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**DRIVERS OF SOIL RESPIRATION OF ROOT AND
MICROBIAL ORIGIN IN GRASSLANDS**

**FATTORI DI CONTROLLO DELLA RESPIRAZIONE DEL SUOLO
DI ORIGINE RADICALE E MICROBICA IN ECOSISTEMI
PRATIVI**

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Abstract

Soil historically has been a major source of atmospheric enrichment of CO₂ and in the same time is one of the biggest storing reservoirs of carbon on the global scale. In fact, soils hold three times as much carbon as the terrestrial biosphere and about twice as much as the atmosphere and exert a large influence on the cycling of carbon between different pools. Soil respiration, which is the flux of CO₂ from soils to the atmosphere, is thus an important component of the ecosystem C budgets and is a major source of CO₂ released by terrestrial ecosystems. Soil respiration is the result of the production of CO₂ from the biological activity of roots and associated microorganisms and the activity of heterotrophic bacteria and fungi living on litter and in the root-free soil. Different sources of soil CO₂ efflux are known to experience high spatial and temporal variation with different controlling factors involved on different time-scales. However, up to now not so many studies have dealt with the interannual variability of soil respiration and its components and only few of them were performed in grassland ecosystems despite the fact that it is one of the world's most widespread vegetation types which comprises 32% of the earth's area of natural vegetation.

This study aimed to advance the understanding of the processes and factors controlling the behaviour of different soil respiration sources in grassland ecosystems. It provides the analysis of the response of soil CO₂ efflux and its components: root- and microbial-derived respiration to different biotic and abiotic factors as well as to widely diffused management activities over a period of three years in a mediterranean grassland site and integrates also different laboratory and *in situ* methodological approaches for deeper studying of the contribution of various respiration sources to total CO₂ efflux from soil and the speed of C cycling within the plant community.

Soil respiration was partitioned in the field using micro (1µm) and macro (1 cm) pore meshes. Soil respiration obtained from the cores with different pore-sized meshes and from the control undisturbed soil were used to calculate values of root-derived and microbial-derived respiration sources. These fluxes were then related to canopy photosynthetic activity, soil temperature, soil moisture and some soil biochemical parameters.

Methodological approach based on pulse labeling of plants in artificial ¹³CO₂ or ¹⁴CO₂ atmosphere was used to found out the speed of the cycling of C in grassland ecosystem (*in situ*) as well as to study the effect of different plant species, plant growing stages, and different nutrient supply on the magnitude of root respiration and on the speed of translocation and respiration of recently assimilated C through roots (on a single species, in laboratory).

The obtained results showed an importance of C assimilate supply in the determination of the variability of root component of soil respiration. It was closely related to gross primary production with a time lag of circa 20h for time scales from daily to annual. Soil temperature which often masks the direct relationship between root respiration and photosynthetic C supply failed to

explain diurnal and seasonal changes in root-derived respiration. Laboratory experiments with a single plant species have shown however that the observed time lag is not stable during the plant ontogenesis, and vary depending on the plant growing stage. The same photosynthetic activity could also result in different magnitude of root respiration, depending on the type of nutrient supply (ex: N in form of NH_4^+ or NO_3^-). All these findings suggest that root respiration is a complex process, tightly coupled to plant canopy activity and could not be explained simply by changes in soil temperature and moisture. Further studies are needed to verify the bonds between aboveground and belowground processes for different species and vegetation types, as well as for various plant growing stages.

Soil temperature and soil water content exerted a significant effect on microbial component of soil respiration. Being a larger part of total CO_2 efflux from soil at Amplerio ($\approx 70\%$), these factors influenced also total soil respiration dynamic on different time scales. Introduction of a management regime have modified however the activity of microbial community by an increase of the quantity of easily available C substrates from the rhizodeposition process, resulting in a general suppression of microbial enzymatic activity and further decrease C mineralization rates. Combination of laboratory studies and *in situ* measurements is necessary for understanding of the effect of changing substrate quality, nutrient and moisture conditions on microbial activity and its C use efficiency.

Sommario

Il suolo è stato storicamente la fonte principale di emissioni di CO₂ nell'atmosfera e al contempo la maggiore riserva di carbonio a scala globale. Infatti i suoli stoccano tre volte la quantità di carbonio presente nella biosfera terrestre e circa il doppio dell'atmosfera esercitando un'importante influenza sul ciclo del carbonio globale. La respirazione del suolo che rappresenta il flusso di CO₂ dal suolo verso l'atmosfera è dunque una componente rilevante del bilancio del carbonio a scala eco sistemica ed è la principale fonte di anidride carbonica emessa dagli ecosistemi terrestri.

La respirazione del suolo è il risultato della produzione di CO₂ dall'attività biologica delle radici e dei microrganismi ad esse associati da una parte e dall'attività eterotrofa di batteri e funghi presenti nella lettiera e nel suolo dall'altra. Le differenti fonti di CO₂ dal suolo sono caratterizzate da un'ampia variabilità temporale e spaziale e sono controllate da diversi fattori che intervengono in relazione alle diverse scale temporali considerate. Tuttavia ad oggi non molti studi si sono occupati della variabilità interannuale della respirazione del suolo e delle sue componenti, inoltre solo alcuni di questi si sono occupati di ecosistemi di prateria nonostante questi comprendano il 32% della superficie coperta da vegetazione sulla Terra.

L'obiettivo del presente studio è il miglioramento della comprensione dei processi e dei fattori di controllo delle diverse origini della respirazione del suolo in ecosistemi pratici. Si analizza la risposta del flusso di CO₂ dal suolo e delle sue componenti: della respirazione microbica e radicale rispetto ai principali fattori biotici ed abiotici così come all'effetto della gestione estensiva del pascolo osservati durante un periodo di 3 anni in una prateria mediterranea.

L'approccio metodologico include l'integrazione di misure in situ con analisi in laboratorio per un'analisi approfondita del contributo delle diverse fonti della flusso totale di CO₂ dal suolo e dei tempi di turn-over del carbonio all'interno della comunità vegetale.

La respirazione del suolo è stata ripartita nelle distinte componenti utilizzando speciali reti con pori fini dal diametro di 1 µm e 1 cm definiti rispettivamente "micro" e "macro" che permettono di escludere selettivamente il contributo radicale alla respirazione del suolo misurata in appositi plot. I flussi delle componenti radicali e microbiche della respirazione del suolo vengono quindi ricavati per differenza con le misure effettuate nei plot manipolati ed in quelli di controllo. I flussi così ricavati vengono messi in relazione all'attività foto sintetica delle piante, alla temperatura ed umidità del suolo e a diversi parametri biochimici del suolo.

La tecnica del "pulse labeling" delle piante in atmosfera arricchita con ¹³CO₂ or ¹⁴CO₂ è stata impiegata per investigare la velocità di turn-over del carbonio assimilato dalla vegetazione (in situ) e per studiare l'effetto della diversità specifica, della fenologia, della nutrizione minerale

sull'intensità del flusso respiratorio radicale e sulla velocità di translocazione e respirazione attraverso le radici del carbonio assimilato (studio di laboratorio, su di una specie).

I risultati ottenuti mostrano l'importanza dell'assimilazione del carbonio come rispetto alla variabilità della componente di respirazione radicale che è risultata correlata alla produzione lorda eco sistemica da base giornaliera fino ad annuale e che ha presentato un *time lag* di circa 20 ore. La respirazione del suolo che spesso maschera la relazione diretta tra respirazione radicale e d assimilazione fotosintetica del carbonio non è in grado di spiegare le variazioni giornaliere e stagionali della respirazione radicale. La sperimentazione in laboratorio su di una sola specie ha comunque che il *time lag* non è costante durante l'ontogenesi della pianta, ma varia in funzione dello stadio fenologico.

La stessa attività fotosintetica è stata osservata anche in associazione a livelli di respirazione radicale diversi, a seconda del del tipo di nutrizione minerale (es.: N in forma di NH_4^+ o NO_3^-). Questi risultati suggeriscono che la respirazione radicale è un processo complesso, strettamente legato all'attività fotosintetica delle piante e che non può essere spiegato esclusivamente da cambiamenti della temperatura e dell'umidità del suolo. Ulteriori studi sono necessari per verificare le relazioni tra processi epigei ed ipogei per diversi tipi di vegetazione, di specie vegetali per diversi stadi di sviluppo.

La temperatura ed il contenuto idrico del suolo esercitano una significativa influenza sulla componente microbica della respirazione del suolo, che per il sito di Amplero rappresenta circa il 70% essendo quindi prevalente. Conseguentemente temperatura ed umidità del suolo influenzano le dinamiche di respirazione del suolo per differenti scale temporali.

L'introduzione delle attività del pascolo ha modificato l'attività microbica aumentando la quantità di carbonio facilmente disponibile derivante dalle rizodeposizioni, con il risultato di una ridotta attività enzimatica della comunità microbica ed un conseguente decremento dell'attività di mineralizzazione del carbonio. La combinazione di studi di laboratorio e di misure in situ è necessaria per la comprensione dell'effetto del cambiamento della qualità del substrato, dei nutrienti e delle condizioni di umidità sull'attività microbica e la sua efficienza nell'uso del carbonio.

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1. INTRODUCTION AND OVERVIEW

1.1. Global C balance: biosphere-atmosphere interactions and human influence.

The earth contains approximately 10^8 Gt of C distributed in several pools which differ in their size and turnover times (Fig.1).

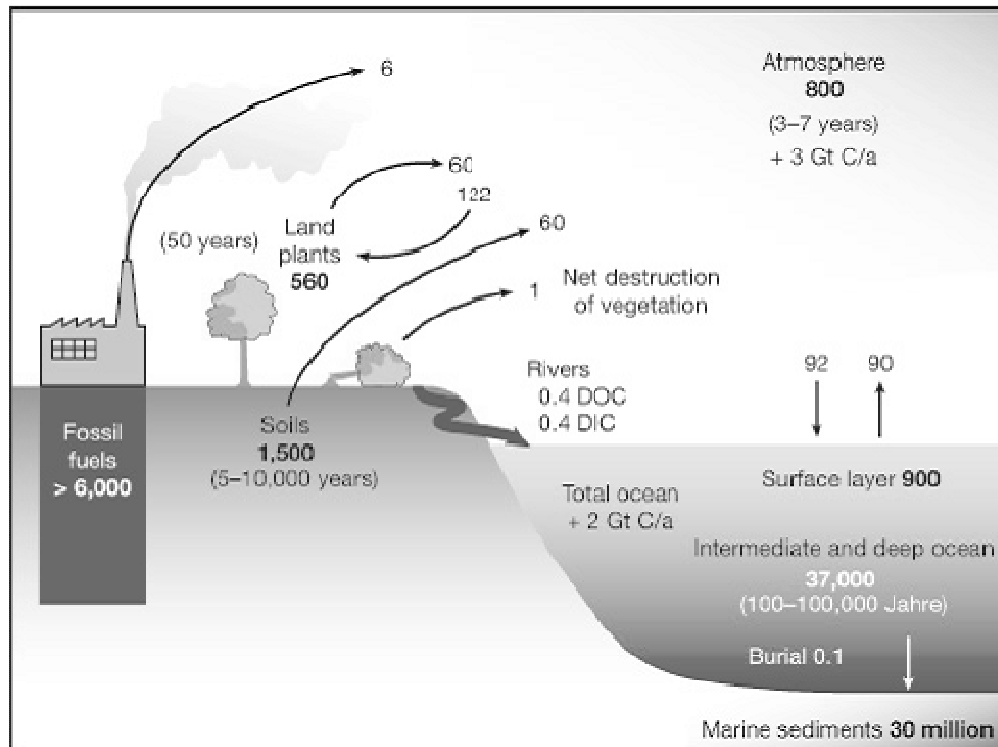


Fig. 1 The global carbon cycle. Values are given in Gt C. **Bold prints represent reservoirs and normal prints represent fluxes.** Mean residence time of the pools is given in parentheses. DOC = dissolved organic carbon, DIC = dissolved inorganic carbon. Source: WBGU (Schubert et al. 2006). Adapted after Schlesinger (1997); WBGU (2003). Numbers expanded and updated for ocean and fossil fuels: Sabine et al. (2003); Raven et al. (2005); for atmosphere: NOAA-ESRL, (2006).

Natural systems and biogeochemical cycles have historically maintained these pools in dynamic equilibrium. More recently, anthropogenic activities such as deforestation, agricultural practices, and the burning of fossil fuels have resulted in large shifts among carbon pools, particularly since the beginning of the industrial revolution (IPCC 1995, 2001) (Fig.2). Annual emissions of CO₂ from fossil fuel combustion are small relative to the natural flows of carbon; nevertheless, these anthropogenic emissions are the major contributor to increasing concentrations of CO₂ in the atmosphere and above all they represent a transfer of carbon from the slow carbon cycle to the active carbon cycle. From 1850 to 1998, 270 ± 30 Gt C were emitted from fossil fuel burning and cement production (Marland et al., 1999). During the same period, emissions from land use change were estimated at 136 ± 55 Gt C (Houghton 2003). These last were related to deforestation, biomass burning, conversion of natural to agricultural systems and the ploughing of soils. World soils historically have been major source of atmospheric enrichment of CO₂: until the 1950s more C was emitted into the atmosphere from land use change and soil cultivation than from fossil fuel combustion (Lal, 2003). Presently, about 20% of the global emissions come from land use change (IPCC, 2001). In fact, soils with a total global storage of approximately 1500 Gt C (Jacobson et al. 2000) hold three

times as much carbon as the terrestrial biosphere and about twice as much as the atmosphere. So even small changes in the decomposability of soil organic matter and the magnitude of soil respiration, could have a large effect on the concentration of CO₂ in the atmosphere.

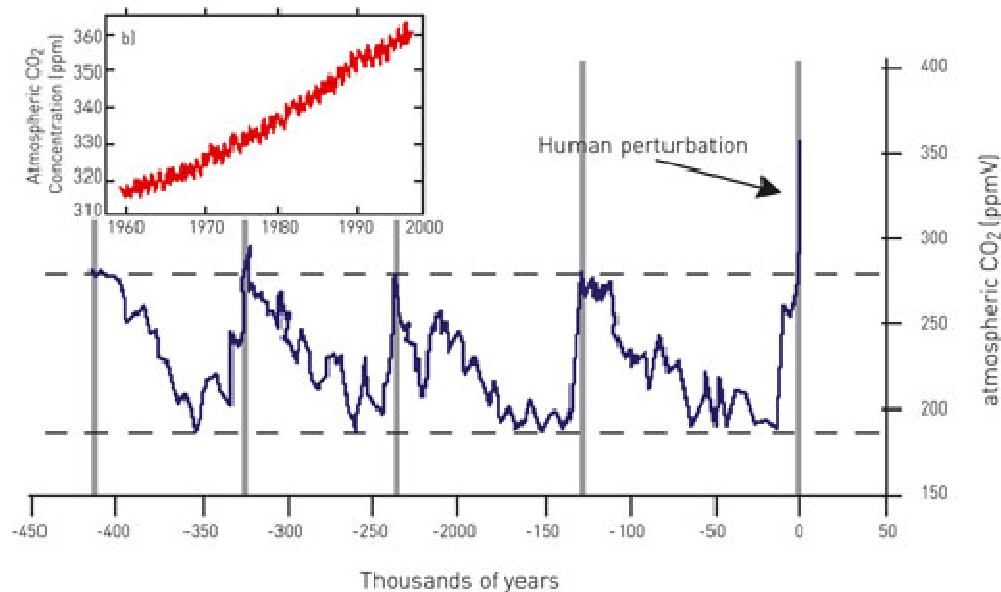


Fig. 2 Recent human influence on the atmospheric CO₂ concentration. Fig. 2 Recent human influence on the atmospheric CO₂ concentration. a) from 1960 to 2000, b) over the last 4×10^5 years. IPCC, 2001.

Moreover, the sequestration of atmospheric CO₂ by vegetation, through photosynthesis is the result of a long and complex process ('slow in'): while standing biomass is thought to be responsible for the enhanced uptake required to balance the global anthropogenic CO₂ budget, the soil organic carbon (SOC) pool provides the longer term transient sink for much of this C (Smith and Shuggard, 1993). This is due to the comparatively long time required for the SOC pool to establish a new equilibrium with the enhanced rates of delivery of C to the soil from standing biomass. By contrast, with combustion and land-use change the release of C into the atmosphere is sudden and unavoidable ('fast out').

1.2. Soil Carbon Pools and Global change

Despite the major role of SOC in the global C cycle, there is still great uncertainty in the size of the SOC pool, its capacity to store additional C sequestered by living biomass, and the response of the SOC pool to changes in climate. Climate related changes in above and belowground CO₂ concentrations, ambient temperatures, and water conditions will have yet largely unknown effects on carbon pools and respiration fluxes. These factors can affect soil CO₂ fluxes directly, as through temperature changes of enzymatic reaction rates (Davidson and Janssens 2006), but they may also have less direct effects through changes in vegetation, nutrient availability, etc.

Reducing this uncertainties will required more robust estimate of the pool size and rates and fluxes through the soil C pool.

Estimates of the size of the global pool of SOC have ranged between 700 Pg (Bolin, 1970) and 2946 Pg (Bohn, 1976), with the value of around 1500 Gt now generally accepted as the most appropriate (Table 1).

Study	Soil C (Pg)
Bolin (1970)	700
Bohn (1976)	2946
Baes et al. (1977)	1080
Basilevich (1974)	1392
Schlesinger (1977)	1456
Aitjay et al. (1979)	2070
Post et al. (1982)	1395
Eswaran et al.(1993)	1576
Batjes (1996)	1500
Jacobson et al. (2000)	1500

Table 1. Estimate of the size of the global SOC pool to 100cm.

From the understanding of the behaviour of the SOC pool, models such as Rothamsted (Jenkinson and Rayner, 1977) and Century (Parton et al., 1993) have been developed and allow the results from the regional validation studies to be extrapolated to a global scale (Schimel et al., 1994). Such models divide the SOC pool into three to five pools with different turnover times ranging from tens to thousands of years, and the sizes of these pools for a given soil texture are determined climate-driven interactions between plant C inputs, nutrients, microbial respiration, and leaching of dissolved organic carbon (DOC) (Fig.3).

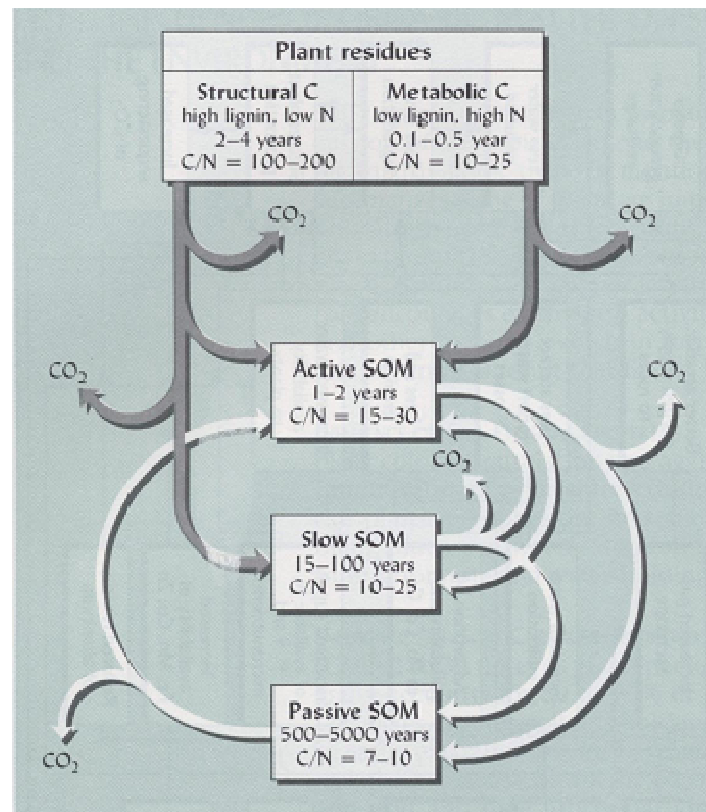


Fig. 3 Different soil C pools with residence time and C/N ratio, derived from plant organic residues, Brady and Weil, 1999.

An important difference between the SOM pools is their turnover rates and means residence time (MRT). Turnover rate is the rate of cycling of C in a pool or a system. If the pool is steady state (input is equal to the decomposition) the value of turnover rate is the ratio of the input amount per time unit per total pool amount. Different turnover rates (TR) results in largely different MRTs of C in the pool. MRT is the reverse of the TR ($1/TR$) and describes the mean period of residence of C in the pool.

Active SOM consists of materials with relatively high C/N ratios, high turnover rates and consequently low MRT of C in the pool. It integrates the living biomass, some of the fine particulate detritus (Particulate Organic Matter, POM), most of the polysaccharides and other non-humic substances, and some of the more labile and easily decomposed fulvic acids. This fraction provides most of the readily accessible food for the soil organisms and most of the readily mineralizable N. Rarely comprises more than 10 to 20% of the total SOM.

Passive SOM (or inert) consists of very stable materials with a very slow decomposition rates. Results of C dating have shown that the MRTs of the pool is thousands of years (Theng et al., 1992; Trumbore, 1997; Rethemeyer et al., 2004). Accounts for 60 to 90% of SOM, it is complex and tightly bound to clay minerals. Being inert it makes only minor contribution to total annual CO₂ efflux from soil.

Slow SOM has intermediate properties between the other two and includes substrates with high content of lignin and other slowly decomposable and chemically resistant components. It is an important source of mineralizable N and other plant nutrients, and is a food source for the steady metabolism of soil microbes.

