - woody plants with perennating buds borne close to the ground; hemicryptophytes (H) - have their buds at or near the soil surface; geophytes (G) - have their buds resting in the soil; and therophytes (T) - annual plants which survive the unfavourable season in the form of seeds. From the vascular plant community assessments in 2007, 2008 and 2011 it was possible to calculate plant richness and evenness (Krebs 1989) for the first and fifth springs of experiment.

Effects of the N additions on aboveground plant biomass and litterfall

Aboveground plant biomass was assessed destructively on June 2011 (the fifth spring of N additions – Fig. 7.1) by cutting all aboveground plant biomass within three 1 m^2 squares, randomly located, per plot. Plant biomass was dried (60°C) to constant weight.

Litterfall was assessed using litter traps (1.5 mm mesh screen with 0.04 m^2 collecting surface; elevated ca. 15 cm above the ground) placed under the canopy of five *C. ladanifer* shrubs (located close to the four corners and the centre of the internal 100 m^2 square) in each plot. Litter collections were made fortnightly from April to December 2010. After drying (60°C), the litter was weighed.

Derived variables and statistics

Changes over time between 2007 and 2011 were calculated for plant richness (Fig. 7.2-b) and evenness (Fig. 7.2-d) and single species (Table 7.2). For single species, changes between 2007 and 2008 were also considered (Table 7.2). Changes between 2007 (the first spring of N additions – t_1) and 2008 or 2011 (the second and fifth spring of N additions – t_2, t_5) were positive (an increase) or negative (a decrease) and were calculated as follows (Sheppard *et al.* 2008; chapter 2, Dias *et al.* 2011a):

$$\text{Parameter Changes (\%)} = \frac{(\text{Parameter}_{2 \text{ or } 5} - \text{Parameter}_1)}{(\text{Parameter}_1 + \text{Parameter}_{2 \text{ or } 5}) / 2} \times 100$$

For calculating the cumulative N, NH_4^+ and NO_3^- (N deposition + N additions) at each plant assessment, we used the above-mentioned EMEP estimates and assumed that N, NH_y and NO_x deposited homogenously over the year (Fig. 7.3). Since the vegetation is dominated by summer semi-deciduous species that shed most of their leaves and twigs in the summer (Correia & Catarino 1994; Cruz *et al.* 2008), the litterfall collected between April and December 2010 was considered to be representative of the whole year litterfall production (Fig. 7.4-b). Because *C. ladanifer*'s aboveground biomass did not respond to the N addition treatments (data not shown) and litter production was not significantly affected by five years of N additions in Californian ecosystems (Vourlitis *et al.* 2007; Vourlitis, Pasquini & Mustard 2009) thus, this was also assumed in the present study. The amount of biomass that was produced and 'lost' every year through litterfall was estimated by multiplying by four (2007 to 2010). The sum of this value and that of aboveground plant biomass (Fig. 7.4-a) was considered to represent the 'total aboveground biomass' produced by the plant community (Fig. 7.4-c).

Summary statistics of soil properties, plant species and community responses (richness, evenness, cover, biomass and litterfall) were compared for the different N additions. The two-way ANOVA was applied to assess the existence of significant interactions between time and treatment for soil and plant variables while differences per treatment in biomass and litterfall were analyzed by a one-way ANOVA, followed by a Bonferroni test ($p< 0.05$ or $p<0.1$), or by a Games-Howell test whenever homogeneity of variances was not confirmed by the Levene's test. Regarding individual plant species, differences between treatments were analyzed by a one-way ANOVA (followed by a Bonferroni test $p<0.1$) and Kruskal-Wallis ($p<0.1$) for normal and non-normal samples respectively. Linear correlations between plant richness and cumulative N, NH_4^+ and NO_3^- were also studied (Pearson's correlations). In all cases, preliminary analyses were performed to ensure there was no violation of the assumptions regarding the tests' application. SPSS software, version 19.0, was used for all tests.

RESULTS

Effects of the N treatments on superficial soil properties

In the first and fifth springs of the N addition treatments (2007 and 2011, respectively), soil total N and C concentrations, and the C/N ratio were similar, irrespective of the N treatment and the year (Table 7.1); the soil was very poor in N (0.1%) and C (~2%), resulting in a low C/N ratio (< 20). In contrast, the soil available N and organic matter responded to the N addition treatments: control plots contained significantly lower concentrations of inorganic N and of organic matter than the 80AN treatment. Plots receiving 40 kg N ha^{-1} yr^{-1} (40A and 40AN) showed intermediate levels of inorganic N and organic matter. Soil NO_3^- and soil inorganic N (these two variables were highly correlated, $r = 0.98$, $p<0.001$) also responded to the year since concentrations were higher on the fifth spring than on the beginning of the experiment. Irrespective of the N treatment, soil was also more acidic on the fifth spring of N additions.

Effects of the N treatments on plant cover

In the first spring of the experiment (2007), plant communities were similar in all treatments (based on richness and evenness – Fig. 7.2). However, differences between treatments were visible by the second (chapter 2, Dias *et al.* 2011a), and fifth springs of the experiment (Table 7.2 and Fig. 7.2). The treatments containing 40 kg NH_4^+-N ha^{-1} yr^{-1} (40A and 80AN) changed plant community (structure and composition) the most. The plant biological types (Raunkier 1934) that were most responsive to N, and to NH_4^+ in particular, were small and ephemeral plants (geophytes, hemicryptophytes and therophytes, Table 7.2).

Table 7.1 – Soil surface (0-15 cm) properties [total N and C – carbon, C/N ratio and concentrations of inorgN – extractable inorganic N, NO_3^- - nitrate, and NH_4^+ - ammonium, OM – organic matter and pH (H_2O)], at the first (May 2007) and fifth (May 2011) springs after the beginning of the experiment according to the N additions (Control, 40A, 40AN and 80AN). Different letters refer to significant differences between treatments (ANOVA $p<0.05$ followed by a Bonferroni test) while * refers to significant differences between the 2007 and 2011 (there were no significant interactions between time and treatment, $p<0.01$). Values represent the mean (n = 3 experimental plots per treatment) ± SE.

Soil properties		Control	40A	40AN	80AN
N (%)	2007	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
	2011	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
C (%)	2007	1.6±0.2	1.6±0.3	1.8±0.1	2.0±0.6
	2011	1.8±0.1	1.9±0.2	2.1±0.1	2.2±0.3
C/N ratio	2007	18.5±0.8	16.6±0.1	17.4±1.9	16.6±1.0
	2011	18.6±0.4	17.4±0.7	18.1±0.5	17.6±0.8
	*	a	ab	ab	b
inorgN	2007	5.9±0.9	9.4±2.0	9.6±2.1	10.5±1.9
(μg g^{-1})	2011	7.9±1.3	12.2±1.9	12.9±1.6	19.2±2.5
	*	a	ab	ab	b
NO_3^--N	2007	5.6±0.8	8.3±1.9	8.9±2.2	8.1±1.7
(μg g^{-1})	2011	7.3±1.3	9.8±1.1	12.0±1.5	17.4±2.3
	*	a	ab	ab	b
NH_4^+-N	2007	0.3±0.1	1.1±0.3	0.7±0.1	2.4±0.3
(μg g^{-1})	2011	0.6±0.1	2.3±1.0	0.9±0.1	1.8±0.5
	*	a	ab	ab	b
OM (%)	2007	5.7±0.7	5.8±0.3	6.4±0.3	7.0±1.2
	2011	4.9±0.5	6.5±0.3	7.0±0.5	7.8±0.5
	*				
pH (H_2O)	2007	5.8±0.2	6.5±0.3	6.4±0.1	6.6±0.4
	2011	5.1±0.2	4.9±0.3	5.1±0.1	5.6±0.3

During the experimental period (2007-2011), the plant community changed both as a result of the ongoing post-fire ecological succession (controls) and the N addition treatments (Table 7.2). In the short-term (2007-2008), *Dittrichia viscosa* was benefited by increased N (irrespective of dose and form) while *Cistus ladanifer* was affected by 80AN. In the longer-term (2007-2011), *C. ladanifer* continued to be affected by 80AN, while *Lavandula luisieri* and *Ulex densus* were affected by 40A. These N-affected species were all perennial shrubs. The N-benefited species were: *Daphne gnidium* (perennial), *D. viscosa* (perennial small shrub), *Carlina corymbosa* (geophyte), *Urginea maritima* (geophyte), *Hypericum* sp (hemicryptophyte), *Pulicaria odora* (hemicryptophyte), *Gastridium ventricosum* (annual), *Solenopsis laurentia* (annual), and *Sonchus* sp (annual). All N-benefited plant species were 'small' and some were annuals (Table 7.2).

Table 7.2 - List of the vascular plant species observed in the three assessments (June 2007, May 2008 - chapter 2, Dias *et al.* 2011a - and June 2011, first, second and fifth springs of N additions) and their respective changes in cover (2008-2007 / 2011-2007 - see materials and methods) according to the N additions (Control, 40A, 40AN and 80AN). Plant species were grouped according to their life form: (i) the first letter represents perennial (P), annual (A) or undetermined (nd); and (ii) the second represents the biological type phanerophyte (P), chamaephyte (C), geophyte (G), hemicryptophyte (H), therophyte (T) and undetermined (nd). Different letters refer to significant differences between treatments (ANOVA *p*<0.1 followed by a Bonferroni test and Kruskal-Wallis *p*<0.1 for normal and non normal samples respectively; n = 3 experimental plots per treatment). + indicates increased cover; 0 indicates no change in cover; - indicated decreased cover; (+) indicates species appearance; and (-) indicates species disappearance.

Life form	Family	Species	Control	40A	40AN	80AN
P (P)	*Anacardiaceae*	*Pistacia lentiscus*	-/0	-/0	+/+	-/0
	Caprifoliaceae	*Lonicera implexa*		/(+)		
	Cistaceae	*Cistus crispus*	/(+)	/(+)	(-)/+	+/-
		Cistus ladanifer	+ b/+ b	+ b/+ ab	+ b/+ b	- a/- a
		Cistus monspeliensis		+/-		(+)/(+)
		Cistus salvifolius	+/+	+/-	+/0	-/-
		Halimium halimifolium	+/0			
	Ericaceae	*Arbutus unedo*		/(+)		-/0
		Calluna vulgaris	+/+	+/0	+/+	+/-
		Erica arborea		+/+	+/+	
		Erica scoparia	+/+	+/+	+/+	+/+
		Erica umbellata				+/+
	Fabaceae	*Genista triacanthos*	+/+	+/+	+/+	0/-
		Ulex densus	+/0 b	-/- a	+/+ b	+/+ b
	Fagaceae	*Quercus coccifera*		+/(-)	+/+	0/+
		Quercus lusitanica	/(+)			/(+)
		Quercus suber			0/0	
	Lamiaceae	*Lavandula stoechas*	+/0 ab	-/- a	+/+ b	0/+ b
		Rosmarinus officinalis		(+)/(+)	/(+)	+/0
		Salvia sclareoides	(-)/(-)			(+)/(+)
	Myrtaceae	*Myrtus communis*	+/0	0/0	+/0	-/0
	Oleaceae	*Olea europaea* var *sylvestris*			+/0	+/0
		Phillyrea angustifolia	/(+)		/(+)	+/+
	Pinaceae	*Pinus pinaster*		-/0		+/0
	Rhamnaceae	*Rhamnus alaternus*			+/+	/(+)
	Rosaceae	*Prunus* sp		/(+)		/(+)

Table 7.2 (continued)