The term “soil C sequestration” implies a net removal of atmospheric CO₂ by plants and its storage as SOC. This process is described by the entering of the plants carbon to the SOC pool in the form of either above-ground litter or root material. In grasslands a significant proportion of plant material is consumed by herbivores and then enters the SOC pool from animal excretion (Bol et al., 2004). High root production by grasses may also explain why pastures accumulate so much soil organic carbon. Most pasture plants (≈80%) are perennial and have well developed root systems that are used as carbon storage of new growth in spring or after grazing (mowing). Hence, the relative belowground translocation of assimilated carbon by pasture plants can reach up to 80% (including C autotrophically respired by roots) but up to only 60% by trees (Kuziakov & Domanski, 2000). Under certain conditions grazing can lead to increased annual net primary production over ungrazed areas (Conant et al. 2001).

Climate (temperature and precipitation) exerts a major influence on SOC at the global scale by controlling the levels of input from live biomass into the soil. Climate also influence the rate at which C delivered to the soil is cycled through the SOC pool and ultimately respired back to the atmosphere by microbial biomass, or is lost from the profile as dissolved organic C (Fig. 3). Climate, in combination with other factors controls initial litter quality (nitrogen content, lignin content etc. Melillo et al., 1982) and processes that modify the nature of organic C. Climate influence the SOC distribution through the soil profile by influencing the efficiency and depth of illuviation and effective bioturbation (Holt and Coventry, 1990) and is a key factor affecting the rate of production and the mineralogy of the soil substrate (Goh et al., 1976).

The role of variety of natural and anthropogenic disturbances in modifying SOC has received increased attention in recent decades owing the large role to the land-use change in determining the magnitude of transfer between the terrestrial C source/sink and atmosphere CO₂ reservoir. In many cases disturbance can lead to long-term changes in local vegetation and soil structure which means that during the period over which the disturbance is maintained, and over which a new equilibrium is established following the cessation of disturbance, the local SOC pool can act as either a source to, or a sink from the atmosphere.

1.3. Soil respiration sources

Soil respiration (R_s) is an important component of the ecosystem C budgets. After the photosynthesis, CO_2 efflux from soil remains the second largest C flux in most ecosystems and can account for 60-90% of total ecosystem respiration (Goulden et al., 1996; Longdoz et al., 2000). On the global basis, the estimate of the contribution of R_s to the atmospheric CO_2 emission is about 75-80 GtC per year. Raich et al. (2002) estimated the mean global CO_2 efflux from soil in the period between 1980 and 1994 to be 80.4 GtC.

Due to the large quantity of C stored in the soil it has been hypothesized that relatively small changes in R_s induced by climate change or land use change could rival the annual fossil fuel loading of atmospheric CO_2 (Jenkinson et al., 1991; Raich and Schlesinger 1992). The realization that the soil could be a possible source of atmospheric CO_2 , together with the continuous increase in atmospheric CO_2 concentration has given a rise to numerous methods to quantify this input.

Soil respiration is the result of the production of CO_2 in soils from a combination of several belowground processes (Ryan and Law 2005; Trumbore, 2006). The most important are the biological activity of roots and associated microorganisms and the activity of heterotrophic bacteria and fungi living on litter and in the root-free soil (Fig. 4). Non biological processes related to chemical weathering in soils are estimated to be a net carbon sink of ca. 0.3 Gt yr⁻¹ (Jacobson et al. 2000), thus being of less significance.

Kuzyakov (2006) suggested five main contributors to total CO_2 efflux (Fig. 4) :

- (1) microbial decomposition of SOM in the root free soil;
- (2) microbial decomposition of SOM in root affected soil, associated with a priming effect;
- (3) microbial decomposition of the dead plant residuals;
- (4) microbial decomposition of the rhizodeposits in the rhizosphere;
- (5) root respiration.

The dynamic of different components of soil respiration is controlled by different biotic and abiotic factors, such as temperature, water availability, photosynthetic activity, or plant phenological development. Heterotrophic processes control soil C storage and nutrient dynamics, while autotrophic component reflect plant activity and supply of organic compounds to roots from canopy (Hogberg et al., 2001; Singh et al., 2003; Binkley et al., 2006). In addition the response of microbial and root components of soil respiration to changes in soil temperatures is different, exhibiting various Q_{10} values (Zhou et al., 2007). Thus, the potential change in soil CO_2 efflux associated with global warming will largely depend on the relative contribution of root and microbial-derived respiration tot total CO_2 efflux. Therefore quantifying the components of soil respiration is imperative for understanding the nature and extent of feedbacks between climate

change and soil processes and to predict ecosystem responses to climate change (Melillo et al., 2002; Ryan and Law, 2005).

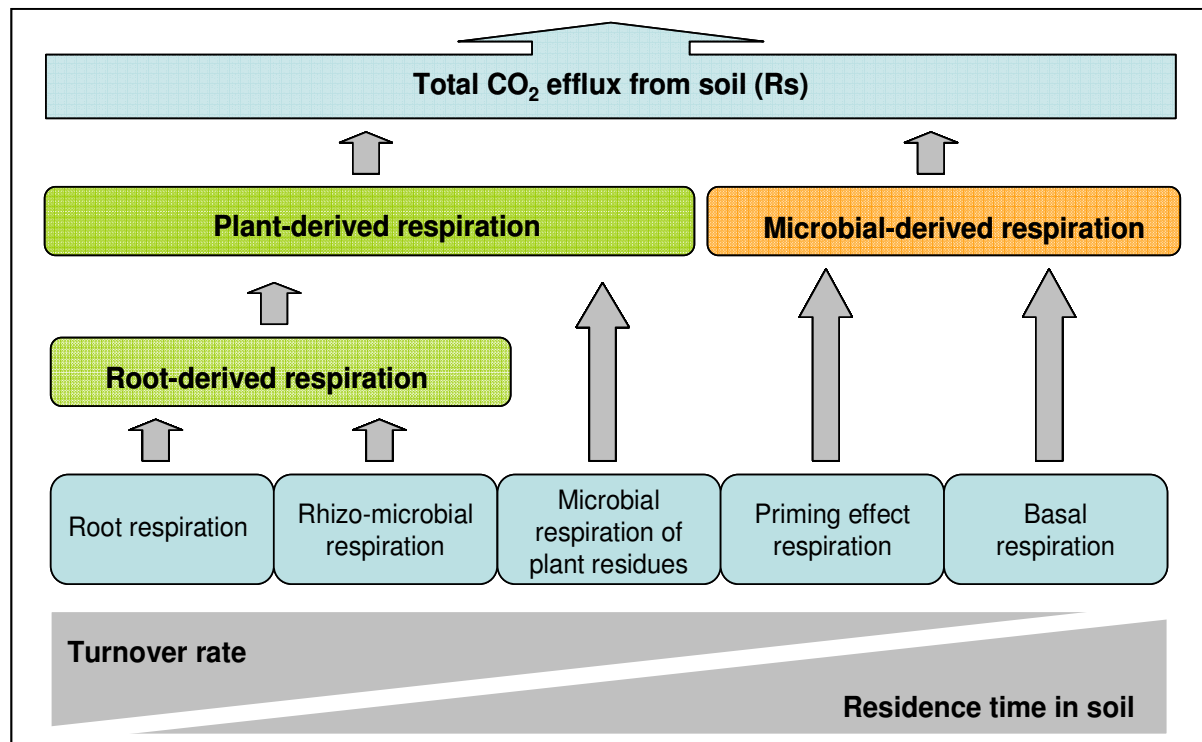


Fig. 4 Five main sources of soil CO₂ efflux from soil, ordered according the turnover rates and residence time in soil. Modified after Kuzyakov (2006).

In regard to the CO₂-driven green house effect, the last three sources of CO₂, because of their fast turn over rates, do not have a significant effect on the C sequestration in the long and short term. The long MRT of SOM and low turn over rates means that it is the only C pool that can be a real long term sink for C in soil. Being a very large reservoir of C, it makes it also a huge potential source of CO₂ if decomposition exceeds humification. Plant-derived respiration in this context masks the contribution of microbial-derived respiration to the total CO₂ efflux, making the total CO₂ efflux unsuitable for direct estimation of the contribution of the soil the changes in atmospheric CO₂.

The fact that total soil respiration is not all SOM-derived and do not provide a sufficient information on whether the soil is a net source or sink of CO₂ have led to an augment of the number of both laboratory and field studies and methods which allow to separate different sources of soil CO₂ and to calculate their contribution to total soil respiration. Partitioning of soil respiration allows researchers to measure the contribution of each respiration source to total fluxes and to account for the individual response of each source to environmental factors. However, the basis assumptions and results obtained by these methods vary significantly among the studies. It remains unclear if the observed variation in results is method-dependant due to the fact that each partitioning approach integrates the biases associated with the proper limitations and shortcomings, or reflects varying experimental

conditions, like soil type, plants cover, equipment, environmental conditions etc. Comprehensive reviews of these methods are given by Hanson (2000), Kuzyakov and Larionova (2005), Kuzyakov (2006), and Subke (2006). Most methods involve a certain degree of disturbance of the soil system that changes natural fluxes to an uncertain degree.

Increasing number of studies have explored soil respiration in relation to environmental factors and across bioclimatic area, pointing out a different role of various ecosystems in the terrestrial C cycle and its feedbacks to climate change. Whilst soil respiration has been well characterized for range of forest ecosystems (recent synthesis by Janssens and al., 2001 Kane and al., 2003; Hibbard et al., 2005; Rodeghiero and Cescatti, 2005) comparatively little is known about grasslands.

1.4. Importance of Grassland ecosystems in global C balance.

Grasslands are one of the world's most widespread vegetation types and comprise 32% of the earth's area of natural vegetation (Adams et al., 1990). Grasslands play a significant role in carbon storage and is an important component of the global carbon cycle. Even so, there have been relatively few long-term studies of grassland at the ecosystem level. At least in part, this is caused by the focus of many scientists on forests (e.g., Dolman et al., 2002; Valentini, 2003). Some researchers have tried to assess the carbon budget in grassland (Kim *et al.* 1992; Dugas *et al.* 1999; Frank & Dugas 2001; Sims & Bradford 2001; Suyker & Verma 2001; Flanagan *et al.* 2002; Sousanna, 2004, Belelli et al. 2007). These studies suggest that grassland ecosystems can be a sink of CO₂ during their growing periods. However the grassland estimate, which is derived from a simple model CESAR (Vleeshouwers and Verhagen, 2002), is the most uncertain (coefficient of variation of 130%) among all land-use types (Janssens *et al.*, 2003). And the contribution of this sink to the global carbon budget has not been adequately clarified.

Grassland ecosystems are particularly complex and difficult to investigate because of the wide range of management and environmental conditions to which that they are exposed. Currently, the net global warming potential (in terms of CO₂ equivalent) from the greenhouse gas exchanges with grasslands is not known. It is clear that an integrated approach, that would allow quantifying the fluxes from all three radiatively active trace gases (CO₂, CH₄, N₂O), would be desirable.

Besides their natural aspect, grasslands have a pure agricultural destination as a primary food source for wild herbivores and domesticated ruminants. Actually, grasslands being a mixture of different grass species, legumes and herbs may act as carbon sinks, erosion preventives, bird directive areas, habitat for small animals, nitrogen fixation (Carlier *et al.* 2004).

The grassland's carbon cycle integrates exchanges of carbon in the form of organic matter among three compartments (soil, vegetation, herbivores) and under inorganic form as CO₂ between

each of these and the atmosphere (Fig. 5 and 6). The vegetation exchanges actively CO_2 with the atmosphere through the biological processes of photosynthesis and respiration and contributes to inputs of organic matter into the soil by the decomposition of the dead tissues. The herbivores consume grass matter, return part of the ingested carbon through excrements, which naturally serve as fertilizing substrate for grasses, and emit CO_2 to the atmosphere as a result of respiration. In managed grasslands the excreted carbon may be incorporated directly into soil as manure by farming practices. For natural steppe ecosystems, in absence of livestock, the fraction of primary productivity consumed by herbivores, typically rodents, is very small and generally does not exceed 1-2% of NPP. Up to 98% of the total carbon store in temperate grassland ecosystems can be found sequestered in the below ground pool (Hungate et al., 1997) which generally has much slower turnover rates than aboveground C (Schlesinger, 1977). Carbon dioxide is lost from grassland soils by root respiration and microbial respiration from decomposition of soil organic matter. Changes in organic carbon content is a function of the balance between inputs to soil of carbon fixed by photosynthesis and losses of soil carbon via decomposition. Soil erosion can also result in the loss (or gain) of carbon locally, but the net effect of erosion on carbon losses as CO_2 for large areas on a national scale is unclear.

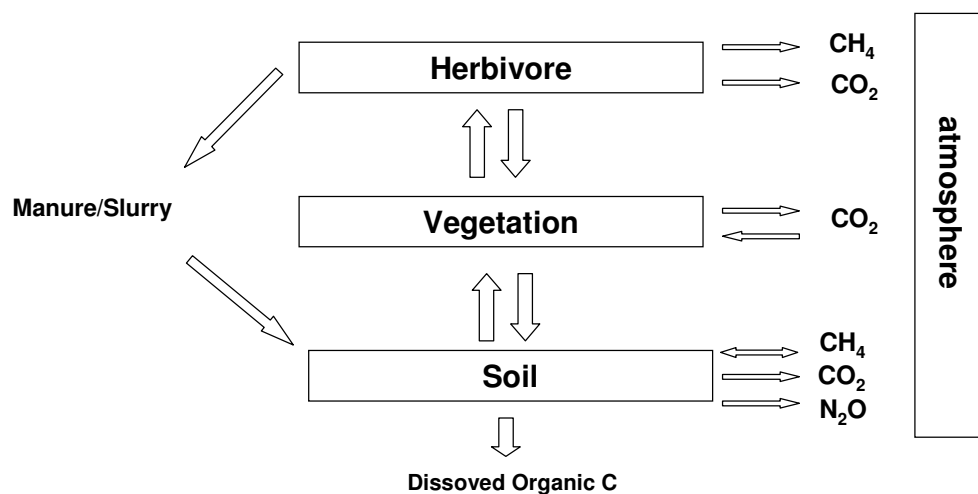


Fig. 5 Schematic diagram of the greenhouse gas fluxes and main organic matter (OM) fluxes in a grazed grassland (modified after Sousanna, 2004).

Moreover, grasslands contribute to the biosphere – atmosphere exchange of non CO_2 greenhouse gases, with fluxes intimately linked to management practices. Of the three greenhouse gases that are exchanged by grasslands, CO_2 is exchanged with the soil and vegetation, N_2O is emitted by soils and CH_4 is emitted by livestock at grazing and can be exchanged with the soil (Fig. 5 and 6).

For grasslands, the nature, frequency and intensity of disturbance plays a key role in the C balance. In agricultural systems, land use and management act to modify both the input of organic matter via residue production, organic fertiliser application, grazing management and the rate of decomposition (by modifying microclimate and soil conditions through crop selection, soil tillage, mulching, fertiliser application, irrigation and liming) (IPCC, 1997). Management practices that increase soil and root respiration cause short-term effluxes of CO₂ to the atmosphere, whilst practices that increase the rate of decomposition of organic matter lead to longer-term losses of soil organic carbon in the form of carbon dioxide. Herbage harvesting by cutting also results in carbon exports from grassland plots. Most of the carbon harvested and stored in hay or silage will be released as CO₂ to the atmosphere shortly after harvest. In a cutting regime, a large part of the primary production is exported from the plot as hay or silage, but part of these C exports is compensated for by farm manure and slurry application. Under intensive grazing, up to 60 % of the above ground dry matter production is ingested by domestic herbivores (Lemaire and Chapman, 1996). However, this percentage can be much lower during extensive grazing. The largest part of the ingested carbon is digestible (up to 75% for highly digestible forages) and, hence, is respired shortly after intake. Only a small fraction of the ingested carbon is accumulated in the body of domestic herbivores or is exported as milk. Large herbivores, such as cows, respire approximately one ton C per year (Vermorel, 1995). Additional carbon losses occur through methane emissions from the enteric fermentation. However, grazing practices which increase grassland productivity have the potential to increase SOC and C sequestration (Conant *et al.* 2001).

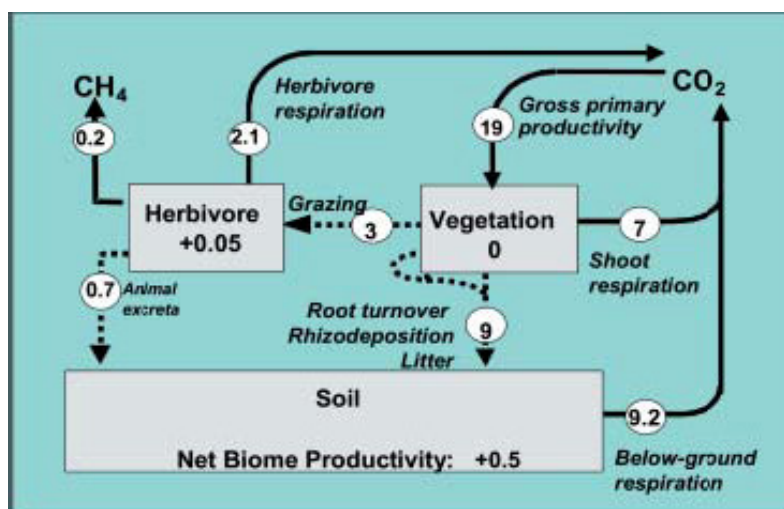


Fig. 6 Carbon cycle in grazed grassland. The main carbon fluxes (t C ha⁻¹ yr⁻¹) are illustrated for grassland grazed continuously by cattle at an annual stocking rate of two livestock units per ha (Soussana *et al.*, 2004)

1.5. Objectives of the study

The general aim of the study was to advance the understanding of the processes and factors controlling the behaviour of different soil respiration sources in grassland ecosystems. The work is subdivided into different topics, according to the following specific objectives and various methodological approaches used to attain them:

- To found out how root- and microbial-derived respiration respond to changes in biotic and abiotic factors on different time scales: from daily to interannual (Chapter 2).
- To found out the delay in the response of root respiration to photosynthetic C supply from aboveground and to calculate to what grade these processes are coupled (Chapter 3)
- To verify how the plant species, plant growing stage and nutrient supply influence the magnitude of autotrophic component of soil respiration and the speed of cycling of C through the plant community (Chapter 5).
- To assess the response of root-derived respiration and soil microbial activity to management based on defoliation practices (mowing and grazing) (Chapter 4).
- To quantify the contribution of individual soil respiration sources to total CO₂ efflux from grassland ecosystems by different *in situ* partitioning techniques. To verify the comparability of the obtained results and discuss the methodological shortcoming of each method (Chapter 6).

The objectives of this study required both *in situ* measurements of soil respiration fluxes and different environmental variables influencing them at the selected grassland site as well as laboratory cultivation and experiments with a single plant species and following analyses of soil and plant material.

Details of methodological approaches will be discussed in the chapters.

1.6. Study approach and summary of main findings

Drivers of soil respiration of various origin (Chapter 2)

Partitioning of soil respiration into root- and microbial-derived components and bimonthly measurements of all respiration fluxes were performed in Amplero, a Mediterranean grassland site located in central Italy (AQ). Amplero is one of the main sites of CarboEurope Integrated Project and is equipped with eddy covariance tower for determining CO₂ exchange between the vegetation and the atmosphere (Fig.7).



Fig.7 Amplero (AQ, Italy): a) satellite image, b) view on the whole territory (May 2007) c) eddy covariance station.

The experiment on partitioning of soil respiration was started in the beginning of the growing season of 2006 and terminated in September 2008. In particular, the following results can be highlighted:

- Average contribution of root-derived respiration to total CO₂ efflux from soil in three years of measurements amounted to 30% with a great variation during the growing seasons (2-70%). An increase in root contribution, observed mainly during the drought periods in summer, was associated with higher sensibility of microbial respiration to changes in the soil water content.
- Daily variation of soil respiration of various origin couldn't be explained only by changes in soil temperature and soil moisture. Other factors are involved in controlling of diurnal variation of soil respiration.
- Seasonal variation of respiration of different origin was controlled by various factors: GPP resulted as a best predictor of changes in root-derived respiration. The correlation between photosynthesis and the effect on respiration was strongest after a lag of 20 hours. Soil temperature which often masks the GPP dependence, failed to explain changes in root respiration at Amplero. In fact, the biomass increment which is usually observed under favourable temperatures and soil humidity was restricted by mowing and grazing. However, changes in soil temperature and soil water content explained well seasonal variation of microbial component of soil respiration. Being a larger part of total CO₂ efflux from soil at Amplero ($\approx 70\%$), these factors exert a significant influence also on total soil respiration dynamic.
- Interannual variability of total soil respiration is controlled by the number of days with SWC<25%

Concentrating on the root-derived respiration source (Chapter 3 and 5)

Two chapters are dedicated to the studying of the effect of other factors than soil temperature and moisture on root-derived respiration component. Methodological approach based on pulse labeling of plants in artificial ¹³CO₂ (chapter 3) or ¹⁴CO₂ (chapter 5) atmosphere was used to found out the speed of the cycling of C in grassland ecosystem (in situ, chapter 3) as well as the effect of different plant species, plant growing stages, and different nutrient supply on the

magnitude of root respiration and on the speed of translocation and respiration of recently assimilated C through roots (on a single species in laboratory, chapter 5).

Speed of C cycling

In situ pulse labeling in $^{13}\text{CO}_2$ atmosphere was performed in Amplero. Raw isotopic values of respired $^{13}\text{CO}_2$, mean residence time and mean age of this C in aboveground and belowground compartments were estimated. Results of two methods for assessment of the time lag between photosynthetic C uptake and its following respiration through the rooting system ((1) in situ pulse labeling and (2) root-derived respiration by mesh exclusion vs. GPP from eddy covariance) were compared. The main results are the following:

- Two distinct pools of C could be recognized: a fast turning over pool, which integrates the assimilates of a current day, and slower turning over pool, which integrates the assimilations during the growing season;
- Aboveground growth and maintenance respiration is fuelled mainly by the assimilates of the current day, while in root respiration the C with higher mean residence time values is involved;
- The peak in root-derived respiration by isotope pulse labeling technique was registered between 16-24h after the label introduction, indicating a general strong and fast link between C assimilation and root activity;
- The time lag obtained by destructive mesh-exclusion technique was confirmed by the non destructive pulse labeling method. The fact that such type of partitioning techniques are widely used in environmental studies and often are coupled with eddy covariance measurements, makes it promising for the estimation of the speed of the C cycling within and between various ecosystems.

Effect of different plant species, growing stage and nutrient supply

Pulse labeling of different plant species in $^{14}\text{CO}_2$ atmosphere under laboratory conditions, varying their growing stage and type of N supply have demonstrated that root respiration is a complex process: the limits of its variation are more likely determined by the photosynthetic C supply from shoots, inside these limits the magnitude of root respiration depends on ion uptake expenses and costs associated with nitrate reduction.

- Nitrate affected negatively the carbohydrate metabolism and energy economy of two plant species: in respect to ammonium, nitrate nutrition increased root-derived CO₂ efflux up to 50%;
- Carbon costs of nitrate reduction were higher for plant species which locate the nitrate reduction site preferably in roots.
- Root contribution to the whole plant nitrate reduction process is not stable during a plant ontogenesis and could be more important during the early phases of plant growth, following by a decrease in nitrate reduction in roots with time. These, consequently reduces C costs associated with nitrate reduction for more mature plants.
- The speed of C cycling through a single plant changes with growing stage. The earlier evolution of CO₂ from the soil corresponded to the later growing stage of corn and lupine, meaning that growth stage could control the metabolic orientation of plants, influencing source (photosynthetically active leaves, which supply a new C) - sink (developing organs of plants, which compete for the new C) interactions, by this accelerating or slowing down the speed of C translocation to roots.

All these should be taken into account while modelling and interpreting the data of CO₂ efflux from soil, particularly separating estimation of individual CO₂ sources which contribute to the total soil CO₂ efflux.

Effect of defoliation management practices on root respiration and microbial activity (Chapter 4)

To study the effect of mowing and grazing on soil respiration of different origin 5 fence areas, which prevent the inclosed plots from mowing and grazing, were installed in Amplero in 2002. Plots for partitioning of soil respiration were established in managed and unmanaged soil with further bimonthly measurements of respiration fluxes during the year 2006 and 2007. In 2006 two soil sampling for further chemical and biochemical analyses were performed: just after the mowing and four months after the mowing. Grassland management, based on plant defoliation appears to be a suitable management practice, influencing positively the below-ground food-web, and thus SOM transformation and nutrient cycling through increasing the quantity of easily available C substrates, shifting to more efficient microbial community with enhanced C use

efficiency, indicating future positive trends of SOM accumulation. The main results of the experiment are:

- Defoliation practices decreased all components of soil respiration in confront with non managed plots under common temperature and soil water content;
- Dependence of root respiration on C supply from shoots is evident from the absence of correlation with soil temperature in managed plots, where the biomass increment under favourable temperatures and SWC was controlled by mowing and grazing. In unmanaged plots root-derived respiration was following temperature patterns, which masks usually the effect of photosynthetic C supply.
- An observed decrease in microbial-derived respiration was confirmed by laboratory measurements of potential microbial respiration rates. Defoliation practices resulted in the increase of the quantity of easily available C substrates through enhanced root rhizodeposition process, which lead to general microbial relax in terms of C gaining and C mineralization rates. Enhancement of N mineralization with positive feedbacks for plant uptake, leaf tissue N content and further benefits for grazers was observed.
- Two methods applied for estimation of the C mineralization activity under different management practices: *in situ* measurements of microbial-derived respiration and potential microbial respiration, measured in laboratory after 28 days of soil incubation, showed comparable trends after accounting for differences in soil temperature and humidity between managed and unmanaged soils.

Contribution of individual soil respiration sources to total CO₂ efflux by different in situ partitioning techniques (Chapter 6)

Were chosen three widely used and perspective partitioning techniques: 1) mesh exclusion technique, a modification of widely used root exclusion method, which was chosen as a reference method in 2007-2008; 2) Combined method: soil induced respiration (SIR) and component integration, applied in 2007; 3) Regression analyses technique, applied in 2008. The estimates of root/microbial contribution to soil respiration obtained by partitioning methods were compared. The main results are the following:

- Three partitioning methods showed comparable results in seasonal variation patterns of root-derived respiration
- The magnitude of root-derived respiration however differed between methods due to particulate shortcomings of each one:

Mesh exclusion: the presence of lateral flow of CO₂ in the soil respiration measured from the nylon mesh bags was confirmed by pulse labeling of the surrounding soil in ¹³CO₂ atmosphere ⇒ overestimation of microbial component of soil respiration;

Combined SIR: a possible boosting of root respiration after the addition of glucose solution, which could be especially true under insufficient soil moisture conditions. This could result in the subsequent overestimation of microbial component of soil respiration. The coefficient of respiration increase after the glucose addition (*k*) differs, depending on the source of microbial respiration: microbial decomposition of dead roots, falloff, detritus or soil organic matter. It was not possible to account for the *k* associated with the decomposition of fine roots. This implies a certain underestimation of the coefficient and again further overestimation of microbial respiration.

Regression technique: Regression technique, given the most uncertain results with low R₂ and non significant regression coefficients, during the whole period of measurements was overestimating the root-derived respiration in confront with mesh exclusion method, sometimes calculating the root contribution as a 100% to total CO₂ efflux from soil. To overcome all the uncertainties, which were mainly associated with the particularities of the grassland ecosystems, the method requires further development and standardization.

1.7. Conclusions

Estimating the contribution of individual sources of CO₂ to the total CO₂ efflux from soil and response of each component to different controlling factors is important for deeper understanding of the terrestrial carbon cycle and its feedbacks to climate change as well as for developing models that can effectively predict the future changes. Whilst measurements of soil respiration and partitioning experiments have been widely diffused for a range of forest ecosystems, comparatively little is known about grasslands.

In this study we have investigated the response of soil CO₂ efflux and its components: root- and microbial-derived respiration to different biotic and abiotic factors as well as to widely diffused management activities over a period of three years in a mediterranean grassland site. A particular attention was dedicated to studying of the aboveground-belowground interactions in terms of C gaining and its translocation velocity within a plant community. Different methodological

approaches for studying of the contribution of various respiration sources to total CO₂ efflux from soil and the speed of C cycling within the plant community were tested.

The use of micro pore mesh bags, utilized previously in forest ecosystems and agricultural crops, resulted as a promising tool for partitioning of soil respiration fluxes in grassland ecosystems, however some specific modifications are necessary. Its combination with the eddy covariance measurements, which is widely used for the monitoring of the CO₂ exchange between vegetation and atmosphere, gives the possibility to study the speed of C cycling within and between various ecosystems. The reliability of time lag between the photosynthetic C uptake and its following evolution through the rooting systems obtained by mesh exclusion technique was confirmed by the results of the pulse labeling of plants in ¹³CO₂ atmosphere. Another promising advantage of the method is an option of variation of the mesh pore size: depending on the scope of the research and changing the aperture diameter it is possible not only to separate root-derived from microbial-derived respiration, but also allowing the in-growth of only mycorrhizal hyphae in the inclosed soil to separate actual root from mycorrhizal respiration sources.

The obtained results showed an importance of C assimilate supply in the determination of the variability of root component of soil respiration. It was closely related to gross primary production with a time lag of circa 20h for time scales from daily to annual. Soil temperature which often masks the direct relationship between root respiration and photosynthetic C supply failed to explain diurnal and seasonal changes in root-derived respiration. Confront between defoliated and non defoliated soils revealed a tight coupling of root respiration with photosynthetic C supply from aboveground. Laboratory experiments with a single plant species have shown however that the observed time lag is not stable during the plant ontogenesis, and varies depending on the plant growing stage. The same photosynthetic activity could also result in different magnitude of root respiration, depending on the type of nutrient supply (ex: N in form of NH₄⁺ or NO₃⁻). All these findings suggest that root respiration is a complex process, tightly coupled to plant canopy activity and could not be explained simply by changes in soil temperature and moisture. Further studies are needed to verify the bonds between aboveground and belowground processes for different species and vegetation types, as well as for various plant growing stages.

Soil temperature and soil water content exerted a significant effect on microbial component of soil respiration. Being a larger part of total CO₂ efflux from soil at Amplerö (≈ 70%), these factors influenced also total soil respiration dynamic on different time scales. Introduction of a management regime have modified however the activity of microbial community by an increase of the quantity of easily available C substrates from the rhizodeposition process, resulting in a general suppression of microbial enzymatic activity and further decrease in C mineralization rates. Combination of laboratory studies and *in situ* measurements is necessary for understanding of the

effect of changing substrate quality, nutrient and moisture conditions on microbial activity and its C use efficiency.

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2. DRIVERS OF SOIL RESPIRATION OF ROOT AND MICROBIAL ORIGIN ON VARIOUS TIME SCALES IN MEDITERRANEAN GRASSLAND

2.1. Introduction

Soil respiration (R_s) is an important component of the ecosystem C budgets. It is a major source of CO_2 released by terrestrial ecosystems and after the photosynthesis, CO_2 efflux from soil remains the second largest C flux accounting for 60-90% of total ecosystem respiration (Goulden et al., 1996; Longdoz et al., 2000; Raich and Schlesinger, 1992). R_s is known to experience high spatial and temporal variation with different controlling factors involved on different time-scales. However, up to now not so many studies have deal with the interannual variability of soil respiration and only few of them were performed in grassland ecosystems (Craine et al., 1999; Mielnick and Dugas 2000; Raich et al., 2002; Zhou et al., 2007; Bahn et al., 2008) despite the fact that it is one of the world's most widespread vegetation types which comprises 32% of the earth's area of natural vegetation (Adams et al., 1990).

R_s integrate the CO_2 produced by soil microorganisms in the root-free and root-affected soil and actual root respiration. Microbial respiration in the root-affected soil, so called rhizomicrobial respiration, is closely coupled to roots distribution and activity and could be hardly separated from the last one (Kuzyakov, 2006). We will call this type of respiration as 'root-derived' (R_a), and the CO_2 originated from the root-free soil as 'microbial-derived' respiration (R_h). According to recent reviews the relative contribution of R_a and R_h generally accounts for approximately one half of the total CO_2 efflux (Hanson et al., 2000, Subke et al., 2006), but varies significantly among studies (10-90%) Quantifying the contribution of these two major respiratory sources to the total CO_2 efflux and understanding the seasonal and interannual variability and their response to climate change is very important for succeed modelling and prediction of the ecosystem C cycling. The potential change in soil CO_2 efflux will largely depend on the relative contribution of R_a and R_h to the total CO_2 efflux.

The dynamic of the two components of soil respiration may be controlled by different abiotic and biotic factors, such as temperature, water availability, nutrient supply, plant phenological development. In addition, the response of R_a and R_h to the temperature changes is different, exhibiting various Q_{10} value (Boone et al., 1998; Rey et al., 2002). Recent studies have shown that apart of well studied effect of soil temperature and soil water content, the supply of assimilates from photosynthetically active plant organs have a significant effect on the root-derived respiration (Moyano et al, 2008; Carbone and Trumbore, 2007). Due to the fact that in many ecosystems the contribution of root respiration is quite high and could arrive up 90 % of total soil respiration (Hanson et al., 2000), the last could be also affected at a high level by the canopy photosynthetic activity. In fact, Bahn et al. (2008), Janssens et al. (2001), Reichstein et al. (2003), Bremer and Ham (2002) have shown that within and across various grassland and forest

ecosystems soil respiration on annual scale is closely related to gross primary production (GPP) and its surrogate leaf area index (LAI).

Isotope studies, applying the isotope pulse labeling techniques have demonstrated that the processes of photosynthetic C uptake and its following evolution through the rooting systems are coupled with a time lag in the range of minutes to days (Gavrichkova & Kuzyakov, 2008; Carbone and Trumbore 2007; Staddon et al., 2003; Ostle et al., 2003), suggesting the diurnal variation in soil respiration is also affected photosynthetic C supply.

Summarizing the above findings and uncertainties the aims of this study were: 1) to partition the soil respiration into root and microbial derived CO₂ efflux in a Mediterranean grassland 2) to characterize what factors are responsible for the variation of respiration of different origin on various time scales: from daily to interannual. We hypothesize that root-derived and total soil respiration would be positively correlated to changes in GPP.

2.2. Materials and Methods

2.2.1. Study site

The study was carried out at the Amplero grassland site. Amplero was established as a main CarboEurope site in central Italy near the city Collelongo (AQ) in the year 2002. A Mediterranean grassland site, located at 900 m a.s.l. Amplero is a nearly flat to gently south sloping (2-3%) doline bottom with an average annual temperature of 10°C and average annual precipitation of 1365 mm. The site is subjected to a long-term management since 1950, which consists in a once-a-year mowing during the peak of the growing season and the rest of the growing season the site is used as a pasture for cattle grazing.

The soil is classified as Haplic Phaeozem (FAO classification) and contains 13% of sand, 33% of silt and 56% of clay, pH_{H2O} of 6.6, total carbon (C) 3.48 % and total nitrogen (N) 0.28%. The plant cover is mainly represented by the following families: *Caryophyllaceae* (19%), *Faseolaceae* (30%) and *Poaceae* (34%).

2.2.2. Partitioning technique

Soil respiration was measured every two weeks in the course of three years: 2006, 2007, and 2008. Measurements were made manually with an infrared gas analyzers (IRGA) operated in the closed path mode. Two closed dynamic systems were involved in the measurements: LI-COR 6400, connected to soil chamber LI-6400 09 (LI-COR Inc., Lincoln, NE, USA), which was used in 2006 and EGM-4, connected to soil chamber SRC-1 (PP systems, UK), which was used in 2007 and 2008. A confront between the systems was performed, to ensure the comparability of the data.

In the year 2005 ten PVC collars for total soil respiration measurements were established in the soil of Amplero inside the main footprint of the eddy covariance station. To avoid root severing, PVC soil collars of 11 cm in diameter were inserted only 2.5 cm into the soil and stabilized with two iron legs to prevent its moving when the chamber was placed on it. In April 2006 five complete partitioning plots were added, aiming for estimation of contribution of autotrophic and heterotrophic soil respiration to the total CO₂ efflux. In April of 2007 the next group of partitioning plots was added to the old one, so that the total amount of old and new added partitioning plots were 10 (Fig. 1).

Soil CO₂ measurements were performed twice a month during the growing seasons from April to November and monthly during the winter months. No data were taken during the winter 2006-2007 as the soil was under the snow cover. Diurnal measurements were performed once a month during the whole growing season 2007 and 3 times during the year 2008. The number of measurements per day differed from month to month and were dependant on the battery status of the instrument, weather conditions etc. Soil temperature was measured near each collar at 5 cm depth using either LI-COR 6400 or EGM temperature probes. Soil water content ($\text{m}^3 \text{m}^{-3}$) at depth of 5 cm was measured with a portable system (ThetaProbe ML2x, Delta-T devices Ltd, Cambridge, UK) at three point around each control collar and once inside each partitioning plot.

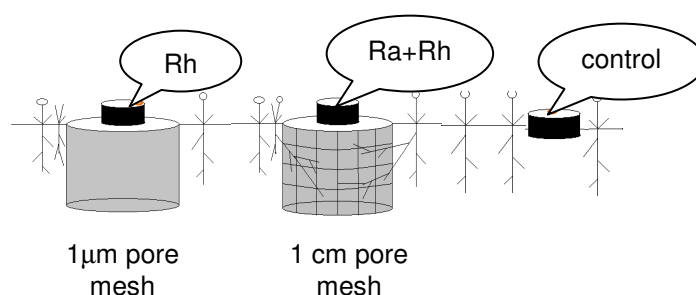


Fig.1 Schematic description of the modified root-exclusion technique. One complete partitioning plot is represented.

A root exclusion technique was modified after Leake et al. (2004) and Moyano et al. (2007, 2008), making it more adaptable for the particularities of grassland ecosystems.

- Soil cores, 20 cm in diameter and 30 cm deep were sampled; the soil was sieved through 4 mm mesh and all the roots were carefully removed from the soil.
- Half of the sampled soil was placed to the nylon meshes with 1µm pore size and returned to the study site. The CO₂ measured from these meshes was considered as respiration associated with microbial decomposition of SOM – microbial-derived respiration (Rh) after disturbance.

- Another half of the sampled and sieved soil was placed back without any barriers for the roots growing (bags with 1.0 cm pore size). The CO₂ efflux, coming from these bags is a sum of microbial and root-derived respiration (Rh+Ra).
- Root-derived respiration was calculated as a difference between these two treatments: ((Rh+Ra)-Rh).
- Total soil respiration (Rs) measured from the undisturbed soil was used as a control and in combination with root-derived respiration was used to calculate the real microbial-derived respiration: (Rs – Ra).

Summarizing:

1 μm = microbial-derived respiration (Rh), after disturbing

1 cm = sum of microbial and root-derived respiration (Rh+Ra)

1 cm - 1 μm = root-derived respiration (Ra)

No mesh = control for total respiration (Rs)

Control - Ra = microbial derived respiration (Rh), excluding the effect of soil sieving

2.2.3. Eddy covariance data

Ecosystem net carbon and water vapour fluxes have been measured at Amplero continuously since 2002. The eddy covariance system integrates a sonic anemometer (Solent R3, Gill Instruments, Lymington, UK) and a LI 7500 open path infrared gas analyzer (LiCor, Lincoln, NE, USA). The flux data were calculated for 30 min intervals by means of the post-processing program Eddyflux.

GPP was calculated from the difference between NEE and TER (Reichstein et al., 2005), where functional dependencies of total ecosystem respiration (TER) with temperatures were determined with CO₂ fluxes measured at night (fluxes measured during low mixed periods were discarded, Aubinet et al., 2000). These functions were then applied to daytime data to derive GPP. GPP was estimated for the year 2006 and 2007, data of 2008 are still under elaboration.

2.2.4. *Biochemical analyses*

Microbial Biomass C

Soil sampling was performed twice in the year 2006: in the middle of the growing season, in June and in the end of the growing season, in October. For each sampling date 8 soil cores were collected.

For MBC determination the Fumigation Extraction method (Vance et al., 1987) was used. In brief, two portions of moist soil (20 g oven-dry soil) were weighed, the first one (non fumigated) was immediately extracted with 80 ml of 0.5M K_2SO_4 for 30 min by oscillating shaking at 200 rpm and filtered with filter paper (Whatman n. 42); the second one was fumigated for 24h at 25 °C with ethanol-free $CHCl_3$ and then extracted as described above. Organic C in the fumigated and non fumigated extracts was determined after oxidation with 0.4 N $K_2Cr_2O_7$ at 100°C for 30 min. Microbial C was calculated as a difference between C content in fumigated and non fumigated extracts divided by a conversion factor (K_{EC} : 0.38).

2.2.5. *Statistics*

Each measurement plot of soil respiration was considered as experimental unit, the replicate measurements were averaged for further analyses, so that soil CO_2 efflux for each date represents the average of all the plots. Standard error (SE) of mean was calculated to provide a measure of the variance for aboveground and belowground biomass and soil respiration flux measurements. Multiple linear regression analysis was used to evaluate the contribution of GPP of various times after the measurements to soil CO_2 efflux from soil. Nonlinear regression analyses were performed to estimate the effect of temperature and soil water content on soil respiration of different origin. The significant of all the regression coefficients were tested using STATISTICA (Stat Soft).

2.3. Results

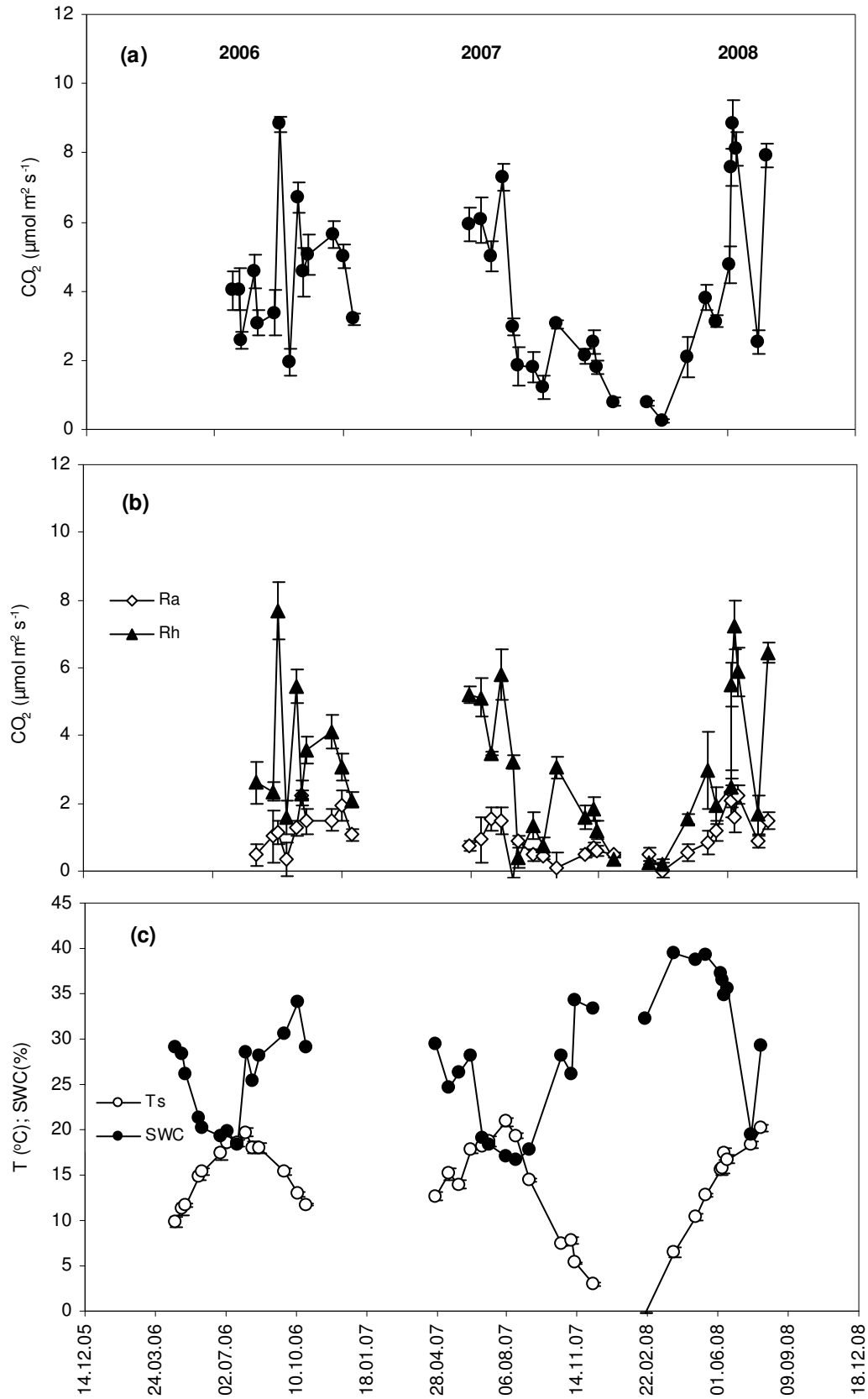


Fig. 2 Seasonal and inter-annual variability of CO₂ efflux originating from a) total soil respiration 2006-2007-2008 b) root-derived (Ra) and microbial-derived (Rh) soil respiration in 2006-2007-2008 ($\pm$ SE) c) Soil temperature (Ts) and Soil water content (SWC) at 5 cm depth in 2006-2007-2008.