Life form	Family	Species	Control	40A	40AN	80AN
P (P)	*Rosaceae*	*Rubus ulmifolius*	(-)/0	(+)/(+)	-/+	
	Thymelaeaceae	*Daphne gnidium*	0/0 ab	+/+ b	-/- a	(+)/(+) ab
P (C)	*Asteraceae*	*Dittrichia viscosa*	- a/- a	+ b/0 b	+ b/- ab	+ b/- b
		Helichrysum stoechas	(-)/(-)	/(+)		-/-
		Phagnalon saxatile		/(+)		(-)/-
	Boraginaceae	*Lithodora prostrata*	0/0		+/0	(+)/
	Lamiaceae	*Thymus villosus*				/(+)
	Rubiaceae	*Rubia peregrina*		/(+)		(+)/(+)
P (G)	*Apiaceae*	*Conopodium* sp			/(+)	
	Asphodelaceae	*Asphodelus ramosus*	(-)/(-)			(+)/(+)
	Asteraceae	*Carlina corymbosa*		/(+)	(+)/(+)	/(+)
	Hyacinthaceae	*Urginea maritima*	/(+) b	(+)/(+) b		(-)/- a
	Iridaceae	*Gladiolus illyricus* ssp *reuteri*		(+)/(+)	(+)/	(+)/(+)
		Iris xiphium				(+)/
	Orobanchaceae	*Orobanche latisquama*	(+)/			
	Ranunculaceae	*Anemone palmata*	(+)/(+)		/(+)	
P (H)	*Apiaceae*	*Daucus carota*			(-)/(-)	
	Asteraceae	*Andryala ragusina*	-/0			
		Cynara sp	(-)/-	/(+)	/(+)	
		Leontodon taraxacoides	-/-	-/-	+/-	0/-
		Pulicaria odora			(-)/0 a	(+)/(+) b
		Senecio jacobaea	(+)/		(-)/(-)	
	Campanulaceae	*Campanula rapunculus*		0/+	(-)/(-)	/(+)
	Cyperaceae	*Carex flacca*	+/-	+/+	+/+	-/-
	Fabaceae	*Anthyllis vulneraria*				(+)/
	Gentianaceae	*Blackstonia perfoliata*	-/-	-/-	(-)/-	0/+
		Centaurium erythraea	-/-	-/-	(-)/(-)	-/-
	Hypericaceae	*Hypericum* sp	-/- a	-/- ab	-/- b	/(+) b
	Lamiaceae	*Prunella laciniata*	/(+)			
	Poaceae	*Agrostis* sp	(+)/			
		Arrhenatherum album		+/(-)		(-)/(-)
		Avenula sp	(+)/	/(+)		/(+)
		Brachypodium phoenicoides	-/(-)	0/0	-/-	+/+
		Dactylis glomerata				(+)/(+)
	Rosaceae	*Sanguisorba hybrida*	/(+)	/(+)	(-)/-	(-)/(-)
nd(H/T)	*Asteraceae*	*Centaurea melitensis*	(+)/			/(+)
		Crepis capillaris		(+)/		

Table 7.2 (continued)

Life form	Family	Species		Control	40A	40AN	80AN
nd(H/T)	*Asteraceae*	*Picris echioides*		/(+)	(-)/+		+/+
	Fabaceae	*Lotus sp*		(+)/(+)	(+)/(+)	+/-	/(+)
		Trifolium sp		(-)/-		(-)/(-)	(-)/(-)
		Vicia sp			(+)/		
	Lythraceae	*Lythrum* sp			/(+)		
A (T)	*Asteraceae*	*Crysanthemum coronarium*			(+)/		
		Evax pygmaea					(+)/
		Filago minima		-/-	(-)/0	(+)/	(-)/-
		Galactites tomentosa		-/(-)	(-)/-	(-)/(-)	(-)/-
		Matricaria recutita		(-)/(-)	(-)/(-)	(-)/(-)	
		Sonchus sp		(-)/(-) a	/(+) b	(-)/0 a	(+)/(+) ab
	Campanulaceae	*Solenopsis laurentia*		/0 a	/(+) b		/(+) ab
	Lamiaceae	*Stachys arvensis*		(+)/	(+)/	(+)/	
	Poaceae	*Briza maxima*				(+)/(-)	(+)/
		Briza minima			(-)/		(-)/
		Briza minor		(-)/(-)		0/(-)	(+)/
		Gastridium ventricosum		/(+) a	/(+) b		/(+) a
	Primulaceae	*Anagallis arvensis*		(+)/(+)	-/-	+/-	+/0
	Scrophulariaceae	*Kickxia cf. cirrhosa*		/(+)		/(+)	/(+)
nd	*Apiaceae*	*Apiaceae*		(+)/		/(+)	(+)/(+)
	Asteraceae	*Asteraceae*		(-)/(-)	(-)/(-)	(+)/	/(+)
	Boraginaceae	*Boraginaceae*					/(+)
		Echium sp			/(+)		
	Cruciferae	*Cruciferae*			/(+)		
	Fabaceae	*Fabaceae*					(-)/(-)
	Liliaceae	*Liliaceae*		(+)/		(+)/	
	Malvaceae	*Malvaceae*		/(+)			
	Poaceae	*Poaceae*		0/-	/(+)	-/(-)	0/-
		Vulpia sp		(-)/(-)	(-)/(-)		(-)/(-)
	Rubiaceae	*Galium* sp			/(+)	(+)/	(+)/(+)
		Rubiaceae					(+)/
Number of species that:		Increased	+	10/5	12/8	19/13	13/9
		Decreased	-	10/10	9/10	5/9	8/15
		Maintained	0	3/10	3/7	2/7	6/7
		Appeared	(+)	11/13	9/24	8/8	18/26
		Disappeared	(-)	11/10	7/5	12/10	10/5

Based on a consistent response after one and 4.5 years of N addition treatments (Table 7.2), it was possible to identify groups of species that responded similarly to the N dose or form. No species appeared to be indicative of low N availability since there was no species that significantly reduced or disappeared from all the N addition treatments but not from the control. The cover of *D. viscosa* and *Sonchus* sp. decreased under all treatments, although N appeared to offset some of the cover loss seen in the control. *Carlina corymbosa*, *Gladiolus illyricus* ssp. *reuteri* and *Galium* sp appeared under all N addition treatments. *Salvia sclareoides*, *Asphodelus ramosus*, *Blackstonia perfoliata* and *Dactylis glomerata* were only present in the high N dose (80AN).

In terms of response to the N form: (i) *Pulicaria odora* was only present in the N treatments containing NO_3^- (40AN and 80AN); (ii) *Sanguisorba hybrida* disappeared (or at least decreased its cover) in the plots receiving NO_3^- (40AN and 80AN); (iii) *Rubia peregrina* and *Brachypodium phoenicoides* were only present with high NH_4^+ (40A and 80AN); and (iv) *Anemone palmate* was not found with high NH_4^+ (40A and 80AN).

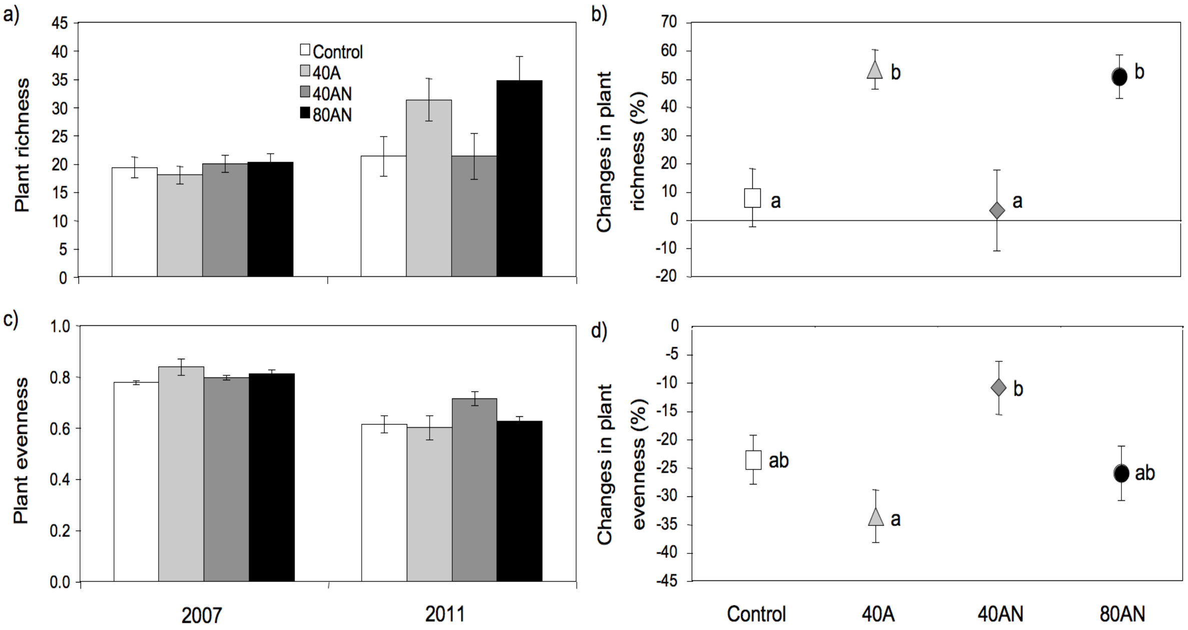

Figure 7.2 – Response of the vascular plant community to the N treatments (Control, 40A, 40AN and 80AN) in terms of plant richness (a-b) and evenness (c-d). Community assessments were performed on the first and fifth springs of N additions: 2007 and 2011 respectively. Changes in each parameter between 2011 and 2007 were calculated as described in materials and methods. Different letters refer to statistically significant differences between treatments (ANOVA $p<0.05$ followed by a Bonferroni test). There were no significant interactions between treatment and time ($p<0.05$). Symbols represent the mean (n = 3 experimental plots per treatment) ± SE.

Effects of the N treatments on plant diversity

From 2007 to 2011 plant species richness (Fig. 7.2-a, b) did not change in control or 40AN plots, but increased (≈ 60% in relation to the control) in both treatments receiving 40 kg

NH_4^+-N (40A and 80AN). Linear, positive and significant ($p<0.001$) correlations were found between plant richness and cumulative N, NH_4^+ and NO_3^-, but the highest correlation was between plant richness and cumulative NH_4^+ addition (Fig. 7.3). During the same period, plant evenness decreased with time (Fig. 7.2-c, d), though least with the addition of 40AN (Fig. 7.2-d).

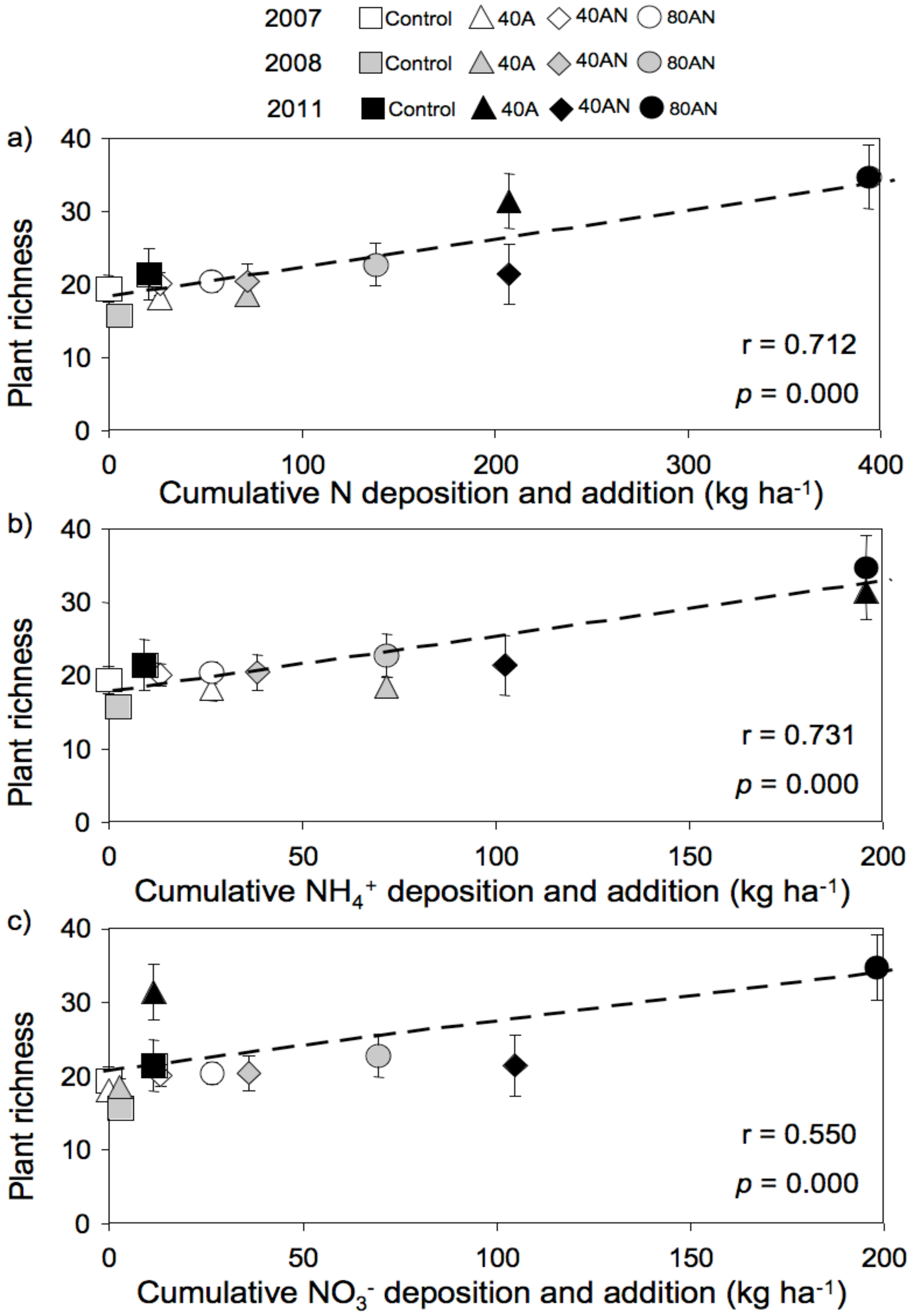

Figure 7.3 - Correlation between plant richness and the cumulative N (a), NH_4^+ (b) and NO_3^- (c) over the experiment and accounting for the estimated background deposition (EMEP- see material and methods). Symbols represent the mean (n = 3 experimental plots per treatment and per year) ± SE but correlations were based on the individual values (n = 36).

Effects of the N treatments on plant biomass and bare soil

The addition of 40AN and 80AN resulted in higher aboveground standing biomass in relation to the control (Fig. 7.4-a). Moreover, taking in consideration the estimated leaf litter produced for the period 2007-2011 (Fig. 7.4-b) the total aboveground biomass produced by the 40AN was higher than that of the control plants (Fig. 7.4-c). In the first spring of the experiment (2007), the % of bare soil in the control plots tended to be higher than in the N addition treatments (Fig. 7.5-a). However, differences between treatments were visible by the second, and fifth springs of the experiment (Fig. 7.5-a). Over the experiment, the % of bare soil in Control and 40AN plots decreased while that in the 40 kg NH_4^+-N ha^{-1} yr^{-1} plots (40A and 80AN), increased. In terms of changes in relation to the beginning of the experiment (Fig. 7.5-b), the addition of 40AN resulted in the most effective reduction of bare soil (almost no bare soil – Fig. 7.5-a) while the addition of 40A led to a loss of plant cover and therefore the % of bare soil increased.

DISCUSSION

Effects of increased N on superficial soil properties

Soils at the experimental site were lower in C (1.6-2.2 %) and N (0.1 % - Table 7.1) than normally reported for the Mediterranean Basin: 2.5-3.5 % C and 0.16-0.2 % N (Gallardo *et al.* 2000; Sardans *et al.* 2008; Rutigliano *et al.* 2009). N addition treatments failed to change soil C and N concentrations, or the C/N ratio (Table 7.1), which remained within the range reported for the Mediterranean Basin (Sardans *et al.* 2008). Soil acidity, likewise was not affected by the N additions, contrary to most studies (Bobbink *et al.* 2010 - Table 7.1), possibly due to the high soil calcium carbonate content (Schultz 2002). By contrast after 4.5 years, application of the highest N dose caused the inorganic N pools and soil organic matter to increase (Table 7.1). Soil inorganic N pools are both spatially and temporally highly dynamic and may reflect more the sampling conditions than soil characteristics (Cruz *et al.* 2008). However, soil nitrate concentrations in the 80AN treatment were higher than in the control in both assessments but especially after 4.5 years of treatment. The higher soil nitrate concentration may reflect an imbalance between nitrate demand and availability in the soil and may indicate the transition from a closed N cycle to an open and leaky one where N may be lost through NO_3^- leaching (De Schrijver *et al.* 2008). High N (80AN) does appear to have increased soil fertility and C retention, contrary to what has been observed in several studies (Knorr, Frey & Curtis 2005). Altogether it can be concluded that the cumulative N inputs have increased the 'N status' of the ecosystem (Padgett *et al.* 1999; De Schrijver *et al.* 2011).

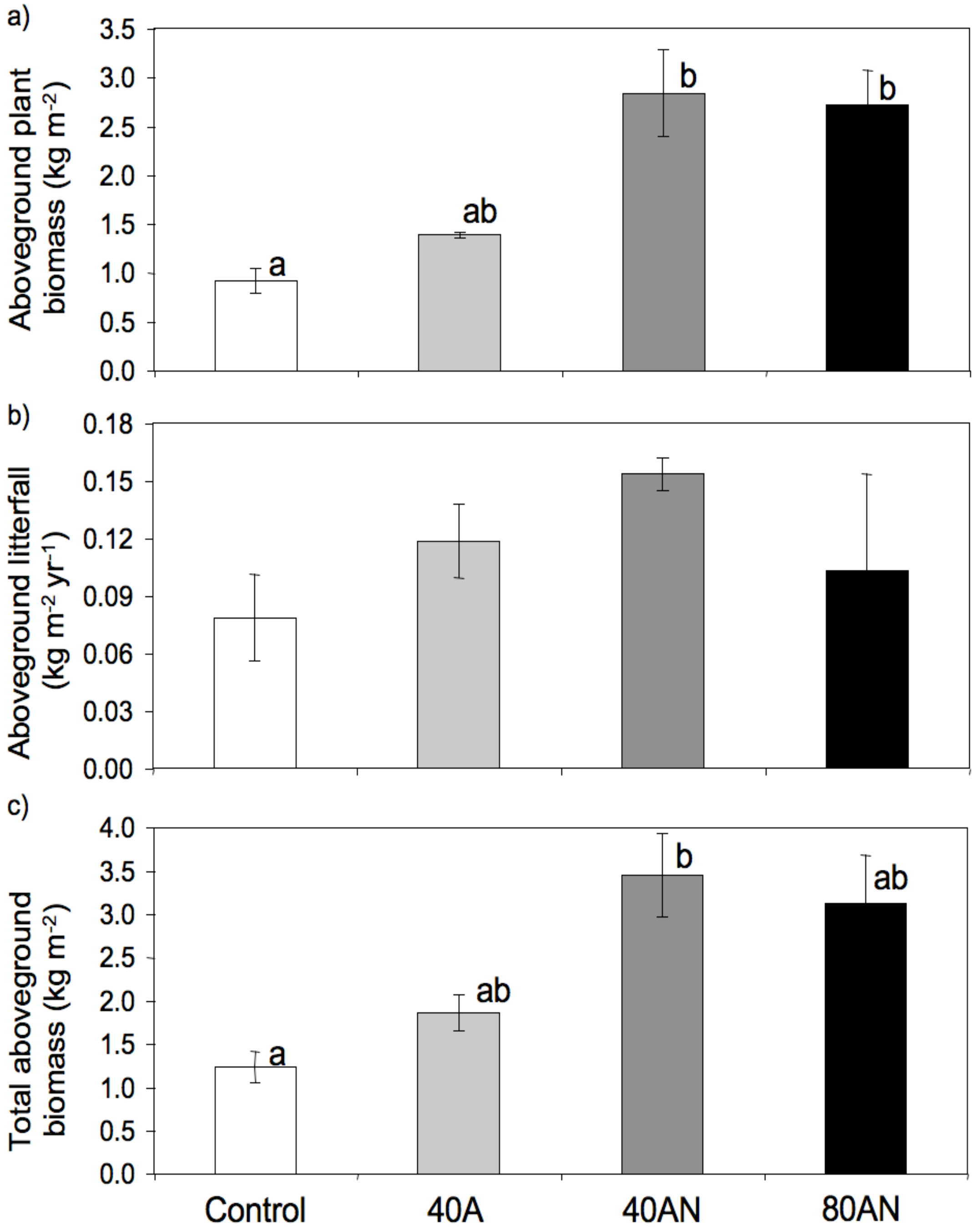

Figure 7.4 – Aboveground plant biomass (a), estimated litterfall production per year (b) and total aboveground biomass (sum of the standing biomass and the cumulative litterfall produced on the previous four years - c) according to the N treatments (Control, 40A, 40AN and 80AN). Aboveground plant biomass was harvested on June 2011 (the fifth spring after the beginning of the experiment) on three 1-m^2 squares per experimental plot. Litterfall was collected from April to December 2010 using litter traps (five 0.04 m^2 per experimental plot). Different letters refer to statistically significant differences between treatments (ANOVA *p*<0.05 followed by a Bonferroni test). Bars represent the mean (n = 3 experimental plots per treatment) ± SE.

Effects of increased N on plant cover

The number of plant species found at the experimental site (Table 7.2) was consistent with what would be expected for a maquis (70 ± 54 species per 1000 m^2, Cowling *et al.* 1996) regenerating after a fire event (summer 2003). After a fire, there is a ‘window of opportunity’, i.e., a period of reduced competition for light, nutrients and water (Lavorel *et al.* 1994; Lavorel, O‘Neill & Gardner 1994), which favours the establishment of ruderal species (Thompson 2005). In a post-fire succession, as the ‘window of opportunity’ passes (~ 5 years after the fire), some plant species tend to disappear from the community (Casal 1987, Trabaud 1987). *Asphodelus ramosus* (Sakar *et al.* 2010), *Dittrichia viscosa* (Lavorel *et al.* 1994) and *Sonchus* sp (Bonet & Pausas 2004) are examples of such species (Table 7.2). This natural dynamic of the plant community (Thompson 2005; Ochoa-Hueso *et al.* 2011) has to be taken into account as a background against which the population dynamics associated with the N additions have to be superimposed.

One year of N additions was sufficient to change the structure and composition of this plant community (chapter 2, Dias *et al.* 2011a). However, even after 4.5 years, the community was still changing (Table 7.2 and Fig. 7.2-a, b). After one year *Dittrichia viscosa* was the only N-benefited species (irrespective of dose and form – Table 7.2). In subsequent years, its cover, together with that from *Sonchus* sp, began to decrease, which is consistent with their disappearance in late succession (Lavorel *et al.* 1994; Bonet & Pausas 2004). However, N additions appeared to offset some of the cover loss seen in the control, suggesting that increasing N availability extends the ‘window of opportunity’ for more nutrient demanding plants. Other plant species responded more consistently along the 4.5 years to the N dose and/or form. The presence of *Carlina corymbosa*, *Gladiolus illyricus* ssp *reuteri* and *Galium* sp may indicate increased N, while that of *Salvia sclareoides*, *Asphodelus ramosus*, *Blackstonia perfoliata* and *Dactylis glomerata* may indicate high N availability (80AN). In terms of response to the N form, *Pulicaria odora* appears to prefer NO_3^-, while *Sanguisorba hybrida* appears to avoid NO_3^- indicating it is characteristic of later phases of succession Finally, *Rubia peregrina* and *Brachypodium phoenicoides* appear to prefer high NH_4^+ availability whereas *Anemone palmate* appears to be rather sensitive to NH_4^+. No species appeared to indicate low N availability.

Effect of increased N on plant diversity

In this Mediterranean Basin ecosystem, enhanced N availability continues to promote vascular plant diversity even after 4.5 years (Table 7.2 and Figs 7.2-a, b and 7.3) unlike most studies (Bobbink *et al.* 2010; De Schrijver *et al.* 2011). Assuming that N limitation represents a severe environmental limitation, the N-driven increase in diversity may be explained by the revised Grime’s humped-back model (Grime 1973; Michalet *et al.* 2006). The diversity increment would reflect the initial alleviation of the stress condition (N limitation), allowing the coexistence of the characteristic site species with the incoming exploitative ones. This rationale is supported by the fact that N-benefited species were those