Variation of soil respiration from different sources together with soil temperature and soil water content during the measurement campaigns 2006-2008 is represented in figure 2. Calculated fluxes of various origin differed significantly in magnitude and showed also different annual course.

Total soil respiration in 2006 peaked in the beginning of July, whereas in 2007 and 2008 the highest respiration was observed in the middle June, with the following decrease. After the period of the low fluxes in July and August which is associated with the low SWC and high soil temperatures soil respiration increased again (Fig. 2a and 2c).

Microbial-derived respiration was generally much higher than the root-derived CO₂ efflux (Fig.2b). Comprising a high proportion of total soil respiration, their annual courses were also similar. Root-derived respiration performed differently during various years. In 2007 and 2008 the maximum value was reached during the month of June followed by a decrease during the dry period. In 2006 root-derived respiration was increasing constantly up to the month of July. The decrease of root-derived respiration associated with drought was less pronounced than for total and microbial-derived respiration, in fact the relative contribution of root-derived respiration to the total CO₂ efflux during this period increases (Fig.3). In the late summer, during the months of August and September root-derived respiration generally experienced the second peak. This period is associated with the mild temperatures and high SWC, which favour the re-growth and new onset of vegetation. Example of above- and belowground biomass changes in the year 2007 are shown in Figure 4

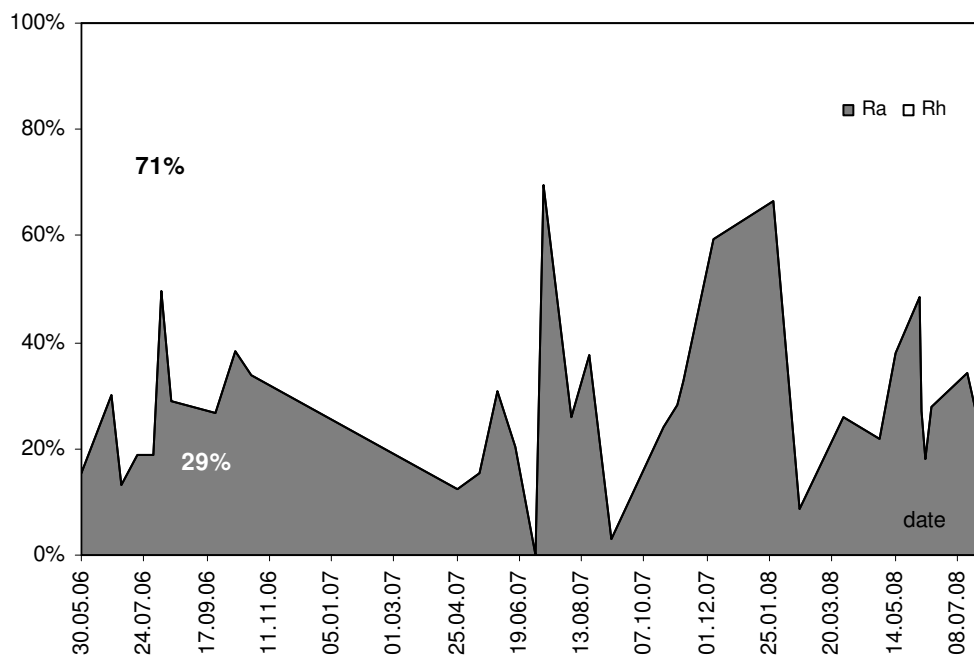


Fig. 3 Relative contribution of root and microbial-derived respiration to total CO₂ efflux during three years of measurements. Percentage in the graph indicate an average contribution.

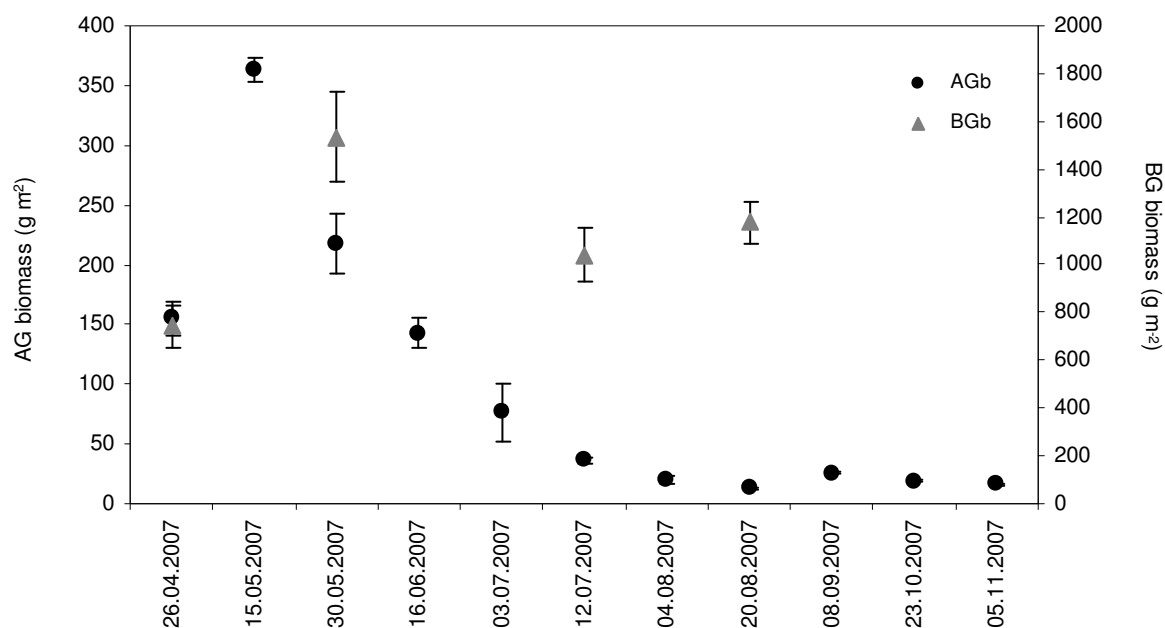


Fig. 4 Changes of the aboveground (AG) and belowground (BG) biomass ($\pm$ SE) in the course of the growing season 2007.

An average contribution of roots to total CO₂ efflux from soil amounted to 30%. However the range of variation in root respiration contribution to Rs varied significantly in course of the growing seasons from 2 to 70% (Fig.3).

2.3.1. Diurnal variation of soil respiration of different origin

Response of soil respiration of different origin to diurnal changes in soil temperature in 2007 and 2008 is represented in figure 5. The first observation which is possible to make from the graphs is that any of respiration sources do not follow diurnal patterns of soil temperature often decreasing suddenly under the high respiration rates, independently from the period of the growing season when the measurements were performed (Fig. 5: ex. 15 June, 11 July, 19 August; 11 November; 5 June; 2 August). Another important observation is that in different times of the day with similar temperatures soil respiration is different. Changes in SWC also failed to explain the diurnal variation in soil respiration fluxes, its fluctuations during the day were negligible (Fig. 6).

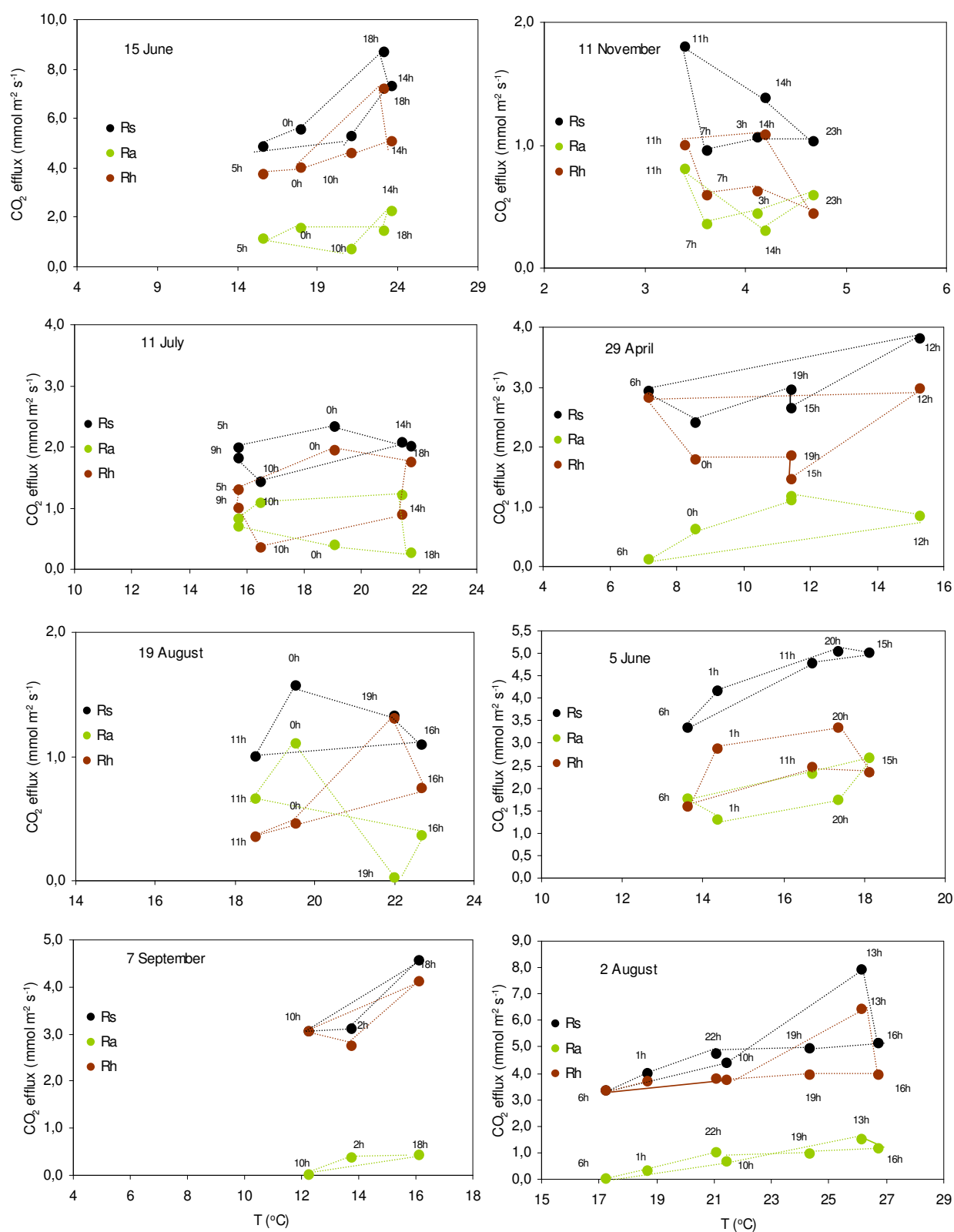


Fig. 5 Diurnal changes of total (R_s), root (R_a) and microbial (R_h) derived soil respiration in response to soil temperature at 5cm depth in the year 2007 and 2008. Consecutive hours were connected by lines, inserts indicate time of the day.

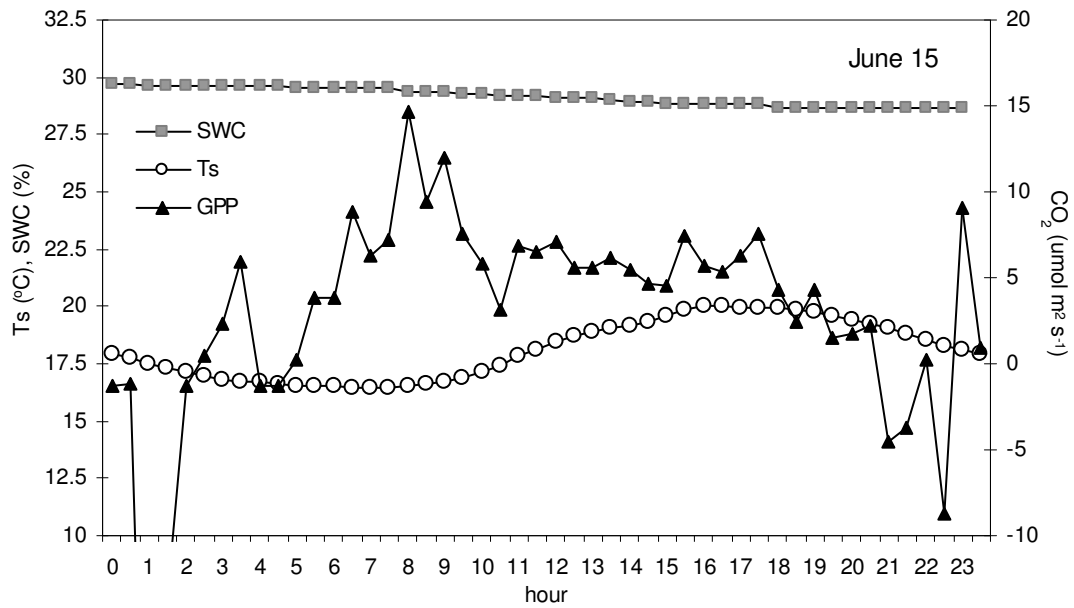


Fig. 6 Diurnal variation of soil temperature (Ts) at 5 cm depth, soil water content (SWC) at 5 cm depth and gross primary production (GPP) on the 15th June 2007. Data are a 30 minutes averages from the meteo station installed in Ampero.

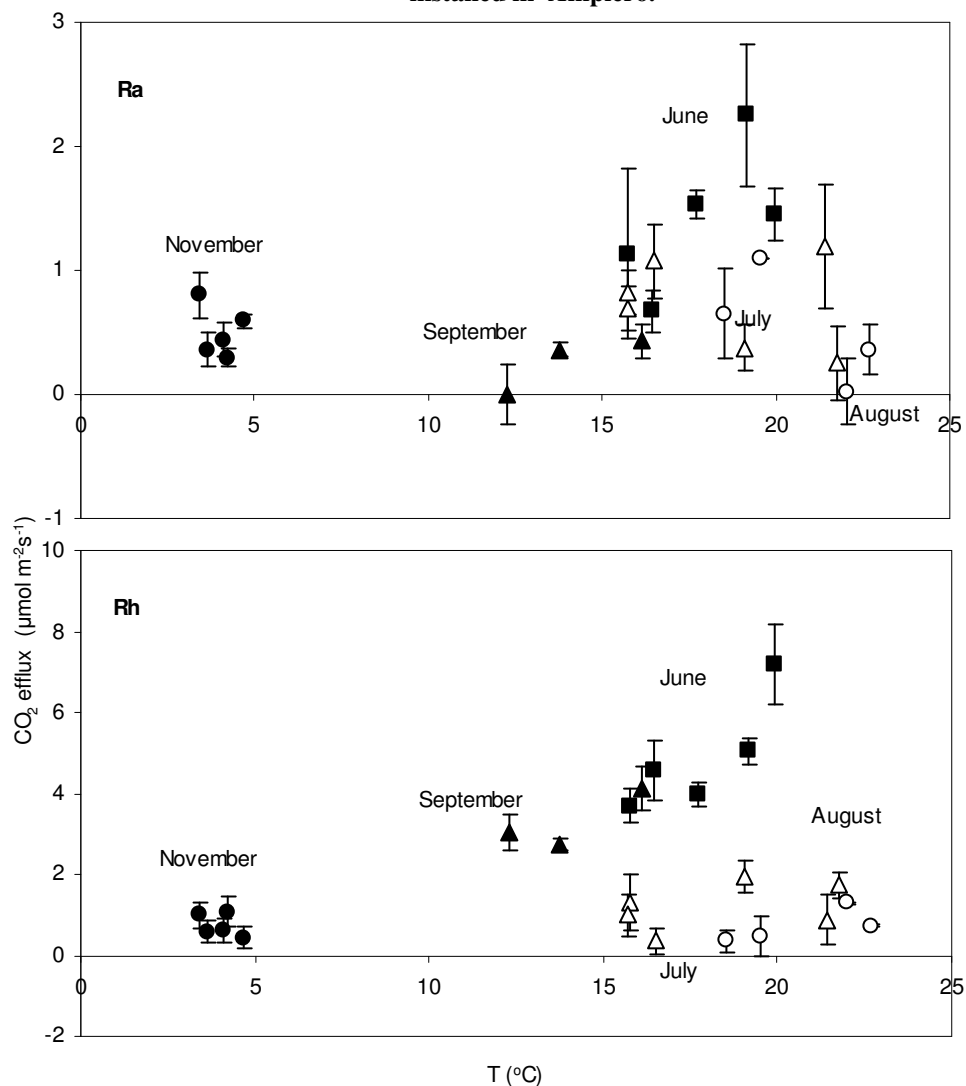


Fig. 7 Variation in soil respiration of root (a) and microbial (b) origin ($\pm$ SE) vs. changes in soil temperature in the course of the vegetation season 2007.

All diurnal measurements of root and microbial-derived respiration performed in various months of 2007 were plotted together against changes in soil temperature (Fig. 7). Microbial respiration clearly increases with increase in the soil temperature: starting from November, September to June. Out of the general trend are the days of July and August with the low microbial activity under the high values of temperature, this period is however characterized by a low SWC. Root-derived respiration didn't show such a clear dependence on seasonal changes in temperature and SWC, with relatively high values also during the driest days of July and August. However, the maximum efflux was observed as for microbial respiration in June.

2.3.2. Seasonal variation of soil respiration of different origin

Although we have found that the diurnal variation in soil respiration was not strongly correlated with soil temperature, increasing the time scale to seasonal level changes a picture. We assessed sensitivity of soil CO₂ efflux of different origin by fitting exponential function to the data from individual treatments.

$$R_x = a \exp^{bT_s}$$

where R_x is soil respiration from the source, T_s is a soil temperature (°C) and a is the intercept of respiration when temperature is 0°C (basal respiration rate) and b represents temperature sensitivity of soil CO₂ efflux. The b values were used to calculate a respiration quotient (Q_{10}), which describes change in fluxes over a 10°C in soil temperature. As the winter measurements were not possible in 2006-2007, we assumed respiration rate, measured in 2008 under 0°C soil temperature as a basal respiration and applied this value to all the other years.

$$Q_{10} = \exp^{10/b}$$

During the period with SWC > 20% total and microbial-derived soil respiration were quite well correlated with seasonal changes in soil temperature at 5 cm depth (Fig. 8), increasing exponentially with increasing of T_s . The exponential relationship in the absence of the water stress accounted for approximately 82% of flux variability for R_s ; 80% for R_h and 60% for R_a (Fig. 8, Table 1). The overall Q_{10} values for the years 2006-2007-2008 are represented in Table 1. Including in the analyses periods with lower SWC resulted in the significant decrease of the Q_{10} 's and loss of the correlation with temperature.

Root-derived respiration was less sensitive to changes in soil temperature, the correlation was not strong and under temperatures higher than 15°C and favourable soil humidity (SWC > 20%) the shape of the regression curve was reaching a plateau, without a characteristic exponential increase, observed for R_h and R_s (Fig. 8).