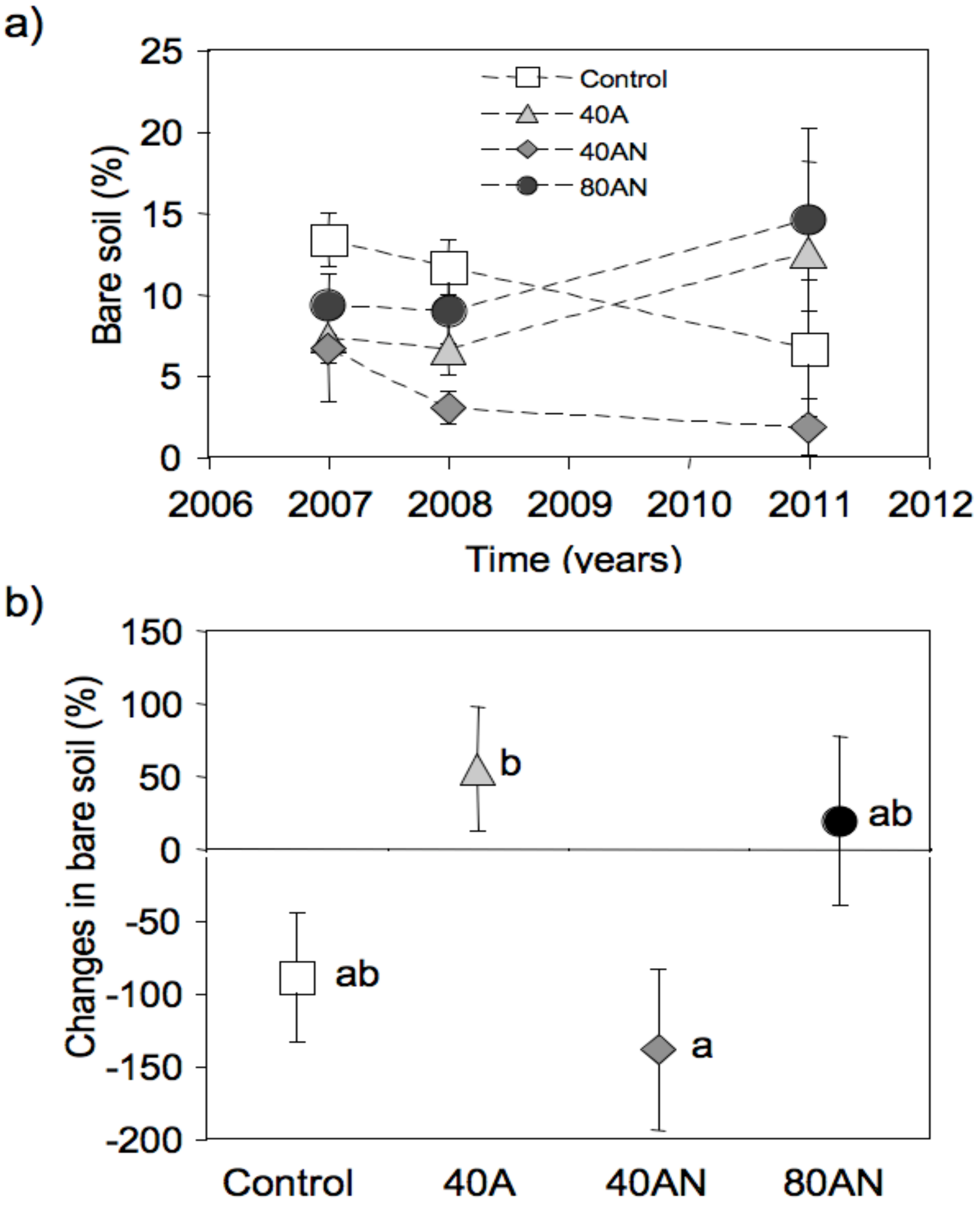

Figure 7.5 – Response of the bare soil to the N treatments (Control, 40A, 40AN and 80AN) in terms of % of bare soil on the first, second and fifth springs of N additions (2007, 2008 and 2011 respectively – a) and its changes between 2007 and 2011 (see materials and methods – b). Different letters refer to statistically significant differences between treatments (ANOVA $p<0.1$ followed by a Bonferroni test). There were no significant interactions between treatment and time ($p<0.05$). Symbols represent the mean (n = 3 experimental plots per treatment) ± SE.

typical of disturbed and/or eutrophied sites (Lavorel *et al.* 1994; Lavorel, O'Neill & Gardner 1994, Table 7.2), while N-affected species were characteristic of nutrient poor sites (Casals, Romanya & Vallejo 2005; Clemente, Rego & Correia 2005 - Table 7.2). This humped effect, when observed, is typically transient (De Schrijver *et al.* 2011). Due to the relatively high N dose added in the 80AN treatment, it was expected that after 4.5 years of N additions plant diversity would not continue to increase, or even start to decrease. But that did not happen.

Some non-exclusive factors may be contributing to this sustained increase in plant diversity: (i) The difference in the life span of the N-benefited species (with short life cycles) in relation to the N-affected ones (perennials), which would allow the detection of the incomers but only drastic effects on the losers. This dichotomy of N-benefited short-lived *versus* N-affected perennials has also been observed by Allen *et al.* (2007) in mixed coniferous forests in California, and by Suding *et al.* (2005) when analyzing the response of more than 900 species from 34 N-addition experiments across nine terrestrial ecosystems in North America; (ii) The ecosystem is also limited by water and phosphorus (P), so that the most aggressive/competitive species do not have the best conditions for their development. Not surprisingly, most N-benefited species were annuals that avoid summer drought in the form of seeds but are highly dependent on adequate supplies of N and P (Fanelli, Lestini & Sauli 2008), which under N additions are more common (chapter 2, Dias *et al.* 2011a). In California, where P availability is higher (Cowling *et al.* 1996), some Mediterranean Basin species (e.g. *Schismus barbatus, Bromus madritensis*, *Erodium cicutarium* - Allen *et al.* 2009 - and *Sonchus* sp – Cione, Padgett & Allen 2002) have become invasive, out competing and displacing endemic vegetation, particularly in wet years (Cione, Padgett & Allen 2002; Everard *et al.* 2010; Rao & Allen 2010); (iii) A large part of the added N is being lost from the ecosystem (De Schrijver *et al.* 2008). This is an obvious hypothesis but one for which there is no supporting data. In the same experimental site after one year of N additions soil inorganic N reflected the cumulative N additions (chapter 5, Dias *et al.* 2012). NO_3^- leaching in similar Mediterranean habitats has only been performed for Californian chaparral (Fenn, Allen & Geiser 2011). In a burnt chaparral, although there was an increase in NO_3^- leaching with increasing N deposition, the N loss was smaller than expected due to the rapidly growing vegetation that immobilised much of the deposited N (Meixner *et al.* 2006). Indeed, accumulation of vegetative biomass in that system was estimated to be73 kg N ha^{-1} yr^{-1} (Riggan *et al.* 1988), which is similar to our higher N dose (80 kg N ha^{-1} yr^{-1}). To quantify how much N (or NH_4^+) the system could potentially ‘process’ before entering the descending zone of the humped model, longer-term/higher cumulative N studies are necessary.

Effect of increased N on plant productivity and soil protection

The aboveground biomass accumulated under control conditions was within the range of the equivalent Californian habitat – coastal sage scrub (Vourlitis, Pasquini & Mustard 2009). Under increased N, and in agreement with what has been reported for other temperate (Bobbink *et al.* 2010) and Mediterranean ecosystems (Fenn *et al.* 2003; Allen *et al.* 2005; Bonanomi, Caporaso & Allegrezza 2006), aboveground plant biomass increased. Positive relationships between plant diversity and productivity have been reported for Mediterranean

(Caldeira, Loreau & Pereira 2005) and several other ecosystems (Tilman, Wedin & Knops 1996; Pfisterer *et al.* 2004; Cardinale *et al.* 2007). However in this study plant diversity (Fig. 7.2-a, b) and plant productivity (Fig. 7.4-c) were not related since productivity and diversity were promoted by particular N sources. Treatments 40A and 80AN promoted species richness (Table 7.2 and Fig. 7.2-a, b), but the incomers were small plants that did not contribute much to productivity or to protect soil from erosion as a consequence of decreased permanent plant cover (Fig. 7.5). In Mediterranean areas, vegetation's spatial structure plays a significant role in preventing soil loss and erosion (López-Bermúdez & García-Gómez 2006). This suggests that agriculture, the main source of enhanced NH_4^+-N availability, may affect the structure of the maquis in the surrounding areas and promote soil erosion. By contrast, the lower NH_4^+-N dose of the combined N form (40AN), with fewer plant species, improved productivity (Fig. 7.4) and prevented soil erosion most effectively (Fig. 7.5). However, in Mediterranean ecosystems increased productivity and biomass accumulation, represent an increased fire risk, which may affect vegetation dynamics, potentially altering species composition, vegetation structure (Zedler, Gautier & McMaster 1983; Keeley 1986; Vallejo & Alloza 1998) and decrease ecosystem resilience (Díaz-Delgado *et al.* 2002). Data suggest that industrial and urban activities, responsible for enhanced NO_3^--N availability may promote biomass accumulation and therefore increase the fire risk.

Altogether results highlight the importance of cumulative N and enhanced N availability (and particularly NH_4^+) as a driving force behind the dynamics and stability in plant community structure and composition of Mediterranean ecosystems (Cruz *et al.* 2008, Fig. 7.3). Accordingly, the effectiveness of the N critical loads (a quantitative estimate of exposure to N deposition below which significant harmful effects on sensitive elements of the environment do not occur according to present knowledge - Nilsson & Grennfelt 1988), which were set for this European habitat, at between 20 and 30 kg N ha^{-1} yr^{-1} (Bobbink *et al.* 2011) would be improved if it included the cumulative N effect (De Schrijver *et al.* 2011).

CONCLUSIONS

As far as we are aware, this is the first integrated study of the effects of different N doses and forms on a Mediterranean Basin ecosystem. Data show that the effects of N on the surface soil properties depended on the treatment and on the cumulative N dose, while plant community richness was particularly responsive to the NH_4^+ dose (40 kg ha^{-1} yr^{-1} as 40A and 80AN) and productivity was improved only in the presence of the smaller NH_4^+ dose (40AN). The N-benefited species were small and/or ephemeral plants (geophytes, hemicryptophytes and therophytes) typical of disturbed and/or eutrophied sites, while the N-affected species were perennial shrubs characteristic of nutrient poor sites. There were groups of species that responded consistently after one and five years to the N dose and/or form, suggesting that they could indicate the N status of the environment.

Data suggest that plant community composition is driven by the amount of NH_4^+-N, highlighting the important role of NH_4^+ as a driving force in Mediterranean ecosystems and suggesting that the calculations of the N critical loads should take into account the form of N and of NH_4^+ in particular. Therefore agriculture, the main source of enhanced NH_4^+-N availability, may affect the structure of the maquis in the surrounding areas and thus promote soil erosion as a consequence of decreased permanent plant cover. By contrast, industrial and urban activities, responsible for enhanced NO_3^--N availability may promote biomass accumulation and therefore increase the fire risk. This information is highly relevant for land use management in biodiverse and fragmented ecosystems such as the Mediterranean ones, especially in Natura 2000 sites.

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Chapter 8

General Discussion

General Discussion

This research was undertaken at a Natura 2000 site (PTCON0010 Arrábida/Espichel) comprising a Mediterranean Basin scrubland. Such sites are biodiversity hotspots and given their low level of nutrient availability, are expected to be highly nitrogen (N) sensitive. However, except for similar systems in California, few studies have been undertaken to determine what the increasing anthropogenic reactive N deposition will do to these ecosystems. Due to the fundamental differences in the patterns of rainfall (dry summers) and of N deposition (the dry N deposition dominates) there is an asynchrony between N availability and biological activity. Also, the distinct soil characteristics (low nutrients and organic matter and high bases such as carbonates) and the natural presence of fire distinguish these ecosystems from the well studied temperate ones, so that lessons learned from them concerning N impacts may not be applicable. Even within Mediterranean-type ecosystems, differences in water and phosporus (P) availability compromise extrapolations from Californian ecosystems to those in the Mediterranean Basin. Thus, in 2007 a field manipulation experiment (N dose and form) was established in a site with low background N deposition (5.2 kg ha^{-1} yr^{-1} = 2.9 kg NO_x + 2.3 kg NH_y - EMEP). The N doses were lower than the N deposition reported for other areas in Mediterranean-type ecosystems (145 kg N ha^{-1} yr^{-1} - Fenn *et al.* 2003; Meixner & Fenn 2004) but high enough to establish 'worst case' scenarios of N enrichment in this type of habitat. The N forms were chosen to mimic the most likely N pollution scenarios within the Mediterranean Basin, i.e., combined inputs from urban/industrial sites and agricultural (addition of nitrate and ammonium) and predominantly agricultural sources (addition of ammonium). Therefore, N availability was modified by the addition of 40 and 80 kg N ha^{-1} yr^{-1} in the form of NH_4NO_3 (designated 40AN and 80AN) and 40 kg N ha^{-1} yr^{-1} as a 1:1 mixture of NH_4Cl and $(NH_4)_2SO_4$ (designated 40A). Control plots were not fertilized. Over the next 4.5 years selected effects on the structure and functioning of above and below ground fractions were monitored.

Key questions addressed included the effects of increased N on above and below ground diversity, plant N use strategies, soil chemistry, N cycling/retention and organic matter decomposition. Based on these data, recommendations concerning N critical loads were made, indicators of N deposition were identified (structural and functional) and the implications of these changes for ecosystem sustainability and functioning were evaluated, bearing in mind that the dynamics of Mediterranean plant communities is closely related to the occurrence of fires. Conceptual scenarios for the effects of increased N availability (mainly NH_y or NO_x) on Mediterranean Basin ecosystems, according to the phase of post-fire succession, were developed.

ABOVE GROUND N-DRIVEN SPECIES CHANGE: A POSITIVE OR NEGATIVE EFFECT ON DIVERSITY?

Mediterranean post-fire succession

The standing vegetation consisted of a dense maquis (Eunis class F5.2 – Mediterranean maquis), which had developed after a fire event (summer 2003), four years before the first N addition. Accordingly, vascular plant diversity was high (70 ± 54 species per 1000 m^2, Cowling *et al.* 1996 - chapters 2 and 7).

Under 'normal' post-fire successions, in the longer-term, summer semi-deciduous plants are progressively eliminated under the canopies of evergreen sclerophylls that characterize late successional phases (Correia & Catarino 1994). In the short-term, and considering the early phase of succession of the standing community, other changes in the vegetation composition occurred: the 'window of opportunity' that follows a fire, i.e., period of reduced competition for light, nutrients and water (Lavorel *et al.* 1994; Lavorel, O'Neill & Gardner 1994), favoured the establishment of many ruderal species (Thompson 2005). However, as this 'window of opportunity' passed, these ruderal species tended to disappear from the community (Casal 1987; Trabaud 1987). *Asphodelus ramosus* (Sakar *et al.* 2010), *Dittrichia viscosa* (Lavorel *et al.* 1994 – chapter 7) and *Sonchus* sp (Bonet & Pausas 2004) are examples of such species. Also, plant evenness decreased (Naeem 2009). These dynamics of the plant community are indicative of an ongoing post fire succession.

Importance of time/dose and N form on N effects on biodiversity

In the present Mediterranean Basin ecosystem, increased N availability promoted vascular plants diversity. After only one year, species changed most in the higher N dose (80AN – chapter 2). In the fifth spring (chapter 7), changes were still being expressed especially in the plots that received more reduced N (40A and 80AN) highlighting the importance of this N form for Mediterranean ecosystem's species diversity.

Dittrichia viscosa was the only species that responded significantly after only one year of increased N (irrespective of dose and form – chapter 2). But in subsequent years, its cover had begun to decrease, although N appeared to offset some of the cover loss seen in the control (chapter 7), which is in agreement with its disappearance in later phases of succession (Lavorel *et al.* 1994). But other plant species responded more consistently to the N dose and/or form and some responses were compatible with the species common habitat and/or with their responses in other N enrichment studies (Fig. 8.1).

The N-driven increased plant diversity, which contrasts with most studies (Bobbink *et al.* 2010; De Schrijver *et al.* 2011), reflected the initial alleviation of the stress condition (N limitation), thus allowing the coexistence of the characteristic site species with the incoming exploitative ones. Assuming the N limitation as a factor of environmental severity, the N-driven increased diversity may be explained by the revised Grime's humped-back model (Grime 1973; Michalet *et al.* 2006). The N-benefited species were those typical of disturbed and/or eutrophied sites, able to profit from the N 'window of opportunity' (Lavorel *et al.* 1994; Lavorel, O'Neill, & Gardner 1994) while N-affected were those characteristic of sites very poor in nutrients (Clemente, Rego & Correia 2005).

Did increasing biodiversity lead to increased productivity?

In this study, the N addition treatments with more plant species (40A and 80AN) did not produce more aboveground biomass (chapter 7), contrasting with results from temperate (Tilman 1996; Pfisterer *et al.* 2004; Cardinale *et al.* 2007) and Mediterranean grasslands (Caldeira, Loreau & Pereira 2005), tundra, estuaries and temperate bryophyte assemblages (Cardinale *et al.* 2007). In our study the 'new' species tended to be small plants (some of which were annuals – e.g. *Solenopsis laurentia*) that did not contribute much to productivity or to protect soil from erosion (chapter 7). The low NH_4^+ tolerance of the standing plant community (dominated by summer semi-deciduous – Cruz, Lips & Martins-Loução 2003; Kronzucker *et al.* 2003; chapter 4) could also have prevented some plants from taking advantage from the alleviation of the N limitation. By contrast the combined N form with less NH_4^+ (40AN) increased aboveground biomass and prevented soil erosion most effectively (chapter 7). But this was achieved at the expense of the small plants which were unable to tolerate the increased shading (Aerts *et al.* 1990). In these plant communities there appears to be a trade-off between plant diversity and productivity/soil protection.

Implications of N deposition for land use management

The N-driven decreased soil cover (chapter 7) may have implications in terms of the risk of soil erosion since in Mediterranean areas, vegetation's spatial structure plays a significant role in preventing soil loss (López-Bermúdez & García-Gómez 2006). This suggests that agriculture, the main source of enhanced NH_4^+-N availability, may affect the structure of the maquis in the surrounding areas and promote soil erosion. The N-driven increased plant productivity (chapter 7) may have implications in terms of the fire risk, interval between fires (likely to decrease) and intensity of burn (likely to increase). This may affect vegetation dynamics, potentially altering species composition, vegetation structure (Zedler, Gautier & McMaster 1983; Keeley 1986; Vallejo & Alloza 1998) and decreasing ecosystem resilience (Díaz-Delgado *et al.* 2002). It has been widely suggested that high fire recurrence maintains early successional communities (Pausas 1999; Walker & Reddell 2007), resulting in the conversion of forests to shrublands (Mermoz, Kitzberger & Veblen 2005; Donato *et al.* 2009) and chaparral to coastal sage scrub (Keeley, Baer-Keeley & Fotheringham 2005; Syphard, Flanklin & Keeley 2006; Baeza *et al.* 2011), so that increased N availability may impact landscape. Therefore industrial and urban activities, responsible for enhanced NO_3^--N availability may promote biomass accumulation and therefore increase the fire risk. These informations are highly relevant for land use management in biodiverse and fragmented ecosystems such as the Mediterranean ones, especially in Natura 2000 sites.

KEY FINDINGS

- Increased N promoted vascular plant diversity (relation between plant richness and cumulative N and NH_4^+);
- Some plant species appear to be consistent responders to N dose and/or form;
- There was a trade-off between plant diversity and productivity/soil protection;
- NH_4^+ enrichment may drive soil erosion via decreased soil protection.
- NO_3^- enrichment may drive landscape changes via alteration in fire regime.

SOIL MICROBIAL N-DRIVEN CHANGES: A POSITIVE OR NEGATIVE EFFECT ON DIVERSITY?

The effect of N depended on dose and form

In the present Mediterranean Basin ecosystem, increased N availability also promoted the diversity of soil bacteria (Dias *et al.* 2011) and fungi (chapter 3). Within one year of N additions, changes in soil microbial diversity were observed especially in the higher N dose (80AN), showing a N-dose response. The effects of the N addition treatments on arbuscular mycorrhizal fungi (AMF) diversity appeared to depend on the N form, since the addition of 40A increased AMF spore richness and evenness proportionally more than the addition of 40AN (chapter 3).

The N addition treatments also changed the species composition of the AMF spores; with some responses being compatible with those obtained in other N enrichment studies (Fig. 8.2). The changes in soil fungal community were not related to changes in AMF spores (chapter 3) because the former integrate other types of mycorrhizal (e.g. ectomycorrhizal and ericoid), saprophytic and endophyte fungi. Based on studies on other ecosystems we can hypothesise that the N addition treatments reduced ectomycorrhizal fungal diversity (Lilleskov *et al.* 2002) while that of saprophytic fungi and/or endophytes (Janssens *et al.* 2010) increased. Based on what was observed, ecosystem functioning was influenced by the alterations in the soil microbial community as hypothesized by Fitter (2005).

How might N be affecting the above and below-ground communities? What are the linkages?

Since soil total carbon (C) was low (~2% - chapters 6 and 7), the concerted response of above and below ground diversity is likely to reflect a C limitation of soil microorganisms (Jackson & Caldwell 1992). Root exudates and root turnover therefore comprise important sources of C for soil microorganisms (Grayston *et al.* 2001). Also, the quality of the C may influence soil microbial communities. In agreement, the addition of litters differing in quality (N and lignin contents) significantly altered the soil microbial community structure (soil fungi and bacteria – chapter 6) and activity (CO_2 and N_2O fluxes – chapter 6). Therefore, increased N may alter soil microbial communities through quantity and quality of the C inputs.

Also, mycorrhizal fungi form ecologically important connections between plants and soils and most herbaceous species form associations with AMF. As a result, the effect of the N form on AMF diversity may be explained by the decrease of herbaceous plants under the 40AN addition treatment (chapter 7), thus highlighting the importance of above ground changes for particular biotic interactions.

Finally, and given that aboveground communities may be influenced by changes in soil microorganisms and vice-versa (Klironomos 2002; Brooker 2006), it is possible that after 4.5 years of N additions, like plant diversity, that of soil microorganisms responded to the amount of added NH_4^+ (chapter 7).

KEY FINDINGS

- Increased N promoted the diversity of soil bacteria and fungi;
- AMF diversity responded to the N form;
- Some AMF species appear to be consistent responders to the N dose and/or form;
- N-driven changes in the quantity and quality of the plant produced C may drive structural and functional changes in below ground communites.

N EFFECTS ON THE SOIL

Irrespective of the N addition treatments and of the duration of the experiment, soils at the experimental site had lower C and N concentrations (~ 2% / ~ 0.1%, respectively - chapter 7) than those reported for the Mediterranean Basin (e.g. > 3.5% / > 0.16%, Gallardo *et al.* 2000; > 2.5% / > 0.2, Sardans *et al.* 2008; > 3% / > 0.2%, Rutigliano *et al.* 2009). There were no effects of the N addition treatments on soil C/N ratio (~18 – chapter 7), which was within the range reported for the Mediterranean Basin (18 - Sardans *et al.* 2008).

Although N-driven acidification has been reported for some Mediterranean-type ecosystems (Californian forests – Breiner, Gimeno & Fenn 2007; Californian shrublands - Vourlitis, Pasquini & Mustard 2009; and Mediterranean Basin shrublands - Ochoa-Hueso & Manrique 2010) it was not observed in the present study (chapters 2, 5 and 7) possibly due to the high soil calcium carbonate content (Schultz 2002).

On the other hand, the effects of N on soil organic matter depended on treatment time/dose. In the spring of years one and two of the experiment (2007 and 2008, respectively), soil organic matter was not related to the N addition treatments (chapter 5). But in the spring of year five (2011), more soil organic matter accumulated in the higher N dose (80AN – chapter 7), which suggest that changes on the diversity of above (chapters 2 and 7) and below ground (chapter 3) communities will delay organic matter decomposition (chapter 6).

Similarly, the effects of N on inorganic N availability depended on the time of:

(i) Experiment; in the first and second springs of the experiment (2007 and 2008, respectively), soil inorganic N pools (NO_3^- and NH_4^+) were not related to the N addition treatments (chapter 5). But in the fifth spring (2011), soil inorganic N pools (NO_3^- in particular) were related to the N dose (chapter 7). Soil inorganic N pools are both spatially and temporally highly dynamic and may reflect more the sampling conditions than soil characteristics (Cruz *et al.* 2008). However, soil nitrate concentrations in the 80AN treatment were higher than in the control in the assessments but especially after 4.5 years of treatment. The higher soil nitrate concentration may reflect an imbalance between nitrate demand and availability in the soil and may indicate the transition from a closed N cycle to an open and leaky one where N is lost through NO_3^- leaching (De Schrijver *et al.* 2008). Altogether it can be concluded that the cumulative N inputs have increased the 'N status' of the ecosystem (Padgett *et al.* 1999; De Schrijver *et al.* 2011); and

(ii) Season; soil inorganic N pools (NO_3^- in particular) strongly reflected the N additions, in the first autumn (late October 2007), when levels almost matched the cumulative N added over the previous three annual additions (middle autumn/winter, spring and summer – chapter 5). Although climates with a pronounced dry season may have high N availability during the summer, a peak of extractable N may occur in autumn/winter as shown in Padgett *et al.* (1999) and Gallardo *et al.* (2000), coinciding with Mediterranean growing seasons (Ochoa-Hueso *et al.* 2011).

KEY FINDINGS

- Increased N did not alter soil's total C, N, the C/N ratio or lead to soil acidification;
- The higher N treatment increased soil organic matter and inorganic N pools.