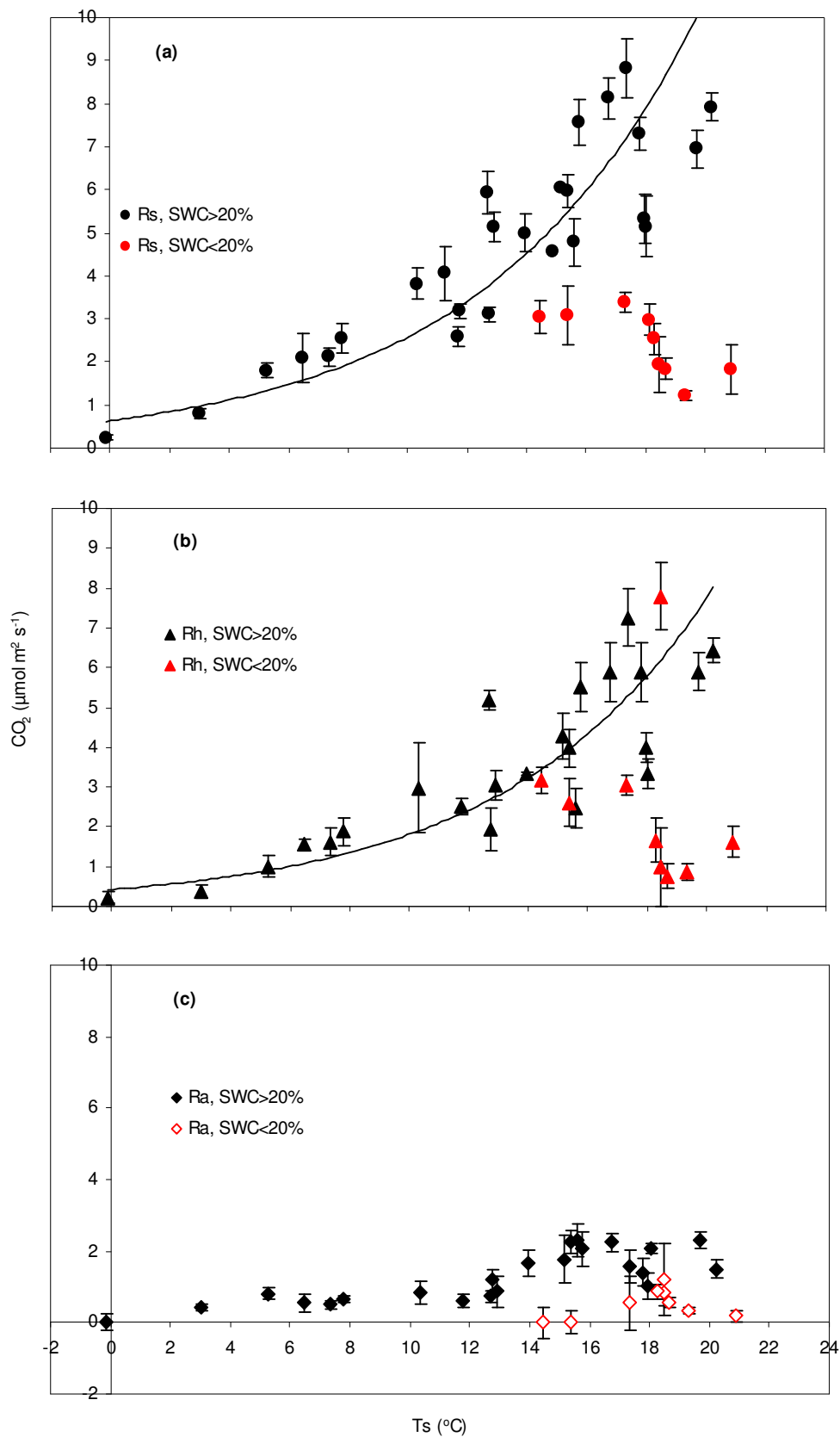


Fig. 8 Relation of total, root- and microbial-derived soil respiration ($\pm$ SE) with soil temperature at 5 cm depth. Data from 2006-2007-2008 were pulled together. The data at $\text{SWC} < 20\%$ were excluded from the regression analyses.

	Q ₁₀	R ²	p
Rs	2.51	0.77	0.000
Ra	2.23	0.60	0.05
Rh	2.72	0.71	0.000

Table 1. Overall (2006-2008) Q₁₀ values obtained from the response of soil respiration of different origin to changes in soil temperature. The data at SWC<20% were excluded from the analyses

Respiration Q₁₀ values obtained from each treatment varied in the course of the growing season. An example of Q₁₀ variation is given in Table 2 for the year 2007. The growing season was subdivided into 3 periods: pre-harvest period (April-June); drought stress period (July-September) and the end of the growing season (October-January). The overall Q₁₀, excluding the drought stress period for Rs and Rh was quite high amounting to ca. 3 and for Ra to 2.2. Values for the shorter time periods varied significantly. The Q₁₀ of the microbial-derived respiration was highest in the end of the growing season, intermediate in spring and lowest in summer. Q₁₀ of total respiration efflux repeated the patterns of microbial one. Root-derived respiration experienced the highest Q₁₀ during the summer drought and lowest in the end of the growing season (Table 2).

Period	Q ₁₀ in 2007					
	Rs		Ra		Rh	
	Q ₁₀	R ²	Q ₁₀	R ²	Q ₁₀	R ²
All	1.23	0	1.21	0.28	1.25	0
All (SWC>20%)	2.97	0.92	2.20	0.71	3.22	0.85
April-June	1.77	0.65	2.27	0.3	1.67	0.28
July-September	0.37	0.52	3.32	0.26	0.18	0.44
October-January	6.69	0.91	1.77	0.42	12.18	0.91

Table 2. Soil respiration Q₁₀ values calculated for each treatment (total soil respiration (Rs); root-derived respiration (Ra); microbial-derived respiration (Rh) for various periods of the growing season 2007.

MBC variation is represented in figure 9. October experienced higher values of MBC in confront with June.

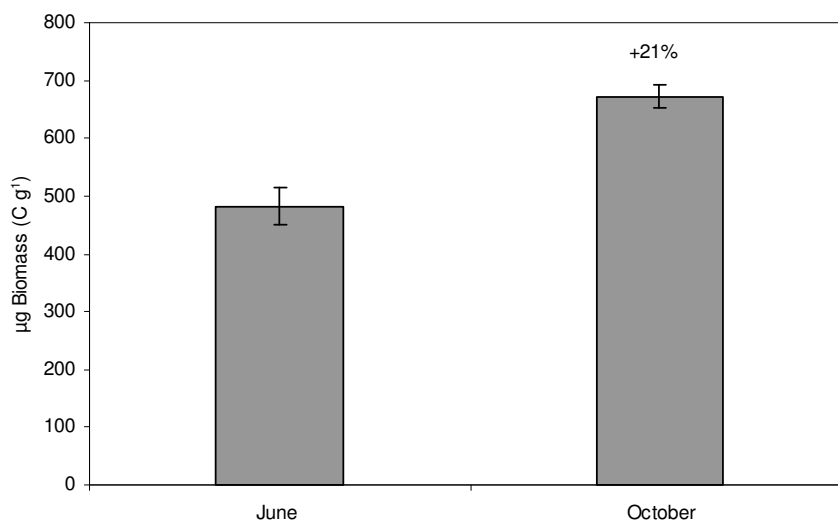


Fig. 9 Changes in microbial biomass C (MBC) during the growing season 2006 (± SE). % indicates an augment in MBC, taking June sampling as a reference.

The influence of moisture on soil respiration was more complex than temperature. In order to remove the temperature effect and examine the single moisture effect on soil CO₂ efflux, soil respiration was normalized by soil temperature at a reference value 15°C:

$$R_{x15} = R_x Q_{10}^{(15-T)/10}$$

where R_{x15} is the normalized respiration flux, R_x is the measured respiration, T is the measured soil temperature.

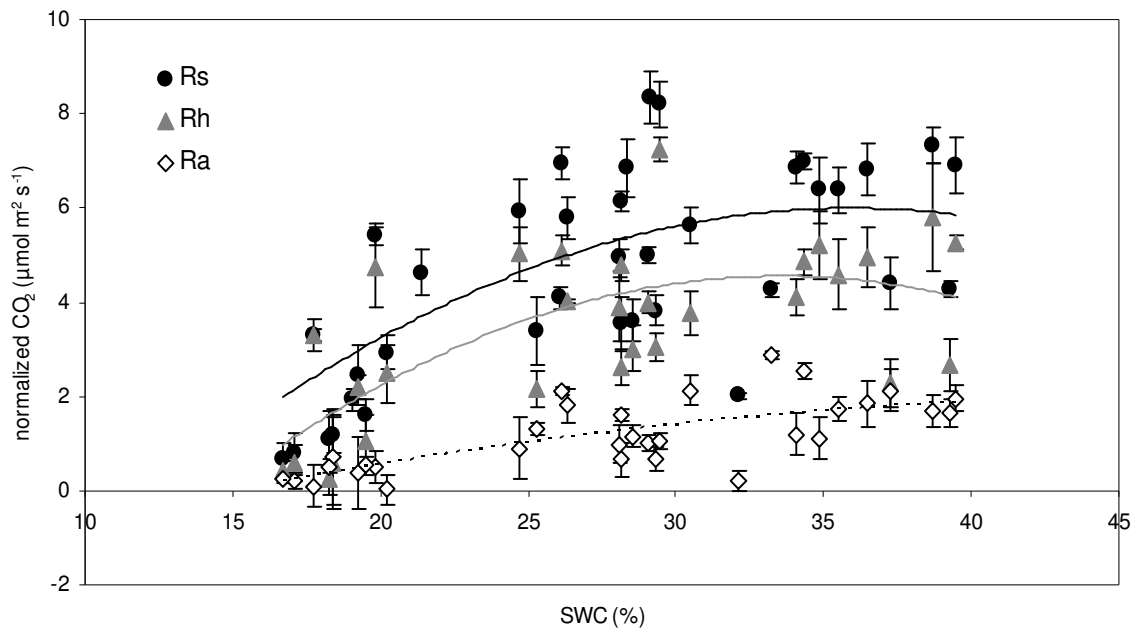


Fig. 10 Normalized total, root- and microbial-derived soil respiration vs. soil moisture measured in 2006-2007-2008.

From figure 10 is clear that soil moisture had two opposite effects on total and microbial soil respiration. When soil moisture was below critical value (below 25%), soil respiration increased with moisture, but it decreased when the soil moisture was greater than 35%. The negative correlation at high levels of SWC is probably associated with a decrease in soil air porosity and oxygen availability in soils which influence negatively microbial decomposition activity. No negative effect of high SWC was observed on root-derived respiration.

To verify the effect photosynthetic C supply on soil CO₂ efflux of different origin the data from the partitioning experiment were plotted vs. GPP from the eddy covariance. Pearson correlations were performed between the means of normalized and non normalized values of root-derived respiration fluxes and the mean values of GPP 0–7 days prior to each respiration measurement date. Linear regressions analyses were performed for the days where Pearson correlations showed peak values. Significant correlations between GPP and root-derived respiration were observed. Peaks in correlation strength correspond to time shifts between the C uptake through

the photosynthesis and its following respiration through roots. Correlation coefficients are shown in figure 11.

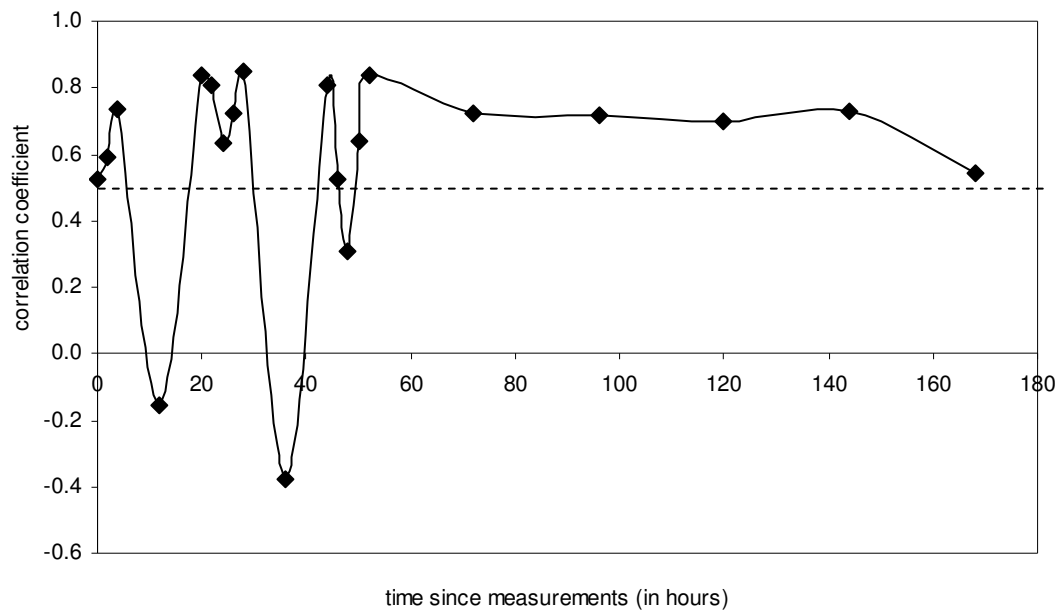


Fig. 11 Pearson correlation coefficients of non normalized root-derived soil respiration against gross primary production (GPP) from 0 to 7 days previous to respiration measurements.

Normalization and excluding days under low moisture content didn't improve the fit with GPP for any of the soil respiration component, making the correlation weaker (data non shown). Results of linear regression for days corresponding to peaks in correlation coefficients are shown in table 3.

Several peaks in correlation strength with GPP were observed. The strongest one correspond to 2, 20, 28, 40 and 46 h. Multiple linear regression analyses showed that the best predictor of the changes in root-derived respiration was GPP 20h after the measurements. (Fig. 11, Table 3). High but not significant correlation coefficient was observed up to 6 days after the measurements.

Changes in root-derived respiration obtained in 2007 versus changes in gross primary production 20h prior to the measurements are shown in figure 12.

Ra vs.GPP				
GPP in h	Beta	B	t	p-level
0	-2.48	-0.22	-1.72	0.16
2	4.24	0.39	2.28	0.08
4	-0.53	-0.05	-0.75	0.50
20	1.60	0.24	2.97	0.01
22	-3.33	-0.30	-2.44	0.07
28	0.36	0.04	0.65	0.55
24	1.17	0.09	1.17	0.31

Table 3. Coefficients of determination for linear regression between soil respiration of different origin and GPP.

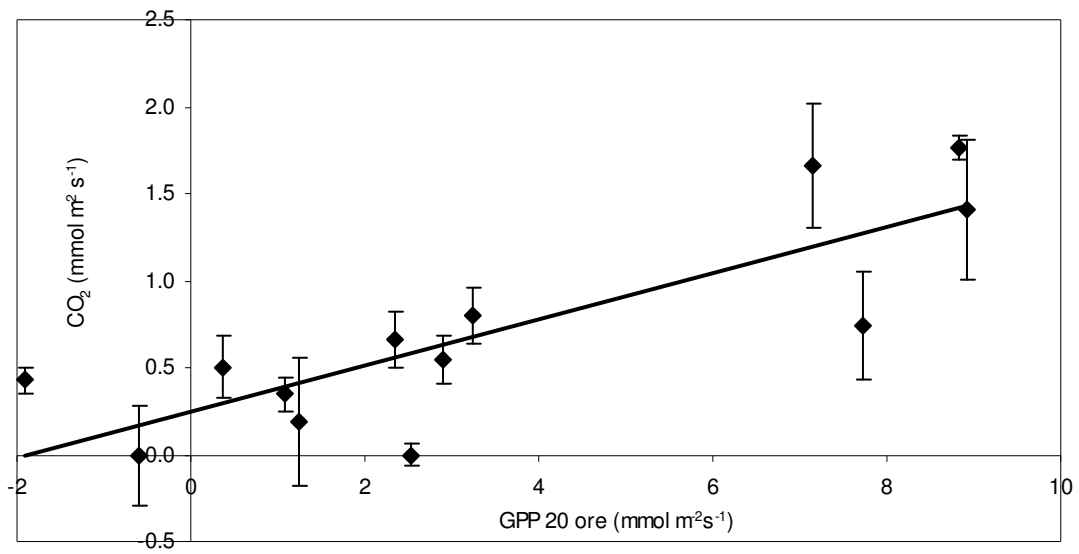


Fig. 12 Root-derived CO₂ efflux versus changes in GPP 20h after each measurement period ($R_2=0.80$, $p<0.001$)

2.3.3. Inter-annual variation of soil respiration of different origin

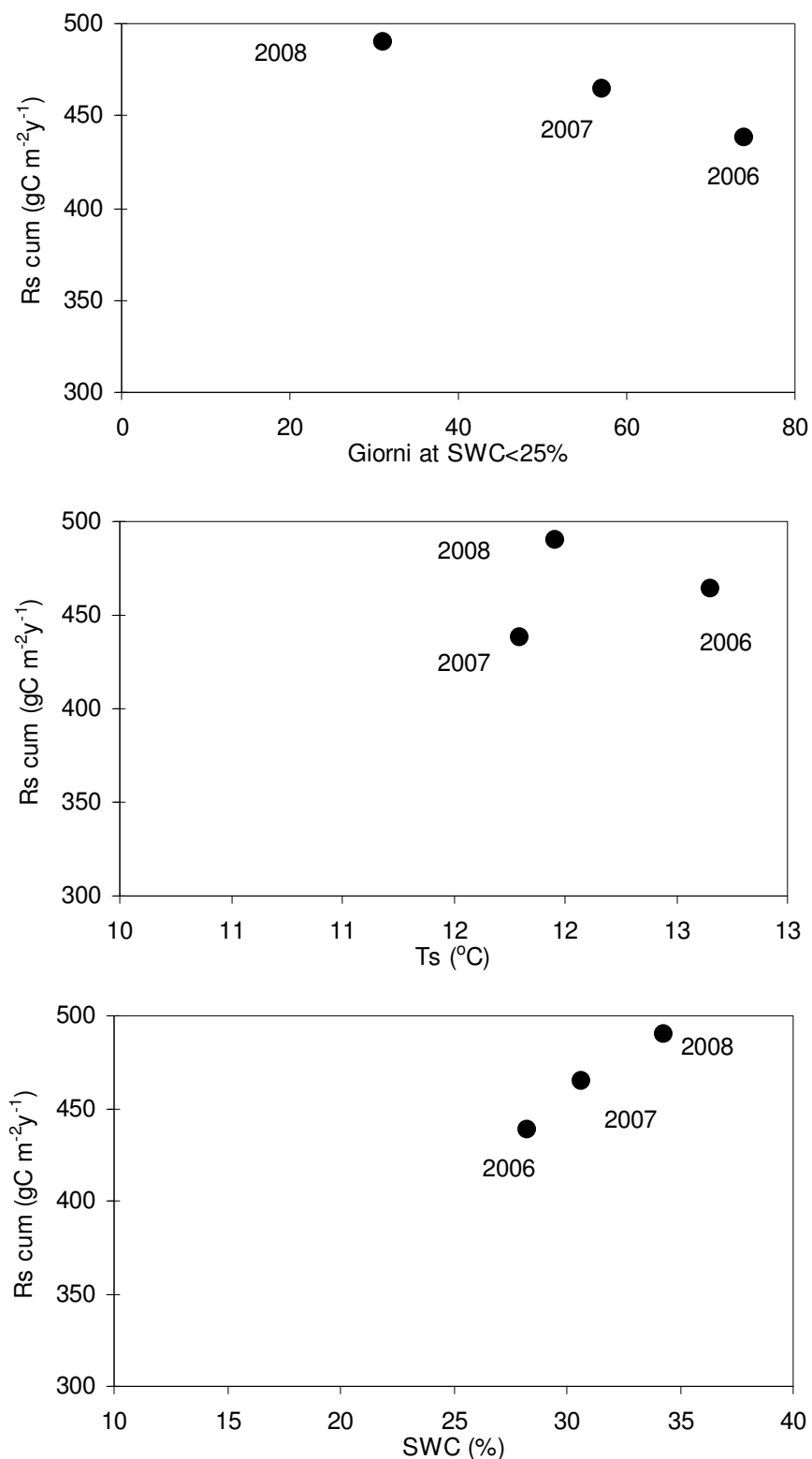


Fig. 13 Cumulative soil respiration (Rs) for the period April- August in relation to a) number of days with SWC<25% with $R^2=0.98$; $p<0.05$ b) mean soil temperature (Ts) (ns) and c) mean soil water content (SWC) with $R^2=0.94$, $p<0.05$.

In 2008 the eddy covariance station installed at Amplero was functioning only up to the beginning of September. To be able to take into account and confront the data of this year, all the cumulative CO₂ soil fluxes, meteorological and physiological parameters were calculated excluding the months of September, October, November and December.

Cumulative CO₂ efflux was estimated by summing the products of soil CO₂ efflux and the number of days between samples. Our measurements, collected between 10:00 and 13:00 hours being 90% of the observed diurnal averages, were assumed to be representative.

Cumulative soil CO₂ efflux from Amplero ranged from 438 to 490 gC m⁻²8months⁻¹ across three years. Interannual variation in Rs was well correlated with the number of days at SWC<25% and mean SWC. Ts failed to explain the interannual variation in Rs (Fig.13).

2.3.4 Modelled soil CO₂ efflux

Soil temperature and soil water content were the main abiotic factors influencing soil CO₂ efflux. To simulate temperature and moisture effect on total and microbial-derived soil respiration during the growing seasons 2006-2007-2008 was applied the following equation:

$$R_x = a * \exp^{(bT)} \exp^{(c*SWC + d*SWC^2)}$$

where R_x is the soil CO₂ efflux of microbial origin or total soil respiration, T is a soil temperature in °C at 5 cm depth, SWC is a soil volumetric water content (m³ m⁻³) and a , b , c and d are the model coefficients. The temperature term takes an exponential form, while the moisture term takes the quadratic form in the exponent to reflect two opposite effects on soil respiration. An example of variation of total soil respiration and microbial-derived respiration with changes in T_s and SWC in the year 2007 are given in figure 14.