EFFECTS OF N ADDITIONS ON N CYCLING IN A MEDITERRANEAN BASIN ECOSYSTEM

Temporal patterns of inorganic N availability and plant growth influence plant community and ecosystem dynamics (Augustine & McNaughton 2004). Therefore, the changes in the temporal pattern of soil inorganic N pools (chapter 5) may explain the short-term N-driven changes in the diversity of above- (chapter 2) and below ground (fungi – chapter 3; bacteria – Dias *et al.* 2011) communities. Under the mild temperatures and the less limiting water availabilities that characterize the Mediterranean spring and permit intense biological activity, most additional N would be taken up by plants and microorganisms and retained within the ecosystem's biotic compartment (chapter 5). Indeed this was demonstrated by the dominant plant species, *Cistus ladanifer*, that significantly changed its cover in response to the added N and increased the N concentration in its leaves and litter (chapter 5). However, during the dry summer, a significant proportion of the N returned to the soil through leaf shedding. In autumn, when water no longer limited biological activity, decomposition was stimulated returning N to the inorganic form through decomposition and mineralization processes causing soil inorganic N pools to peak in autumn. By transforming the applied N doses over the first year of experiment (40 and 80 kg N ha^{-1}) into soil inorganic N concentrations (20 and 40 µg N g^{-1} soil, respectively) the 'total N added' could be compared with the 'measured N' in autumn. These data suggest that N added as NH_4NO_3 (40AN and 80AN) was retained by the system and reflected on soil inorganic N pools. Whereas for the N added as NH_4^+ (40A) a significant proportion was not measured in the soil (chapter 5) but is not clear if the 40A-N was lost from the system (e.g. volatilization) and/or if this treatments' litter mineralization took longer and therefore it was not detected as inorganic N.

This was the first integrated field study on how a European Mediterranean ecosystem retains increased N, comparing different forms and doses: longer-term studies are needed to explore the generality of these observations. Although N retention was not measured beyond three years, plant diversity continued to increase even after 4.5 years of N addition treatments (chapter 7), with no loss so far, of the slow growing conservative plant species (Emmett 2007). Together, this may indicate that a significant part of the added N continues to cycle through the biotic compartment. As far as we are aware, there are no measurements on N retention/NO_3^- leaching in similar Mediterranean habitats, only for Californian chaparral. Meixner *et al.* (2006) showed that the N retention capacity of the fire-regenerating chaparral was high due to the accumulation of vegetative biomass, which was estimated to be similar to our higher N dose (73 kg N ha^{-1} yr^{-1} - Riggan *et al.* 1988). Also, the soil C/N ratio did not change over the experiment (chapter 7) so that even after 4.5 years of the N addition treatments, a significant fraction of the added N is expected to have been retained by this Mediterranean maquis. Given that it is mainly the vegetation that stores the added N, soil C/N ratio may not be a good indicator of N retention. If the N was retained, it is expected to alter ecosystem functioning.

KEY FINDINGS

- Soil inorganic N pools reflected cumulative N additions in autumn;
- The biotic compartment plays a very important role in N retention;
- Soil C/N ratio may not be a good predictor of N retention because the added N is mainly stored in the biotic compartment.

N-DRIVEN CHANGES IN ECOSYSTEM FUNCTIONING – DECOMPOSITION

Considering the integrated changes in the plant community (chapter 2), and not just that of individual species (chapter 5), on the effects of increased N availability on decomposition, provides an innovative and more integrative approach than has been employed up to now (chapter 6). After 2.5 years of N addition treatments, soil from the control plots and leaf litter from the four treatments (control, 40A, 40AN and 80AN) were collected from the N-manipulation field experiment for a laboratory controlled decomposition study. As expected from chapters 2 and 7, leaf litter consisted mostly of leaves from summer semi-deciduous shrubs. However, there was a change in the composition of the leaf litter produced only under the high N treatment (80AN), i.e., it had twice the amount of evergreen sclerophyll leaf litter compared to the other treatments (chapter 6) reflecting that in the short-term, N dose drives the changes in plant community (chapter 2). The leaf litter produced with 80AN had both higher lignin (due to the higher fraction of evergreen sclerophylls litter) and N concentration, resulting in lower C/N and lignin/N ratios. At the ecosystem level, the N-driven changes in plant community affected leaf litter traits (e.g. increased litter lignin and N content and decreased lignin/N ratio, all powerful determinants of litter decomposition rates - Aber & Melillo 1982; Parton *et al.* 1987; Taylor, Parkinson & Parsons 1989; Aber, Melillo & McClaugherty 1990; Knorr, Frey & Curtis 2005). As a result, organic matter decomposition in the microcosms containing 80AN litter was slower (with concomitant reduction in microbial biomass and activity) than in the 40 kg N ha^{-1} yr^{-1} (40A and 40AN) treatments (chapter 6). This implies that increasing N availability in Mediterranean maquis would select for plants with litter traits that delay decomposition and potentially increase soil organic matter accumulation. Although it is important to also quantify *in situ* leaf litter decomposition (e.g. litter bags), which would also account for the N-driven changes in the soil microbial community (Dias *et al.* 2011; chapter 3), at the end of the N-manipulation field experiment, soil organic matter concentration was higher in the 80AN plots (chapter 7). Thus, the accumulation of organic matter in the field confirms the slower decomposition rates observed in the laboratory. In addition, under natural conditions, organic matter accumulates much more in the soil patches under the influence of evergreen sclerophylls (~16%) than under that of summer semi-deciduous (~10% - Cruz *et al.* 2008).

KEY FINDINGS

- At the community level, the N treatments resulted in a trade-off in litter quality traits: increased N but also increased lignin;
- The N-driven integrated changes in plant community delayed organic matter decomposition;

INDICATORS OF INCREASED N AVAILABILITY IN MEDITERRANEAN MAQUIS

Structural indicators of increased N availability

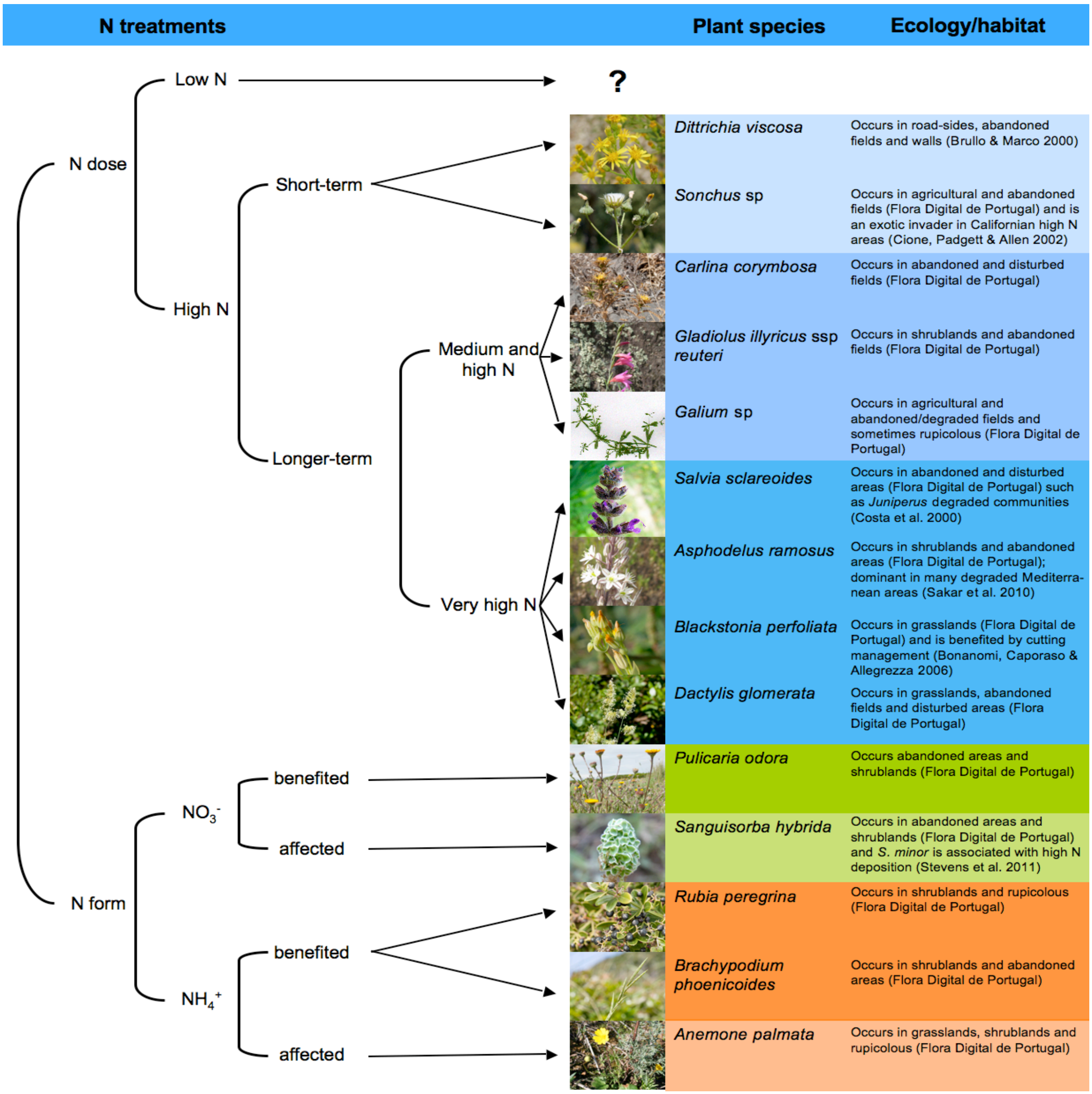
If the observed changes in plant (Fig. 8.1) and AMF (Fig. 8.2) species composition are robust over the longer-term, these may provide a sensitive indicator of N enrichment, at least in Mediterranean ecosystems.

Are there any consistent plant responders to low/high N or NH_4^+?

Based on a consistent response after one and five years the N addition treatments (chapter 7), there were groups of species that responded similarly to the N dose or form (Fig. 8.1). No species appeared to be indicative of low N availability. *Carlina corymbosa*, *Gladiolus illyricus* ssp *reuteri* and *Galium* sp may indicate high N availability since they were present under all N addition treatments. *Salvia sclareoides*, *Asphodelus ramosus*, *Blackstonia perfoliata* and *Dactylis glomerata* may indicate very high N availability since they were only present under the high N dose (80AN). Some species did show preferences for the form of N: *Pulicaria odora* preferred NO_3^- whereas *Sanguisorba hybrida* appeared to avoid it, suggesting that it is characteristic of later phases of succession. The presence of *Rubia peregrina* and *Brachypodium phoenicoides* was associated with high NH_4^+ availability while that of *Anemone palmate* may indicate low NH_4^+ availability.

Do species of AMF respond to low/high N or NH_4^+?

The N addition treatments also changed the species composition of the AMF spores which could be grouped according to their response: those only observed in control plots (*Glomus etunicatum* and *Acaulospora mellea*); those only observed in N fertilized plots (*Glomus geosporum-like*, *Acaulospora excavata* and *Paraglomus occultum*); those only observed in the plots receiving NH_4^+ (40A – *Glomus mosseae* and *Acaulospora morrowiae*); and those



Plant species	Ecology/habitat
Dittrichia viscosa	Occurs in road-sides, abandoned fields and walls (Brullo & Marco 2000)
Sonchus sp	Occurs in agricultural and abandoned fields (Flora Digital de Portugal) and is an exotic invader in Californian high N areas (Cione, Padgett & Allen 2002)
Carlina corymbosa	Occurs in abandoned and disturbed fields (Flora Digital de Portugal)
Gladiolus illyricus ssp *reuteri*	Occurs in shrublands and abandoned fields (Flora Digital de Portugal)
Galium sp	Occurs in agricultural and abandoned/degraded fields and sometimes rupicolous (Flora Digital de Portugal)
Salvia sclareoides	Occurs in abandoned and disturbed areas (Flora Digital de Portugal) such as *Juniperus* degraded communities (Costa et al. 2000)
Asphodelus ramosus	Occurs in shrublands and abandoned areas (Flora Digital de Portugal); dominant in many degraded Mediterranean areas (Sakar et al. 2010)
Blackstonia perfoliata	Occurs in grasslands (Flora Digital de Portugal) and is benefited by cutting management (Bonanomi, Caporaso & Allegrezza 2006)
Dactylis glomerata	Occurs in grasslands, abandoned fields and disturbed areas (Flora Digital de Portugal)
Pulicaria odora	Occurs abandoned areas and shrublands (Flora Digital de Portugal)
Sanguisorba hybrida	Occurs in abandoned areas and shrublands (Flora Digital de Portugal) and *S. minor* is associated with high N deposition (Stevens et al. 2011)
Rubia peregrina	Occurs in shrublands and rupicolous (Flora Digital de Portugal)
Brachypodium phoenicoides	Occurs in shrublands and abandoned areas (Flora Digital de Portugal)
Anemone palmata	Occurs in grasslands, shrublands and rupicolous (Flora Digital de Portugal)

Figure 8.1 – List of the plant species that responded consistently (after one and 4.5 years of N addition treatments) to the N dose and/or form (chapter 7). These species' most common habitats and, when available, their responses to N enrichment in other studies are shown on the right column. Photos by Miguel Porto and Ana Júlia Pereira.

only observed in the plots receiving 40 kg NH_4^+ ha^{-1} yr^{-1} (40A and 80AN – *Glomus coremioides* and *Glomus glomerulatum*). Some of these AMF species have been reported to display compatible responses to N enrichment in other studies but the presence of *Glomus etunicatum* in control plots was not expected (Fig. 8.2).