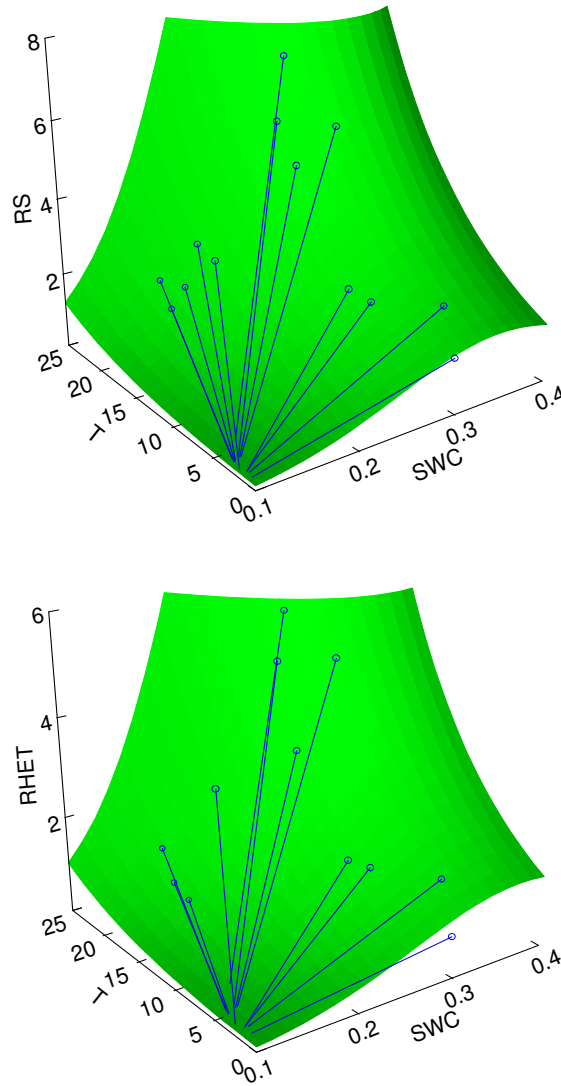


Fig. 14 Relationship of total (Rs) and microbial-derived (RhET) soil respiration with soil temperature (T) and soil water content (SWC) at 5 cm depth for the year 2007.

In figure 15 the residuals (measured value – fitted value) of Rs estimation were plotted vs. gross primary production of the day before each measurement. The model clearly underestimates the soil respiration under high values of GPP and overestimates under low. After correction of the residuals for the GPP, the model's fitting have slightly improved.

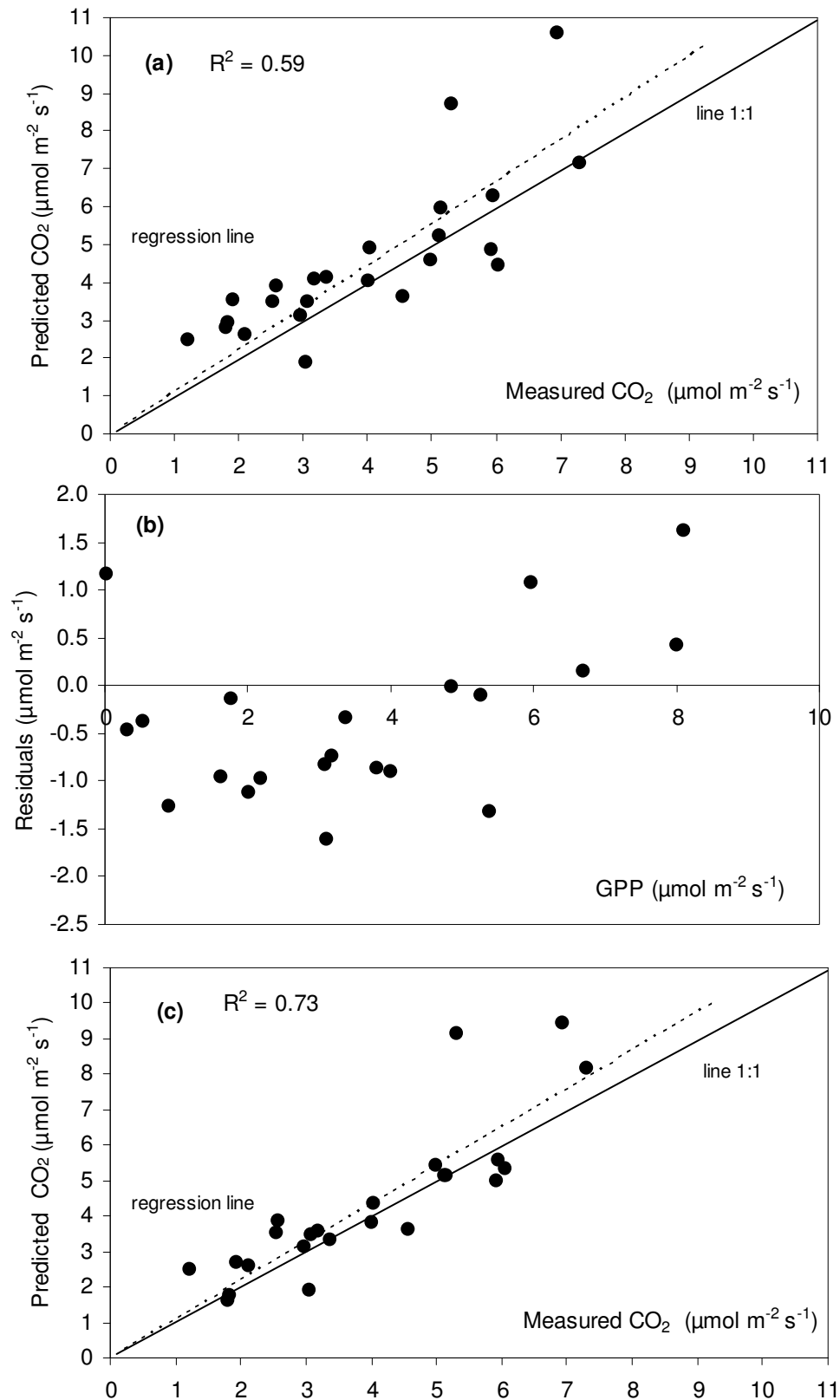


Fig. 15 a) Measured vs. predicted CO₂ efflux b) Residuals vs. Gross primary production (GPP); c) Measured vs. predicted CO₂ efflux including GPP in the model equation. Data of 2006 and 2007 were used.

2.4. Discussion

2.4.1. Partitioning of soil respiration

In our study the relative contribution of Ra to total CO₂ efflux over the 3 years amounted to 29%. During the growing seasons the relative contribution of Ra varied from 2 to 70%, however an increase in the relative input of root respiration to the total one was associated mainly with a considerable decrease in microbial respiration under the water stress conditions (Fig. 3). The observed average value of root contribution to soil respiration is less than 38-40% and 43-56% reported respectively by Craine et al. (1999) and Wang et al. (2007) for temperate grasslands, and 39-41% reported by Tang et al. (2005) for oak-savannah. The lower value could be attributed to the fact that Amplero is subjected to the annual mowing and grazing, which influence the photosynthetic C supply to roots and thus root respiration itself (see below). In fact, our estimate is consistent with the results obtained in semi-arid grazed grassland of China (15%-37% by Li et al., 2002). The methods used for partitioning also vary between various studies, each with its own shortcomings and limitations, which could also influence the final result, making the comparison between studies especially difficult.

As all soil respiration partitioning methods, the calculated here fluxes are an approximation of real field fluxes. The mesh-exclusion technique we used to partition soil respiration is associated with various limitations and assumptions, which result in a possible overestimation of microbial-derived respiration and underestimation of root-derived one. Namely, the shortcomings of the method are: disturbance of the soil structure by sieving, lateral diffusion of CO₂ (Jassal and Black, 2006) to the mesh bags from the surrounding soil and inability to separate root respiration from rhizomicrobial one. The advantage of the nylon mesh bag technique over other trenching and root-exclusion methods is that it permits to exclude and control the roots in-growth inside the plot and in the same time allows the exchange of water and other substances with an exterior soil.

We tried to minimise the effect of the soil disturbance on microbial activity and respiration fluxes by installing an additional mesh of 1cm, and obtaining the value of root-derived respiration as a difference between two meshes with the equally disturbed soil. However, here we make an assumption that there is no difference in the in-growth patterns of roots between non disturbed soil and soil which was previously sieved. Microbial-derived respiration was calculated further from the difference between control (non disturbed soil) plots and root respiration. The rate of the influence of the lateral diffusion of CO₂ from the surrounding soil is difficult to estimate. Moyano et al. (2008) reported a value of ca. 10%. It results in a systematic overestimation of the microbial-derived respiration. Here we make a second assumption: the influence of the lateral flow of CO₂ is constant during the growing season, and doesn't modify the seasonal trend in contribution of soil respiration components to the total one.

2.4.2. Diurnal, seasonal and inter-annual variability

Soil CO₂ efflux varied significantly during the growing seasons with lower respiratory losses during the winter months (around 0.25 $\mu\text{mol m}^{-2} \text{s}^{-1}$), accompanied by low ambient temperatures and low gross primary production and during summer drought period (around 1.5 $\mu\text{mol m}^{-2} \text{s}^{-1}$) with high temperatures and low SWC. Low CO₂ efflux was compensated by high respiration rates in early and late summer (up to 8 $\mu\text{mol m}^{-2} \text{s}^{-1}$) with comparatively high GPP and favourable Ts and SWC. This variability yielded the cumulative respiration for the period January - September in three years of measurements in the range of 440-490 gC m^{-2} . Model application resulted in the increase of cumulative respiration for the same measurement period to the range of 722-875 gC m^{-2} , indicating that a simple summing of the respiration assuming linear changes in soil CO₂ efflux between the measurement days, used in some studies as an estimate of the year efflux, result in a two fold underestimation of the cumulative fluxes. Care should be taken while comparing annual fluxes, calculated with different approaches. The modelled values are in agree with the one, reported by Bahn et al. (2008) for European grasslands and Raich and Schlesinger (1992) for temperate and tropical grasslands and savannas. Evidently, seasonal variability of soil respiration was much higher than the inter-annual one.

Diurnal variation of soil respiration of root and microbial origin showed different patterns (Fig. 5). Throughout the experimental period soil respiration peaked in different times of the day. However, morning peaks were registered only in the very end of the growing season and in the most cases respiration reached its maximum in the afternoon or late evening. Notably, root and microbial derived respiration peaked in various times of the day, indicating the possibility of different controlling factors or different response-time to the same one. All the respiration sources were out of phase with changes in soil temperature, demonstrating that it is a weak indicator of diurnal variation in soil respiration. SWC showed weak diurnal fluctuations (Fig 6), and was not influencing the soil CO₂ efflux on diurnal basis. During most of the measurement days was observed some kind of a loop after plotting soil respiration versus changes in soil temperature, meaning that under the same soil temperature soil respiration was different, changing with the time of the day. All these suggest that another factor was driving diurnal variation of soil respiration of various origin in addition to soil temperature, which is more likely a photosynthesis. Soil respiration didn't show an instantaneous correlation with photosynthesis, however after shifting it 6 h backward, soil respiration of root origin was proportional to photosynthesis in months of June, August and July, with R₂ around 0.70, though the correlation didn't result significant (data not shown). We have expected to observe a coupling of root-derived respiration with GPP with some time lag on a diurnal basis, however, due to technical limitations: the number of diurnal measurement was too low, ranging from 3 to 6 also with different time periods between the

measurements, the possibility to find a reliable time lag between soil respiration and GPP was limited. Continuous measurements of soil respiration are perspective in this case. In fact, Tang et al. (2005) using solid-state CO₂ sensors and flux-gradient method have demonstrated that photosynthesis is controlling diurnal variation of soil respiration with the time lags changing from 7 to 11 h in the course of the growing season.

A tight relationship of root-derived respiration with assimilate supply was observed on the seasonal basis. Photosynthetic C supply resulted as a best predictor of seasonal changes in root respiration in confront with Ts and SWC, which were however influencing greatly the fluxes of total and microbial-derived respiration. It is generally assumed that the major part of the energy derived from respiration in higher plants is used for growth and maintenance processes (Hansen et al., 1977; Veen, 1981; Amthor, 1994; Desrochers et al., 2002). The sensitivity of root CO₂ efflux to soil temperature is determined by the maintenance component of root respiration. Maintenance respiration is responsible for keeping of the existing root cells and tissues alive, it requires considerable amounts of carbohydrates (Kozlowski and Pallardy 1997) and is also highly temperature-dependent (Sprugel and Benecke 1991). In late autumn and during the winter in the absence of plant growth, root respiration is reduced to the level of maintenance respiration (Desrochers et al., 2002; Wieser and Bahn, 2004). During this period respiration rates experience immediate changes with changes in soil temperatures by a direct effect on enzymatic activity, soil water and nutrient availability. However, during the growing season also the maintenance respiration could be limited by the inputs of the photosynthetic C products from aboveground, due to a high affinity between enzymes and their substrates (Wassink 1972; Hunt and Loomis 1979), it could be a case for the managed grasslands as Amplero. The growth respiration is associated with the costs of the production of a new plant material. It is unaffected by temperature, depending mainly on the supply of non-structural C (Penning de Vries et al., 1974; Desrochers et al., 2002; Xu et al., 2008).

It was shown by numerous studies that newly assimilated C cycles quickly within the ecosystem, being found in root respiration already some hours after its assimilation. In the field and laboratory studies using a pulse labeling technique in ¹⁴C and ¹³C atmosphere the time lags between photosynthetic C uptake and its following respiration from roots varied from minutes to days. In fact, Horwarth et al. (1994), Eklab and Hogberg (2001) and Knohl et al. (2005) reported for different tree species the time lag in the magnitude of days (1d to 5d), while Carbon and Trumbore (2007) and Ostle et al. (2003) for grasslands and shrublands found the time lag being in the magnitude of hour (less than 4 hours up to one day). The time lag of 20 hours obtained in our study corresponds well to the one reported for grasslands. The higher absolute magnitude of lags obtained for tree species in confront with grasses, suggest that a plant height and thus a phloem pathway

length could determine the range in which varies the delay between the photosynthetic C uptake and its following respiration. Inside this limits the speed and the quantity of the translocated C to belowground is more likely determined by plant growing stage (Kuzyakov et al., 1999; Kuzyakov and Domanski, 2000; Gavrichkova and Kuzyakov, 2008; Carbone and Trumbore, 2007). The speed and quantity of C flow to roots will be regulated depending on the current size of rooting system, its growth rate, metabolic activity and relative contribution of fine and coarse roots to total belowground biomass (Dickson, 1991; Desrochers et al., 2002; Larionova et al., 2006; Carbone and Trumbore, 2007) as well as by level of nutrients supply (Veen, 1981; Lambers et al., 1981; Kuiper 1983; Gavrichkova and Kuzyakov, 2008).

The effect of photosynthetic C supply on root respiration is often masked by temperature because root biomass typically peaks in summer (Lyr and Hoffmann, 1967), however roots can only respire a proportion of what they are allocated, so the effect of temperature on root respiration is likely to be constrained by GPP (Janssens et al. 2001). In fact, at Ampler, subjected to management based on the defoliation practices, the decrease in C supply from defoliated plants resulted in further decrease in correlation strength between temperature and root-derived respiration.

The effect was less pronounced for total soil respiration as the root component account for a minor part of soil CO₂ efflux at Ampler (av.30%, Fig. 3). Microbial respiration is more sensitive to changes in soil temperature and soil water content and more likely is independent of GPP, as in the soil there are large amounts of substrates waiting to be decomposed. Microbes however prefer the easily available C substrates, the quantity of which is determined by the litter input and root rhizodeposition processes (Parton et al., 1987; Trumbore et al., 1990; Schimel et al., 1994; Schulze et al., 2000; Bahn et al., 2006), and thus indirectly or with a considerably higher time lags could be dependant on the site productivity.

Temperature sensitivity of microbial respiration decreased from winter to summer (Table 2), which could be explained by physiological acclimation of soil microorganisms to high temperatures as well as by the increase in the microbial population in autumn period (Fig. 9). Similar seasonal changes were observed also by Luo et al (2001), Janssens and Pilegaard (2003) and Moyano et al. (2007). Increasing temperature in winter time could also activate dormant microbes and thus augment its C mineralization activity (Andrews et al., 2000), resulting in a sudden increase of respiration rates. Andrews et al. (2000) reported a stronger temperature effect on microbial population at lower temperatures. Low value of Q₁₀ during the summer drought indicates as well a reduction in the diffusion rates of organic molecules and thus in microbes nutrient availability.

Q₁₀ of microbial derived respiration in the end of the growing season, was however unproportionally high than the overall one and the Q₁₀ reported in literature (Schleser, 1982; Raich

and Schlesinger, 1992; Moyano et al., 2007). This largest Q_{10} , was accompanied by only a modest increase in respiration rates. In fact, as expected from the exponential relationship between soil respiration and temperature the increase in R_h per temperature unit is considerably smaller at lower fluxes in winter than at higher fluxes in summer, despite the higher Q_{10} . This originates from the relative nature of Q_{10} . The very high autumn-winter Q_{10} 's are probably partly related to the low basal respiration rates and therefore are hardly comparable with summertime Q_{10} (Janssens and Pilegaard, 2003).

The Q_{10} of root-derived respiration was highest during the drought period, associated though with a low respiration rates. Changes in soil temperature during this period explained however only 30 % of variation in root-derived respiration, whereas it was perfectly correlated with changes in GPP ($R^2=0.99$, $p<0.001$), which experienced low values after the June harvesting and continued to decrease during the summer drought.

2.4.3. *Modelled soil CO₂ efflux*

The above findings suggest that site productivity in the form of the gross primary production should not be ignored while predicting the changes in soil respiration. This could be especially true for the sites where root-derived respiration accounts for a significant part of total CO₂ efflux from soil, or for ecosystems subjected to any kind of disturbance, which apportion the usual coupled course of biomass and temperature. In fact, residuals of the applied model were linearly correlated with changes in GPP. Adding the GPP of 2006 and 2007 to the model equation have slightly improved the model fitting to observed soil CO₂ efflux. Data obtained in the year 2008 were excluded from the residuals analyses as the GPP data are still under elaboration and will be introduced further.

We however do not point towards a simple connection between photosynthetic activity and root respiration. Other methods, like $^{13}\text{C}/^{14}\text{C}$ pulse labeling or continuous measurements of soil respiration fluxes are promising for deeper studying of particularities of aboveground-belowground relations in different ecosystem types and the physiological mechanisms involved in driving these relations.

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3. CONTRIBUTION OF PHOTOSYNTHETIC CARBON INPUTS TO PLANT RESPIRATION USING DESTRUCTIVE AND NON-DESTRUCTIVE TECHNIQUES

3.1. Introduction

Soil respiration (R_s) is the largest terrestrial source of CO_2 . Quantifying the processes that control the dynamic of R_s is thus critical to understand the global carbon (C) cycle. R_s in most related studies is determined primarily by soil temperature (T_s) (Lloyd and Taylor, 1994); soil moisture (SWC) often plays a role at the dry and wet ends of its distribution (Davidson et al., 1998; Palmroth et al., 2005; Tang and Baldocchi, 2005). In addition to well studied effects of T_s and SWC on R_s dynamic, some recent studies have investigated the role of photosynthetic C inputs in determining the magnitude and variability of R_s , suggesting that within-ecosystem, photosynthetic C transport and allocation could be critical for understanding the biosphere-atmosphere exchange of C and for the accurate prediction of terrestrial ecosystem respiration sources (Table 1). The allocation of recently assimilated C to below- vs. aboveground plant components and to storing vs. respiration are key uncertainties in global terrestrial ecosystem C models (Friedlingstein et al., 1999). How plants allocate C determines how long that C may remain in the plant or soil before returning to the atmosphere by respiration.

If models of R_s are to incorporate photosynthetic C inputs it is necessary to understand how these two fluxes are coupled. Studies employing stable isotope, radioactive isotope or automated ecosystem flux measurements are perspective for these purposes as they permit to determine the time lag between photosynthesis and C evolution from rooting-system, avoiding disturbance of the soil surface and natural growing conditions of plants. However, the reported time lags between photosynthetic C uptake and its evolution through the rooting systems vary in the order of hours to days. The possible reason is that up to now the most part of the mentioned experiments were performed in the laboratory conditions: in soil solution or in filled with soil narrow pots, which limits the root growth and do not reflect the real plant growing conditions. Studies *in situ* are still not numerous, covering few plant functional types and ecosystems. The methodology is not unique, destructive and non-destructive techniques with different shortcomings are involved and is not clear if the obtained results could be compared.

Additionally, not much is known about the sources of C that support plant metabolism, but there is clear evidence that both new and old stored C sources contribute (Dickson, 1991; Czimczik et al., 2006; Schuur & Trumbore, 2006; Carbone et al., 2007; Carbone and Trumbore, 2007). Three C pools fuelling plant respiration were recognized in grassland and shrubs: the fast pool, composed of the assimilates of the current day, intermediate pool, which integrates assimilates during the growing season and a storage pool, which is mobilized when necessary, such as during initial leaf growing stage in spring, with mean residence time (MRT) from month to years (Carbone & Trumbore, 2007). To be able to detect it, a longer chase period should be chosen after the labeling: ^{14}C label measured by accelerated mass spectrometer allows to detect a low-level

quantities and changes of label in CO₂ respired from weeks to month, otherwise a high concentrations of ¹³C or continuous isotope labeling application in ¹³C atmosphere should be applied, as it is a less sensitive tracer due its relatively high natural abundance.