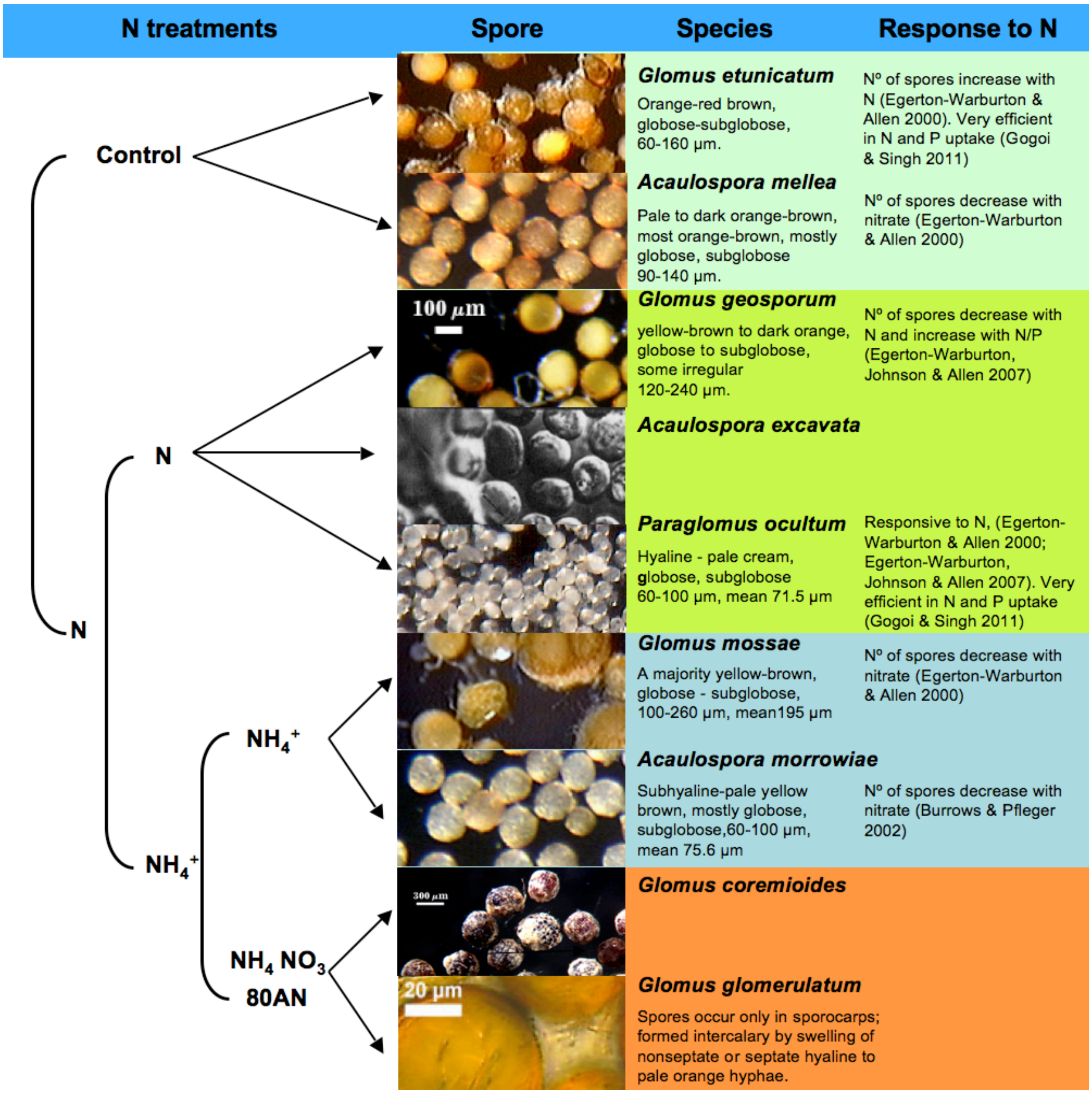
Functional indicators of increased N availability

Soil inorganic N availability

- Soil inorganic N pools reflected the N addition treatments especially when measured in autumn.

Litter traits

- At the species level, the N addition treatments increased the concentration of N in leaf litter;
- At the ecosystem level, the N addition treatments altered leaf litter composition and proportion and increased lignin and N contents.



Species	Response to N
Glomus etunicatum Orange-red brown, globose-subglobose, 60-160 µm.	Nº of spores increase with N (Egerton-Warburton & Allen 2000). Very efficient in N and P uptake (Gogoi & Singh 2011)
Acaulospora mellea Pale to dark orange-brown, most orange-brown, mostly globose, subglobose 90-140 µm.	Nº of spores decrease with nitrate (Egerton-Warburton & Allen 2000)
Glomus geosporum yellow-brown to dark orange, globose to subglobose, some irregular 120-240 µm.	Nº of spores decrease with N and increase with N/P (Egerton-Warburton, Johnson & Allen 2007)
Acaulospora excavata	
Paraglomus ocultum Hyaline - pale cream, globose, subglobose 60-100 µm, mean 71.5 µm	Responsive to N, (Egerton-Warburton & Allen 2000; Egerton-Warburton, Johnson & Allen 2007). Very efficient in N and P uptake (Gogoi & Singh 2011)
Glomus mossae A majority yellow-brown, globose - subglobose, 100-260 µm, mean195 µm	Nº of spores decrease with nitrate (Egerton-Warburton & Allen 2000)
Acaulospora morrowiae Subhyaline-pale yellow brown, mostly globose, subglobose,60-100 µm, mean 75.6 µm	Nº of spores decrease with nitrate (Burrows & Pfleger 2002)
Glomus coremioides	
Glomus glomerulatum Spores occur only in sporocarps; formed intercalary by swelling of nonseptate or septate hyaline to pale orange hyphae.	

Figure 8.2 – List of the AMF species that responded to the N dose and/or form (chapter 3), their characteristics and, when available, their responses to N enrichment in other studies are shown on the right column.

OUTPUTS

N critical loads

This dataset permitted the first estimation of an N critical load for this European habitat, at between 20 and 30 kg N ha^{-1} yr^{-1} (Bobbink *et al.* 2011). For the equivalent Californian habitat, coastal sage scrub, the N critical load was set much lower (<10 kg N ha^{-1} yr^{-1} – Fenn, Allen & Geiser 2011). However, given the short duration of this experiment and the lack of other available information, this new value may need to be revised downwards toward the Californian value. Plant cover and plant and AMF diversity studies on coastal sage scrub were done along an N deposition gradient (Fenn, Allen & Geiser 2011), which constitutes realistic N inputs despite other confounding variables. Also, the soils at the present experimental site are poorer in N and P (chapters 5 and 6) than Californian soils (Cowling *et al.* 1996) potentially muting the effects of N through P co-limitation (Ochoa-Hueso *et al.* 2011).

Scenarios of the effects of increased N deposition on Mediterranean maquis

Usually, change scenarios are developed for the year 2050 (e.g. N deposition - Galloway *et al.* 2004; vascular plant diversity - van Vuuren, Sala & Pereira 2006). But the occurrence of periodic fires in Mediterranean ecosystems result in very dynamic plant communities (Naveh 1974; Trabaud 1994) so that it is difficult to predict at where along the stage of succession a given habitat will be in 2050. This has implications for predicting the effects of increased N availability because these vary according to the dominant plant species and consequently to the phase of succession (e.g. Fenn *et al.* 2010). The effects of increased N deposition within Mediterranean Basin ecosystems were hypothesized according to the phase of post-fire succession. Two scenarios for each phase were considered: increased NH_y deposition (large inputs from agricultural areas); and increased NO_x deposition (large inputs from urban/industrial areas). The response variables considered were vascular plants diversity (lichens or soil microorganisms were not considered), biomass production, soil organic matter and NO_3^- leaching (other N losses were not considered). The effects of increased N deposition were analyzed qualitatively (positive, negative or null) in comparison to low N deposition conditions, and were based on the present study (chapters 2-7) and others in Mediterranean-type ecosystems in Europe and California (e.g. Bobbink *et al.* 2011; Pardo, Robbin-Abbott & Driscoll 2011).

Effects of increased N deposition on early phases of a post-fire succession

In early phases of a post-fire succession, Mediterranean communites are dominated by summer semi-deciduous species (Fig. 8.3-a) and are functionally equivalent to the Californian coastal sage scrub. Under increased NH_y deposition (Fig. 8.3-b), there will be a trade-off between the alleviation of the N limitation and the low NH_4^+ tolerance of the dominant plant group (chapter 4). This will allow the establishment of 'small' plant species that will increase plant richness but will not contribute as much to promote biomass producti-

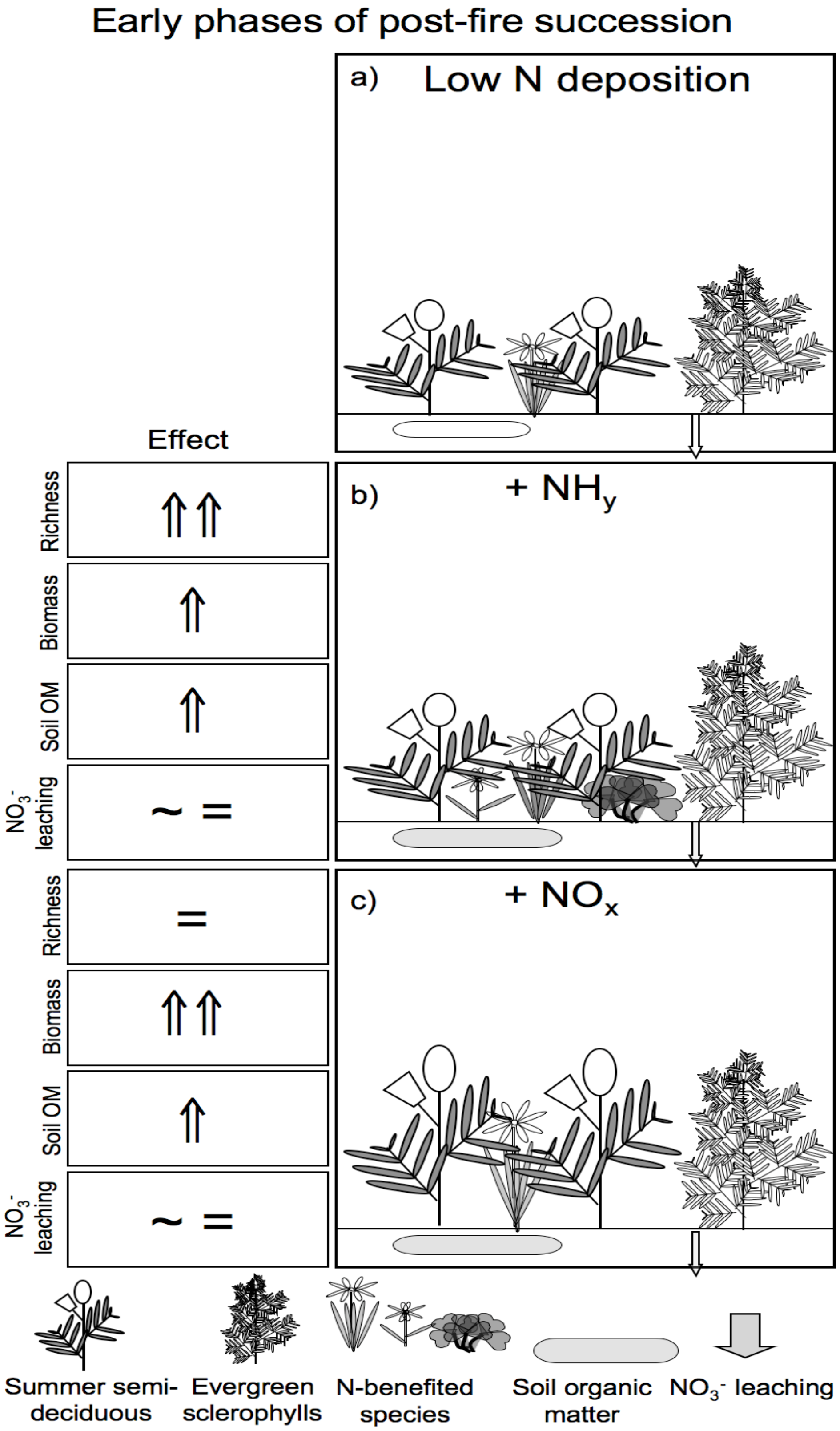


Figure 8.3 – Scenarios for effects of low N deposition (a) and increased deposition of NH_y (b) and NO_x (c) on a Mediterranean maquis in an early phase of post-fire succession. The response variables considered were vascular plant richness, biomass production, soil organic matter and NO_3^- leaching. The effects of increased N deposition were assessed qualitatively (positive, negative or null) in comparison to low N deposition conditions, and were based on the present study (chapters 2-7) and others in Mediterranean-type ecosystems in Europe and California.

on (chapter 7). Given that evergreen sclerophylls are more NH_4^+ tolerant than summer semi-deciduous (chapter 4), litterfall will be enriched in evergreen sclerophylls' material. This shift in litter traits will delay organic matter decomposition and will therefore increase soil organic matter (chapter 6) at least in the soil patches under the influence of evergreen sclerophylls (Cruz *et al.* 2008). Given the rapid growth of the standing plant community, NO_3^- leaching will not increase (Meixner *et al.* 2006).

Under increased NO_x deposition (Fig. 8.3-c), the alleviation of the N limitation will promote biomass production of most plant species (chapter 7), and summer semi-deciduous in particular. Given this plant group's shallow root system (chapter 4), water stress will increase thus increasing the fire risk and intensity (Rao & Allen 2010). This N-driven growth will prevent the establishment/persistence of most 'small' plant species due to increased shading (chapter 7), so that plant richness will not change. Thus, no changes in litter traits will occur. However, litter production will tend to increase, which, depending on the balance between amount of litter input and increased decomposition rate (due to lower C/N ratio of the litter - chapters 5 and 6) will correspond to a slight increase in soil organic matter. As previously mentioned, the rapid growth of the standing plant community will act as a sink for the deposited NO_3^- restricting NO_3^- leaching losses (Meixner *et al.* 2006).

Effects of increased N deposition on later phases of a post-fire succession

In later phases of a post-fire succession, Mediterranean communites are dominated by evergreen sclerophylls species (Fig. 8.4-a) and are functionally equivalent to the Californian chaparral. Under increased NH_y deposition (Fig. 8.4-b), the alleviation of the N limitation may enable the appearance of a few plant species with high NH_4^+ and shade tolerance, resulting in a slight increase in plant richness. Although, if epiphytic lichens were included, diversity would decrease (Fenn *et al.* 2010; Pinho *et al.* 2011). Due to the alleviation of the N limitation, biomass production will increase slightly. Thus, the amount of litter produced may increase slightly but litter traits will not change. Nevertheless, and because more inorganic N will be available, decomposition will slow down (Alarcón-Gutierrez *et al.* 2008; Liu *et al.* 2010) and soil organic matter will accumulate slightly. In Californian chaparral soils nitrification is high (Fenn *et al.* 1998; Fenn *et al.* 1993; Riggan, Lockwood & Lopez 1985; Vourlitis & Zorba 2007) and so is NO_3^- leaching. However, within the Mediterranean Basin, the presence of allelopathic compounds leached from plants (Gallardo & Menino, 1992), and the low quality of the sclerophyllous leaves (chapter 6) have been shown to inhibit nitrification (Cruz *et al.* 2008). Therefore, within the Mediterranean Basin, N losses through NO_3^- leaching will be small.

Under increased NO_x deposition (Fig. 8.4-c), the alleviation of the N limitation will especially promote biomass production of the remaining summer semi-deciduous. Because the domi-

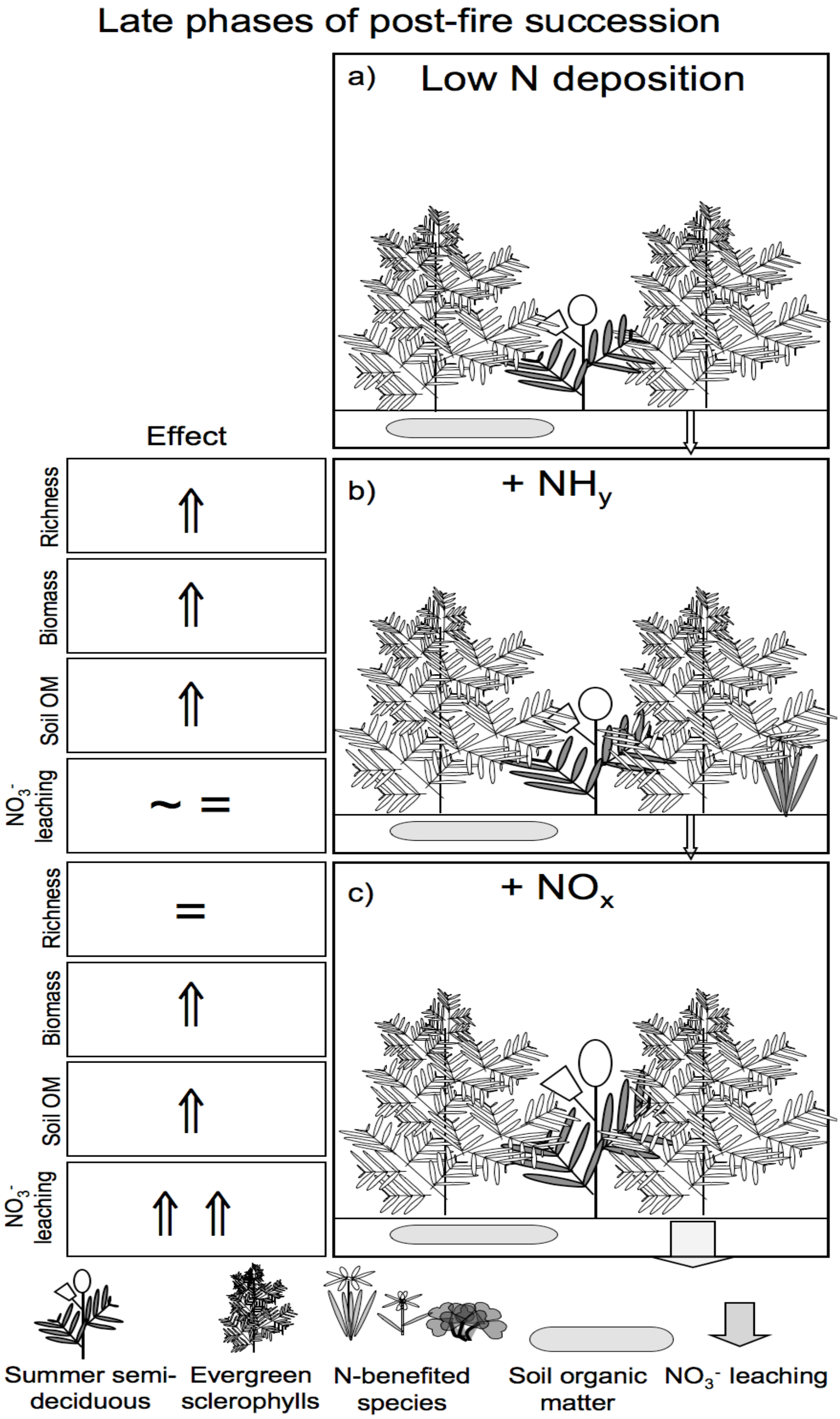


Figure 8.4 – Scenarios for effects of low N deposition (a) and increased deposition of NH_y (b) and NO_x (c) on a Mediterranean maquis in a late phase of post-fire succession. The response variables considered were vascular plant richness, biomass production, soil organic matter and NO_3^- leaching. The effects of increased N deposition were assessed qualitatively (positive, negative or null) in comparison to low N deposition conditions, and were based on the present study (chapters 2-7) and others in Mediterranean-type ecosystems in Europe and California.

nant form of inorganic N (NO_3^- in early successional phases and NH_4^+ in later ones – Kronzucker, Siddiqi & Glass 1997) and light (no light limitation in early successional phases but much in later ones – Aerts & Bobbink 1999) change along succession, few shade-tolerant species prefer NO_3^-. Therefore plant richness will not change. If lichens were considered, these would change less since they are less sensitive to NO_x (Pinho *et al.* 2011). As previously explained for the scenario of a late phase for a post-fire succession subjected to increased NH_y deposition (Fig. 8.4-b), soil organic matter will accumulate slightly and because evergreen sclerophylls grow slowly (Dawson & Lucas 2005), the N retention capacity of the vegetation will be low and NO_3^- leaching will be high (Meixner *et al.* 2006; Fenn *et al.* 2010; Pardo, Robbin-Abbott & Driscoll 2011).

Implications of the phases of a post-fire succession for the effects of increased N deposition on 'downstream systems'

A single N-containing molecule can have a series of impacts, sometimes contradictory, because reactive N can easily move among the different media of air, soil and water in the so called 'N cascade' (Galloway *et al.* 2008). Therefore, even though different types of ecosystems (or at different phases of succession) may be more or less responsive to N enrichment, this only means that the reactive N will impact another ecosystem 'downstream' from the studied one. Based on the present study, and on what is known from Californian coastal sage scrub and chaparral (Fenn, Allen & Geiser 2011), Mediterranean ecosystems in early succession are most responsive to increased N availability. This is related to a higher N retention capacity within 'young' ecosystems. As ecosystems mature, the growth rate decreases and with it the system's N retention capacity, thus explaining why a regenerating chaparral loses less NO_3^- than a mature one (Meixner *et al.* 2006). Therefore there is a trade off between N retention and 'evolution' of the ecosystem, i.e., the reactive N is either retained and alters the ecosystem's structure and functioning (chapters 2-7) or is lost and will alter another system 'downstream', namely aquatic ones. Although the processes are not clear, Mediterranean Basin water bodies have a high N retention capacity as evidenced by the fact that almost all N (~90%) that enters the Ebro River Basin (Spain) is retained and does not reach the Mediterranean Sea (Lassaletta *et al.* 2012).

CAVEATS, GAPS AND FUTURE WORK

Our findings, based on a field N manipulation study, provide new important insights into the effect of N on the structure and functioning of Mediterranean maquis. However, in order to extrapolate the results, and use them to predict future anthropogenic N deposition regimes on the Mediterranean Basin we must understand their limitations. The form and N doses used (NH_4NO_3 or [NH_4Cl + $(NH_4)_2SO_4$]) do not wholly simulate real world N inputs (missing gaseous, particulate and organic N) or it's regime. Mature stands with their very different characteristic (e.g. plant species composition, plant age, rate of plant growth, soil pH, predominance of inorganic N in the reduced form, presence of allelopathic/recalcitrant materials) may respond very differently from the post-fire succession stage used in this experiment. Also this experiment is short with respect to the lifetime of the ecosystem so longer-term studies are needed to explore the generality of the findings. This is especially relevant for the response of the soil microbial community, whose data has been collected but has not been included in the present work, and for the N retention capacity of the system. Many of the key N pools and fluxes, such as microbial immobilization, plant annual N uptake and storage, and losses through gaseous emissions and volatilization (NO, N_2O, NH_3) and leaching remain unquantified. The mechanisms of how N additions alter N storage and cycling are still poorly understood. Finally it should be acknowledged that reactive N deposition is only one of many drivers currently changing these ecosystems (e.g. climate change, invasive exotic species, ozone – Ochoa-Hueso *et al.* 2011; and land-use change – Sala *et al.* 2000). Ideally, future experiments should try to incorporate some of these interactions and also address the critical role of co-limitation by water and P in controlling the response of Mediterranean ecosystems to N enrichment, including that of above and below ground fauna to N enrichment (Ochoa-Hueso *et al.* 2011).

As an attempt to address some of these issues, a new project has been proposed and funded: In-nitro. The aims of the In-nitro are to: (i) maintain the research site and research topics developed in the present study to obtain longer-term results (ii) incorporate the importance of the co-limitation by water and phosphorus in the integrated response of Mediterranean ecosystems to N enrichment; and (iii) integrate the results obtained in the distinct projects to improve the conceptual framework for the Mediterranean ecosystems responses to N enrichment.

CONCLUSIONS

This study provided an integrated framework for better understanding the effects of increased reactive N on nutrient poor ecosystems such as Mediterranean Basin ones. Distinct approaches were applied: (i) a structural one, which showed that N enrichment promotes plant and soil microbial diversity in ecosystems with very low N status; (ii) a functional one, which highlighted the importance of increased N in alterations in ecosystem functions such as organic matter decomposition and plant productivity/soil protection; and (iii) an integrated one, which related the ecosystem's N retention capacity with the its structural and functional N-driven changes. Based on these data, recommendations concerning N critical loads were made, indicators of N deposition were identified (structural and functional) and the implications of these changes for ecosystem sustainability and function were evaluated. However, longer-term studies are needed to explore the generality of the findings and ideally, the interaction with other global change drivers and with other co-limiting factors (water and P) should be studied.

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