Study	Lag	Method	Ecosystem
Horwath <i>et al.</i> , (1994)	2 to 3d	14C	<i>Populus eumERICANA</i>
Mikan <i>et al.</i> (2000)	<1 d	14C	<i>Populus tremuloides</i>
Warembourg&Estelrich (2000)	20 h	14C	<i>Bromus erectus</i>
Tang <i>et al.</i> (2005)	7-12h and 5-6d	Rs/GPP	Oak-grass savanna
Baldocchi <i>et al.</i> (2006)	multiple	EC / flux gradient	Oak-grass savanna
Ekblad & Hogberg (2001)	1 to 4 d	13C	Boreal mixed coniferous
Bowling <i>et al.</i> (2002)	5 to 10 d	13C	US coniferous forests
Barbour <i>et al.</i> (2005)	3 d	13C	<i>Nothofagus solandri</i>
Knohl <i>et al.</i> (2005)	4 to 5 d	13C	Mixed deciduous
Mortazavi <i>et al.</i> (2005)	3 to 4 d	13C	PP and HW
Andrews <i>et al.</i> (1999)	<7 d	13C	PP / FACE experiment
Kuzyakov <i>et al.</i> (2001)	9h	14C	<i>Lolium perenne</i>
Kuzyakov&Domanski (2002)	~10h	14C	<i>Lolium perenne</i>
Domaski <i>et al.</i> (2001)	~12h	14C	<i>Lolium perenne</i>
Kuzyakov <i>et al.</i> (1999)	<1d (10-24h)	14C	<i>Lolium perenne</i>
Kuzyakov&Cheng (2001)	6h and 36h	14C	spring wheat (<i>Triticum aestivum</i>)
Cheng <i>et al.</i> (1993)	<1 h	14C	winter wheat and rye
Gavrichkova&Kuzyakov (2008)	<6 h	14C	<i>Lupinus albus</i>
Gavrichkova&Kuzyakov (2008)	1,25 d	14C	<i>Zea mays</i> (V6 stage)
Gavrichkova&Kuzyakov (in press)	<6 h	14C	<i>Zea mays</i> (V8 stage)
Carbone <i>et al.</i> (2007)	4 d	14C	black spruce (<i>Picea mariana</i>)
Carbone and Trumbore (2007)	<4 h	13C	meadow plant community
Carbone and Trumbore (2007)	<4 h	13C	deciduous perennial shrubs
Xu <i>et al.</i> (2008)	<1 h	14C	rice seedlings
Moyano <i>et al.</i> (2007)	4 d	Mesh bag/GPP	Beech forest
Moyano <i>et al.</i> (2008)	5 d	Mesh bag/GPP	Spruce forest
Staddon <i>et al.</i> (2003)	<2 h	13C	Natural grassland: turf removed from the field and grown in pots
Ostle <i>et al.</i> (2003)	1 d	13C	Natural grassland: turf removed from the field and grown in pots

Table 1. A summary of studies that investigated the relationship between aboveground processes and soil respiration (Rs) or ecosystem respiration (Reco) in forest, savannah and grassland ecosystems or on a single plants in the laboratory conditions using destructive and non-destructive methods.

Our experiment was conducted in a mediterranean grassland (Amplero, Italy), aiming to cover the gap in the understanding of allocation patterns and speed of cycling of recently assimilated C in such type of ecosystems. Summarizing the above findings and uncertainties, the aims of the study were:

- To monitor the dynamic of the $^{13}\text{CO}_2$ evolution from the soil, with and without shoots, aiming to get the peak of respiration of recently assimilated ^{13}C of root- and shoot-origin *in situ* under the real plant growing conditions.
- To study temporal variation of source (photosynthetically active leaves)-sink (developing shoots, roots, leaves) interaction within the plant community: the fraction of new assimilated C respired below- vs. aboveground.
- To compare the observed time lags obtained by non destructive technique of isotope pulse labeling with the results of widely diffused destructive technique, where the value of root-derived respiration is obtained by root-exclusion technique and is plotted vs. GPP from eddy covariance (performing both experiments in the same time).

3.2. Materials and Methods

3.2.1. Site description

Amplero was established as a main CarboEurope site in central Italy near the city Collelongo (AQ) in the year 2002. A Mediterranean grassland site, located at 900 m a.s.l. Amplero is a nearly flat to gently south sloping (2-3%) doline bottom with an average annual temperature of 10°C and average annual precipitation of 1365 mm. The site is subjected to a long-term management since 1950, which consists in a once-a-year mowing during the peak of the growing season and the rest of the growing season the site is used as a pasture for cattle grazing.

The soil is classified as Haplic Phaeozem (FAO classification) and contains 13% of sand, 33% of silt and 56% of clay, $\text{pH}_{\text{H}_2\text{O}}$ of 6.6, total carbon (C) 3.48 % and total nitrogen (N) 0.28%. The plant cover is mainly represented by the following families: *Caryophyllaceae* (19%), *Faseolaceae* (30%) and *Poaceae* (34%).

3.2.2. *In situ pulse labeling procedure and gas sampling*

Pulse labeling was performed in the first days of June 2008.

1. Three labeling zones of 60cm² were established on the study site two months prior to the labeling procedure. Inside each zone two PVC collars (Ø20cm) were inserted to the soil. One of the collar was placed on the planted soil (total respiration, R_{tot}) and another one - on the bare soil with previously clipped plants (belowground respiration, R_b).
2. A Plexiglas labeling chamber (LC) was designed and constructed (Fig. 1 and 2) in the laboratory of forest ecology, University of Tuscia. LC was placed on soil to cover the chosen labeling zones. Air was circulating between LC and an infrared gas analyzer (CIRAS DC, PP-System, UK) using its internal pump at a constant rate. Some of the circulating gas stream was by-passing the IRGA by the use of adjacent pump, connected to the column with soda-lime for CO₂ scrubbing.
3. A labeling solution (Na₂¹³CO₃) was placed in 100 ml jar and sealed with a lid containing two valves and a septum port. Plastic tubes were connecting the jar to the IRGA-soda lime system, in the entrance of it to LC. A 5M lactic acid was introduced to the jar through the septum port with a syringe, after that the valve on the lid was opened letting the label to enter the LC. To each LC was introduced 350 mg of the Na₂¹³CO₃.
4. To stimulate the photosynthesis when the ¹³CO₂ was assimilated an additional unlabeled pure CO₂ was released (by acidifying Na₂CO₃) into LC, keeping the level of CO₂ in the chamber close to the ambient by regulating the rate of the flow of gas through soda-lime. The hole labeling procedure took 1 h.
5. After the opening of LC was started the trapping of recently assimilated CO₂ from the PVC collars: each collar was sealed with a lid for 1 hour (Fig. 3). Each chamber headspace was connected through a valve to a 2L flask, which previously had been evacuated to a high vacuum. A 2L vacuum flask were collected after 1 hour of CO₂ accumulation in the chamber space. After that the lid of the PVC chambers remained open, up to the next accumulation. A 2L flask from bare plot was collected before the pulse labeling to estimate the δ¹³C-CO₂ background signal. Gas sampling was performed ten times during the chase period which lasted ten days. The timing of the sampling was the following: 1h-2h-4h-10h-16h-24h-35h-48h-96h-240h.
6. Along with the pulse labeling, measurements of soil respiration from mesh bags which were installed previously to separate heterotrophic and autotrophic respiration were performed. Both time lags, obtained with ¹³C labeling and meshes experiment were confronted.

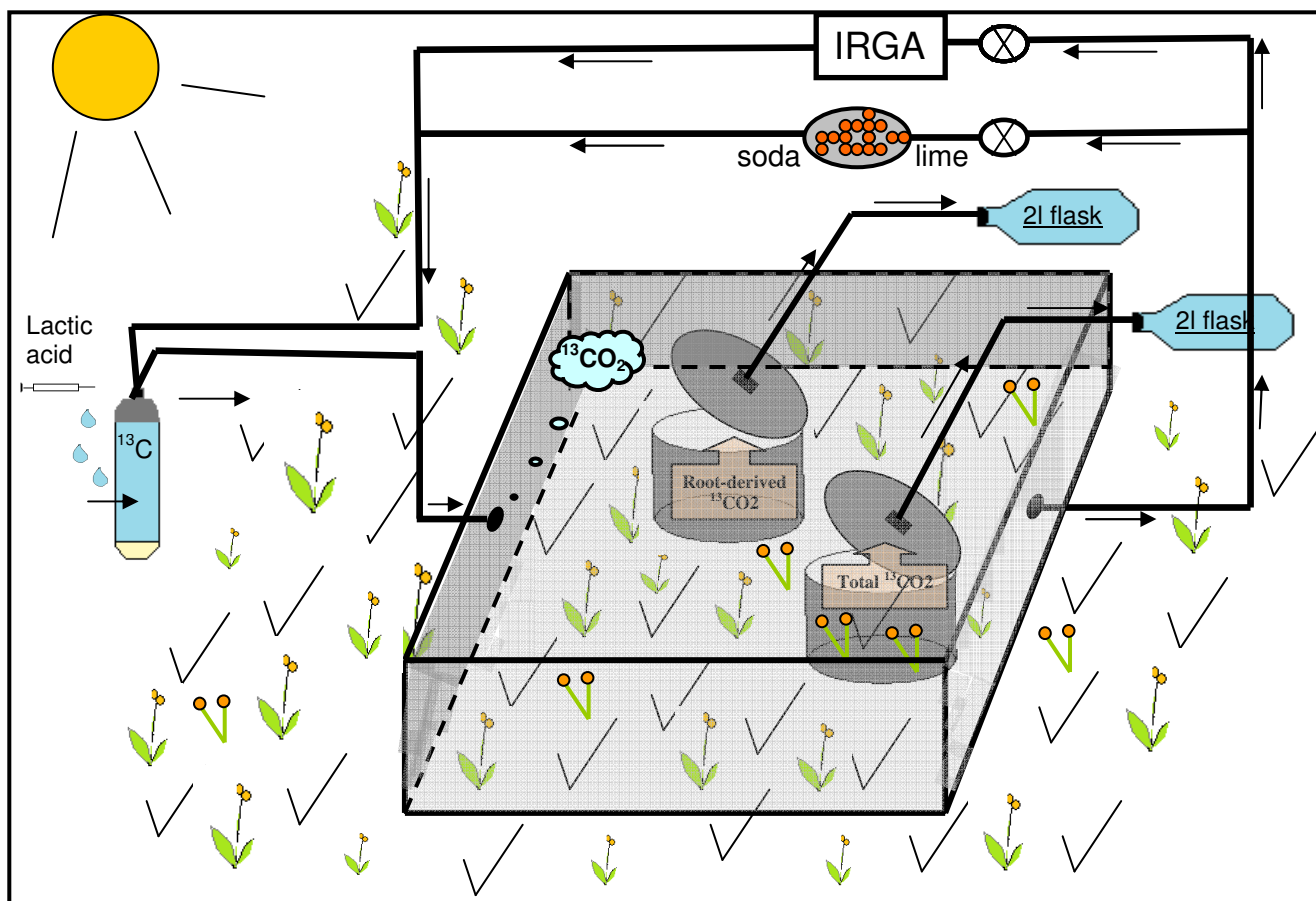


Fig. 1 In situ ^{13}C pulse labeling: chamber construction and experiment schematic view.

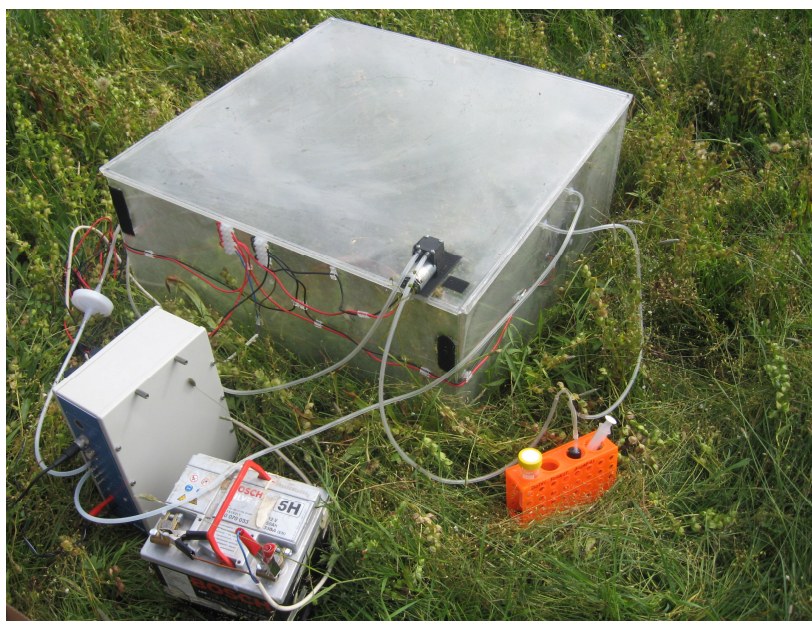


Fig. 2 Labeling chamber (LC) during the pulse labeling procedure, June 2008.

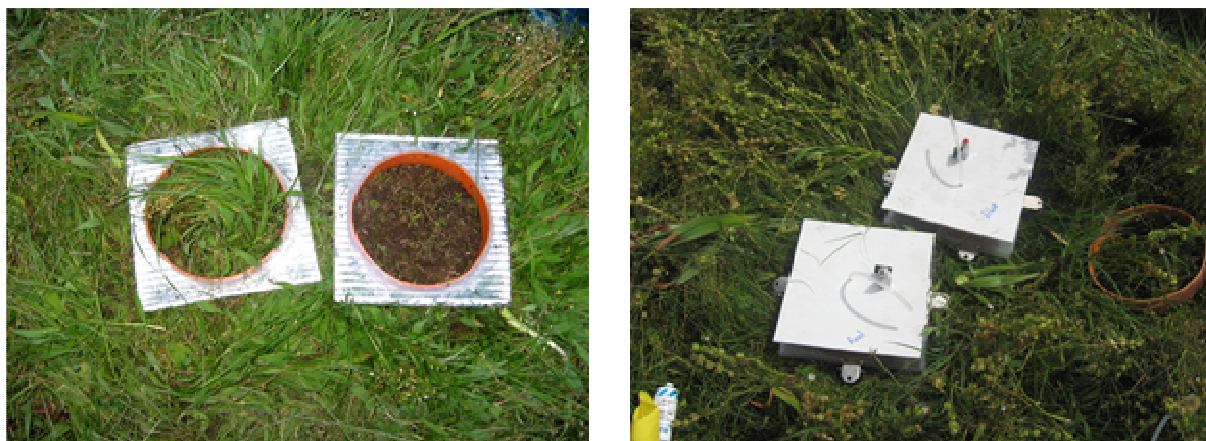


Fig. 3 Chambers for CO₂ accumulation with opened (left) and closed (right) lids.

3.2.3. *Sample preparation and analyses*

The CO₂ from the air samples was extracted using the cryogenic line (Fig. 4) less than one week after the collection to minimize the storage effect. Collected CO₂ then could be stored for a longer periods in the perfectly sealed test-tubes for the following analyses.

In brief, gas entering the cryogenic line passes firstly through a water trap, cooled with dry ice ethanol mixture and then through the CO₂ trap, cooled with liquid nitrogen. A pumping system (VARIAN, TURBO-V250, FR) at the end of the line forces the gas to pass through the tubes. Multiple loops are used to ensure that the gas molecules of CO₂ and H₂O are caught on their way, The pumping system is connected to the line with a needle valve. The flow rate is measured by a mass flow meter (Omega, UK) and regulated through the needle valve.



Fig. 4 Cryogenic line: 1-sample 2L flask with CO₂, 2-mass flow meter and controller; 3-H₂O trap; 4-CO₂ trap; 5-pumping system; 6-dry ice ethanol mixture; 7-liquid mixture; 8-pressure gauge; 9-test tubes

At the end of the process, the sublimated CO₂ is cryogenically collected in a test tube connected to the line. This test tube can be ‘safely’ sealed, melting it by a butane flame, and removed from the cryogenic line to be used for the analyses with the dual inlet isotope ratio mass spectrometry techniques (IRMS).

The isotopic measurements were performed at the Mass Spectrometry Laboratory of the Department of Environmental Science (Second University of Naples) on the mass spectrometer (MS) Thermofinnigan Delta^{plus}.

The IRMS technique used is a standard one. Briefly, the sealed test tubes were broken by tube-crackers and the gas was injected into the MS through a multiport section. The sample and reference gases then are stored in bellows, which allow also the regulation of the pressure of the gases. The pressure of the sample bellow was registered for each gas sample and then used to obtain the CO₂ concentration of the sample, by comparison with the pressure value of the reference gas of known CO₂ concentration (CO₂ 400 ppm, calibrated with respect to RM 8564 (IAEA), with ¹³CVPDB = -11.02 +/- 0.05 ‰). After entering to the ion source via the changeover valves (connected to the bellows through a capillary), the gases are ionized and accelerated by the 3 kV acceleration section of the MS. The ion beams are then focused by an electrostatic quadrupole and linear momentum analyzed by a 90° magnet operated with the adapted magnetics. Three Faraday cups positioned at right angles are used to measure the charge carried by the beams corresponding to masses 44, 45 and 46. The charge signals are then converted to voltage signals, amplified, digitized and acquired by a computer-based control and acquisition system.

The ratios ⁴⁵R=(⁴⁵CO₂/⁴⁴CO₂) and ⁴⁶R=(⁴⁶CO₂/⁴⁴CO₂) are measured for both sample and reference gases from the average on eight sample/reference cycles. Finally, the isotopic ratios for carbon are expressed in the usual δ (‰) notations:

$$\delta^{13}\text{C} = ({}^{13}\text{R}_{\text{sample}} / \text{R}_{\text{reference}} - 1) \times 1000$$

The δ ¹³C were measured with respect to international standards for carbon isotopic analyses: V-PDB (Vienna-Pee Dee Belemnite). As a working standard was used a gas with a known concentration of CO₂.

3.2.4. Data analyses and definition of terms

The ^{13}C signature of belowground respiration and total respiration were combined with the measurement of CO_2 concentration in the respective flask. These allowed estimating the fractional contribution of the belowground respiration to the total one, and then applying a two-source mixing model, to estimate the ^{13}C signature of the aboveground respiration:

$$\delta^{13}\text{C}_{tot} = ((f * \delta^{13}\text{C}_b) + ((1-f) * \delta^{13}\text{C}_a)) / I \quad (\text{eqn. 1})$$

where, $\delta^{13}\text{C}_{tot}$, $\delta^{13}\text{C}_b$ and $\delta^{13}\text{C}_a$ is the ^{13}C signature of total, below and aboveground respiration respectively, and f is a fractional contribution of each of the component to the total respiration.

As the continuous measurement of the soil and total CO_2 efflux were not available, we assumed that the respiration rates and ^{13}C contributions from all the sources varied linearly between the measurement events. These allowed estimating the total amount of ^{13}C respired during the period from 1 hours to 10 days following the application of the label (Carbone *et al.*, 2007).

The total label recovered (TLR) was defined as the calculated sum of the label (^{13}C , g) from below- and aboveground components between 1h and 240h after pulse labeling. This quantity was defined as a 100% for each labeling. The below and aboveground components were then partitioned into percentage of TLR.

The fraction of respiration from label (FRL) for below and aboveground components was defined using the same equations as described by Carbone *et al.* (2007) and Carbone and Trumbore (2007). The isotope mass balance approach was used to partition the fraction of respiration coming from the label:

$$\text{FRL} = (\delta^{13}\text{C}_s - \delta^{13}\text{C}_B) / (\delta^{13}\text{C}_L - \delta^{13}\text{C}_B) \quad (\text{eqn. 2})$$

where $\delta^{13}\text{C}_s$ is the measured sample respiration; $\delta^{13}\text{C}_B$ - is the background respiration signature, $\delta^{13}\text{C}_L$ is the label signature. $\delta^{13}\text{C}_L$ was estimated as the measured mean $\delta^{13}\text{C}$ of CO_2 during the 1h labeling period.

The mean residence time (MRT) of the label in the below and above ground components was calculated fitting an exponential decay function to the FRL. All the data were separated in two periods for the better fit of the exponential curve. MRT represent the time which is required for the amount of label to be reduced to 1/e times of its initial value. Using the data of TLR and MRT was calculated the mean age (MA) of respired C (Carbone and Trumbore, 2007):

$$\text{MA}_s = (\text{TLRs}(0-24h) * \text{MRTs}(0-24h) + \text{TLRs}(24-240h) * \text{MRTs}(24-240h)) / \text{TLRs}(0-240h) \quad (\text{eqn.3})$$

where, MA_s – the mean age of the component; $TLRs(0-24h)$, $TLRs(24-240h)$, $TLRs(0-240h)$ is the percentage of total label recovered from the component for the first 0-24 hours, for 24-240 hours and total (0-240h) respectively. $MRTs(0-24h)$ and $MRTs(24-240h)$ is a corresponding mean residence time.

3.2.5. Time lag by mesh bag technique

Partitioning experiment aiming to separate the contribution of root-derived and microbial-derived respiration from the total CO_2 efflux was established in Amplero in 2006. New partitioning plots were added in 2007. Biweekly measurements were performed during the growing seasons 2006-2008. Soil respiration was partitioned by excluding roots from soil cores using nylon mesh bags of 1 μ m pore size (Leake et al., 2004; Moyano et al., 2007; Moyano et al., 2008). An advantage of the micro pore nylon bag is that it permits to maintain an exchange of water and other substances between the enclosed plot and exterior soil, keeping the conditions inside the mesh equal to the surrounding one. Briefly, the procedure was the following: soil cores, 20 cm in diameter and 30 cm deep (the depth of the soil core depends on the rooting depth in the study site) were sampled from the study site. Half of the sampled soil was placed to the nylon meshes with 1 μ m pore size and was returned to the site. The CO_2 measured from these meshes was considered as microbial respiration (R_h). Another half of the sampled and sieved soil was placed back without any barriers for the roots growing (bags with 1.0 cm pore size). The CO_2 efflux, coming from these bags is a sum of microbial and root-derived respiration (R_h+R_a). Root-derived respiration was calculated as a difference between the above treatments: $((R_h+R_a)-R_h)$. Additionally, total soil respiration (R_s) was also measured from the undisturbed soil and was used as a control and to determine the true value of microbial respiration by subtracting from the value of R_a from R_s .

Ecosystem net carbon and water vapour fluxes have been measured at Amplero continuously since 2002. The eddy covariance system integrates a sonic anemometer (Solent R3, Gill Instruments, Lymington, UK) and a LI 7500 open path infrared gas analyzer (LiCor, Lincoln, NE, USA). The flux data were calculated for 30 min intervals by means of the post-processing program Eddyflux.

GPP was calculated from the difference between NEE and TER (Reichstein et al., 2005), where functional dependencies of total ecosystem respiration (TER) with temperatures were determined with CO_2 fluxes measured at night (fluxes measured during low mixed periods were discarded, Aubinet et al., 2000). These functions were then applied to daytime data to derive GPP.

To calculate the time lag between the photosynthetic CO_2 uptake and its following respiration, the data from the partitioning experiment were plotted vs. GPP from the eddy

covariance. Pearson correlations were performed between the mean of respiration values of different origin from each date and the mean values of GPP 0–7 days prior to the respiration measurement date. Linear regression analyses were performed for the periods where Pearson correlations showed peak values.

Root-derived respiration was normalized to soil temperature of 15°C using the mean annual Q_{10} :

$$R_s = a \cdot \exp(b \cdot T_s),$$

$$Q_{10} = \exp(10 \cdot b)$$

$$Ra_{15} = Ra \cdot Q_{10}^{(15-T_s)/10} \quad (\text{eqn.4})$$

where, Ra_{15} is normalized respiration rate, Ra is measured respiration rate, T_s is soil temperature at 5 cm depth and a and b are coefficients.

3.3. Results

3.3.1. Raw isotopic values

The raw isotopic values of total, above-, and belowground respiration are shown in figure 5. For total and aboveground respiration were observed three peaks in $^{13}\text{CO}_2$ efflux. The first peak occurred before the first sampling was performed, the second one was registered between the 2-4 hour after the pulse labeling and the next one falls on the end of the first day after the label injection: from 16 to 24h. The $^{13}\text{CO}_2$ signature of belowground respiration decreases sharply from time 0 after the pulse labeling, but on the end of the first day a small peak could be distinguished in all three replicates.

3.3.2. Label partitioning

Plants invested more $^{13}\text{CO}_2$ to the aboveground respiration during the first hours after the label injection, however with time, starting from the 10th hour, significantly more $^{13}\text{CO}_2$ was recovered from the belowground compartment (Fig.6). The chase period was separated into two time periods: 0-24h and 24-240h after the label injection. The majority of the TLR was respired in the first day after labeling (Fig. 7, Table 2). During the first 24 hours 13.9% and 16.3 % of TLR were respired from above and belowground respectively. Between the first and the tenth day the rest of the TLR was respired in proportion 20.6% from aboveground and 49.2% from belowground compartments.

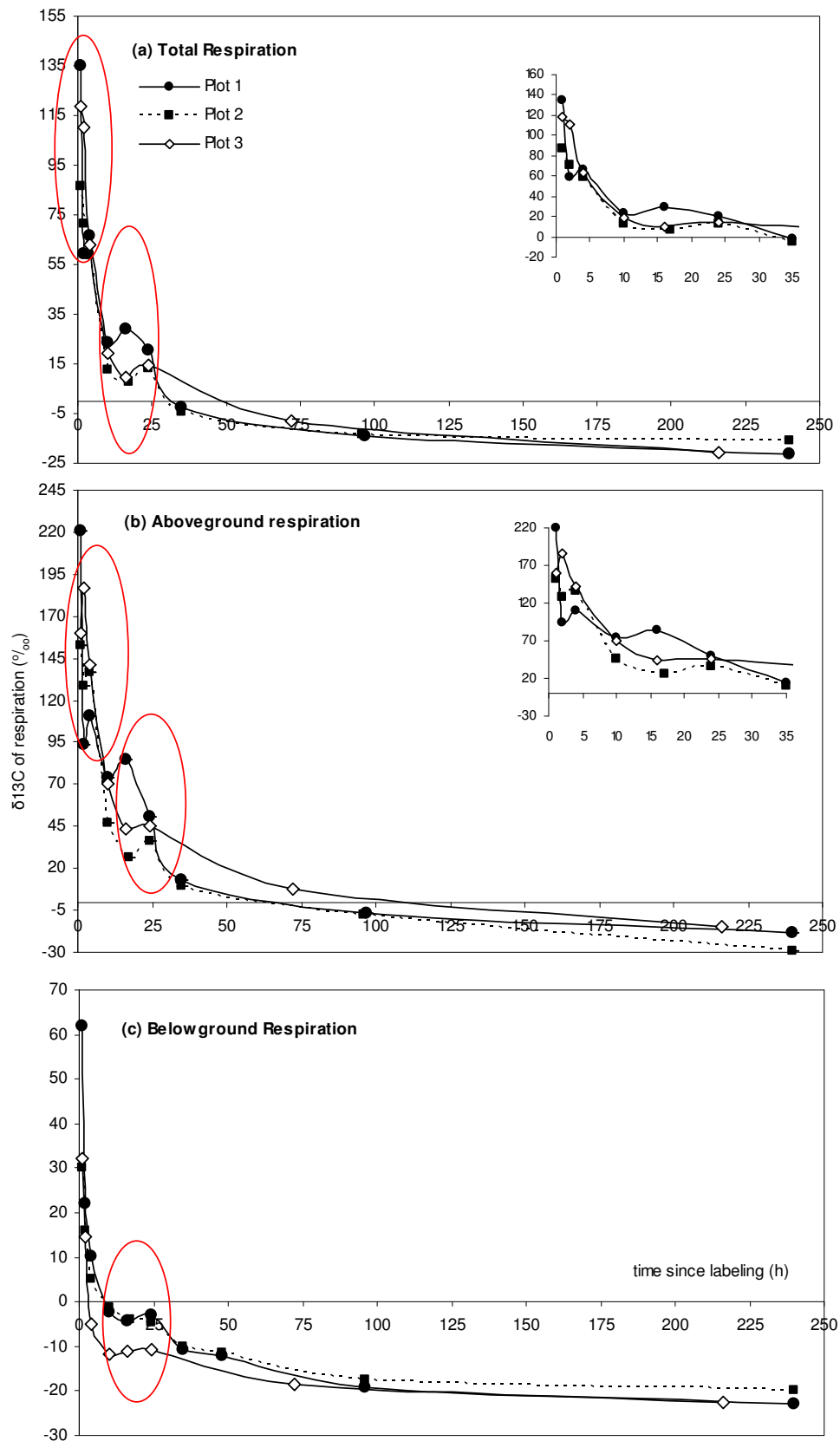


Fig.5 Raw isotopic values for total (a), above- (b) and belowground (c) respiration. For primary and secondary plots: all x-axes are time elapsed since pulse labeling (h), and y-axes are $\delta^{13}C$ values (‰). Peaks in evolution of $^{13}CO_2$ from all the sources are indicated in red.

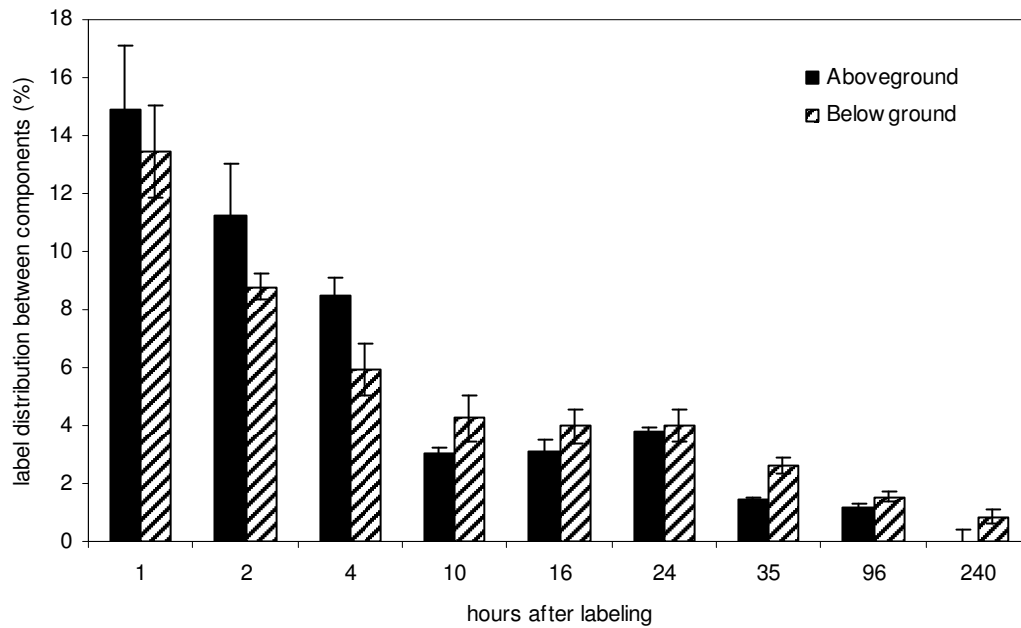


Fig.6 Allocation of the recovered label between aboveground and belowground respiration during the gas sampling periods, taking the sum of the recovered label as a 100%.

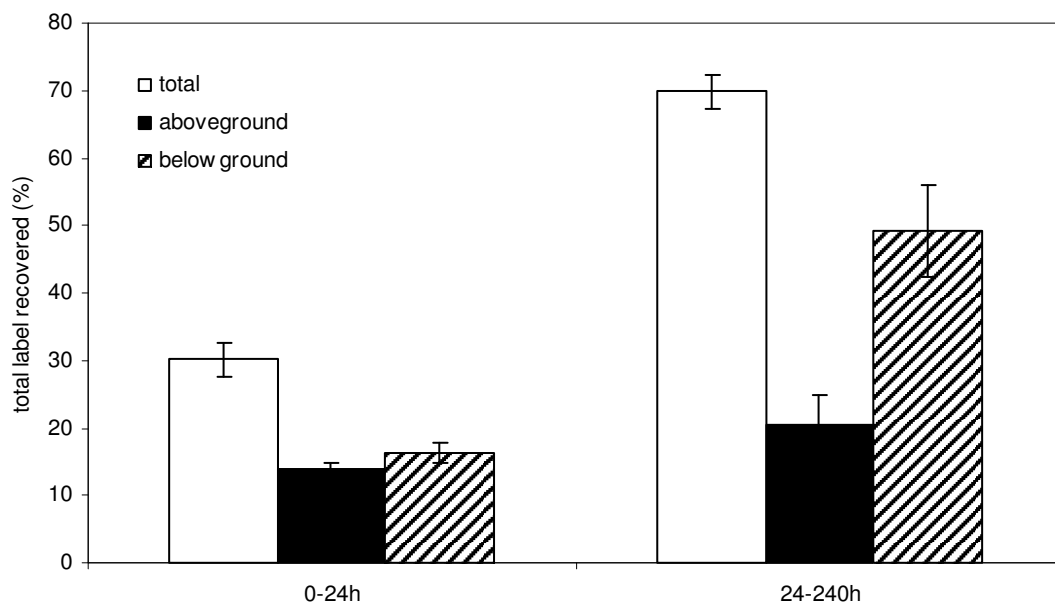


Fig.7 Allocation of total label recovered between above and belowground respiration, the chase period was sub divided into two time periods: 0-24h and 24-240h.

3.3.3. Mean Residence Time

The MRT for the label to be respired showed through below and aboveground plant parts are presented in Table 3. For the first 24 hours after respiration the labeled C cycled rapidly, with the MRT of less than one day for both components. For this period the MRT was generally shorter for belowground (0.38 d) than for aboveground (0.74 d). Much slower cycling of recently assimilated C was observed for the time period of 24-240h after the pulse labeling with an average values of 6.3 for both above and belowground respiration.

Total Label Recovered (TLR, %)			
	Ra	Rb	Total
0-24h	13.9±1	16.3±1.6	30.2±2.5
24-240h	20.6±4.3	49.2±6.8	69.7±2.5
Total	34.5	65	100

Table 2. Allocation of total label recovered between above and belowground respiration for two time periods: 0-24 and 24-240 after pulse labeling in three replicates.

Mean residence time, (days)				
	Plot 1	Plot 2	Plot 3	Average
Ra				
0-24h	1.2	0.5	0.6	0.7±0.21
24-240h	4.2	9.9	4.8	6.3±1.81
0-240h	3.4	5.1	3.8	4.1±0.54
Rb				
0-24h	0.3	0.5	0.3	0.4±0.1
24-240h	4.8	7.9	6.2	6.3±0.9
0-240h	3.6	5.3	4.6	4.5±0.5

Table 3. Mean Residence Time (MRT) for the label in below and aboveground respiration for two time periods: 0-24 and 24-240 after pulse labeling.

3.3.4. Mean Age of new C

In table 4 is reported the mean age of C in below and aboveground compartments. C was cycling slowly in belowground component. An average MA for ^{13}C in aboveground was 3.8 and for ^{13}C in belowground 4.9 days.

Mean age (MA, days)				
	Plot 1	Plot 2	Plot 3	Average
Ra	3.1	5.2	3.2	3.84±0.7
Rb	3.4	6.6	4.6	4.85±0.9

Table 4. Mean Age (MA) for the label in below and aboveground respiration.

3.3.5. Time lag by mesh bag technique

Significant correlations between GPP and root-derived respiration were observed. Peaks in the correlation strength responded to the time shifts between photosynthetic activity and respiration CO_2 evolution through the source (Fig.8).

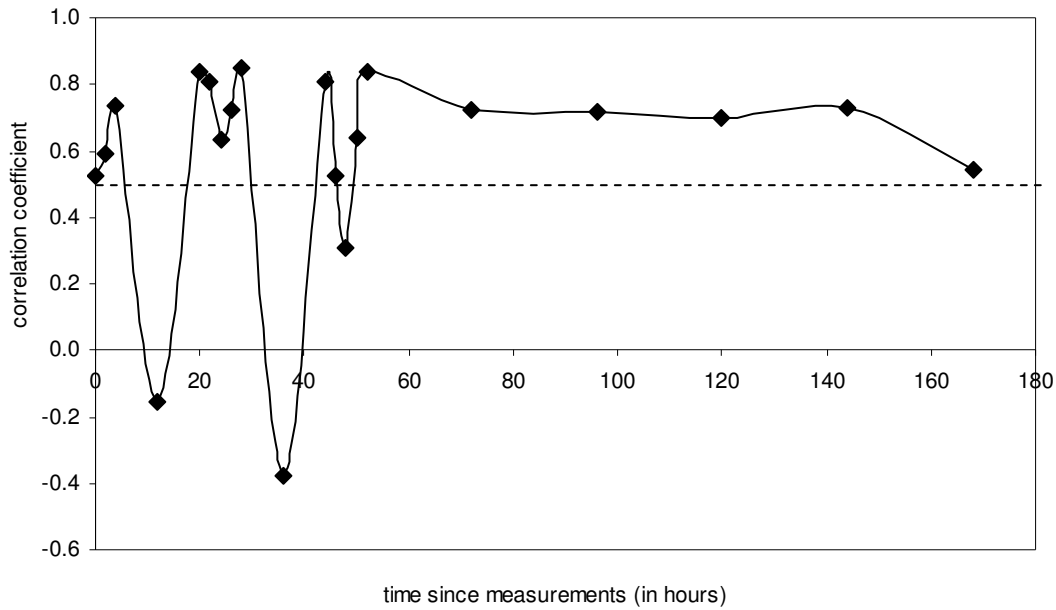


Fig.8 Pearson correlation coefficients of temperature normalized root-derived respiration against gross primary productivity (GPP) from 0 to 7 days prior to respiration measurements.

GPP in h	Ra vs.GPP			
	Beta	B	t	p-level
0	-2.48	-0.22	-1.72	0.16
2	4.24	0.39	2.28	0.08
4	-0.53	-0.05	-0.75	0.50
20	1.60	0.24	2.97	0.01
22	-3.33	-0.30	-2.44	0.07
28	0.36	0.04	0.65	0.55
24	1.17	0.09	1.17	0.31

Table 5. Multiple linear regression analysis. Coefficients of regression determination: Ra vs. GPP in hours after the measurements. Significant is marked in red.

Correlation with GPP for root-derived respiration was stronger for non-normalized respiration data, confirming a tight relation between the photosynthetic C supply and autotrophic component of soil respiration. Correlation coefficient was especially high on the second and third day after measurement of Ra, giving the peaks on the 20th, 28th, 44th and 52^d hours after the measurements were performed, decreasing slowly with time.

Example of diurnal variability of GPP and Ts is shown on the figure 9. Clear, that day peaks of GPP and Ts appear in different time. For the GPP often could be distinguished two peaks with the midday depression in between.

Multiple linear regression analyses indicated that the best predictor of changes in root-derived respiration with significant coefficient of regression determination resulted was GPP 20h after the measurements of soil respiration (Table 5).

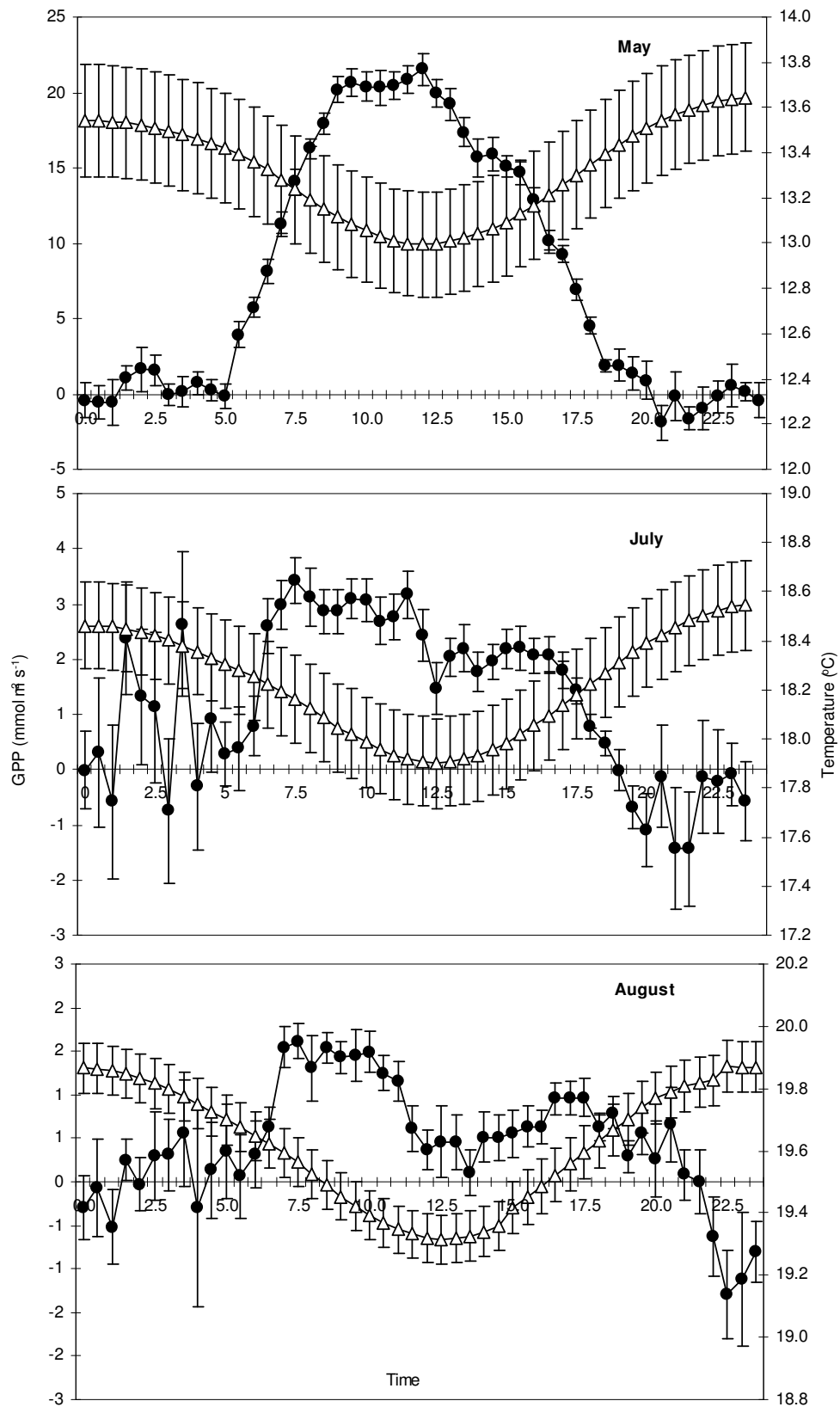


Fig.9 Diurnal patterns of Gross Primary Production (GPP) and Soil Temperature (Ts) for May, July and August at Amplero. Values represent daily month averages.

3.4. Discussion

3.4.1. Speed of C cycling

New C cycled quickly within the ecosystem, with a great proportion of recently assimilated C respired within the first day (Fig.7). The first peak in evolution of $^{13}\text{CO}_2$ occurred less than one hour after the opening of the LC, both in above and belowground respiration, followed by the other peaks in the course of the day (Fig.5). These initially high values in the ^{13}C signature of respired CO_2 is possibly attributed to the residuals of the label which may have diffused to the soil pores during the pulse labeling procedure and consequently is not of a plant-origin. In fact some studies, confirm this phenomenon for the *in situ* pulse labeling experiments (Ostle et al., 2003, Leake et al., 2006). Ostle et al. (2003) by increasing the number of the replicates (from 3 to 6) and the sampling frequency improved the results reported by Staddon et al. (2003). The greater replication gave more consistent results with peak ^{13}C enrichment of soil respiration occurring 1 day after the labeling, rather than 2 hours, reported by Staddon et al. (2003). In fact, laboratory conditions permit to isolate the soil compartment during the labeling procedure, avoiding the uncontrollable penetration of the label into the soil, while *in situ* its quite impossible. The ^{13}C signature of the aboveground respiration experienced a small decrease during the first hour after the pulse labeling, but the first peak originating from plant respiration occurred between 2 and 4 hour of the chase period (Fig.3b). This time lag is similar to the one reported by Carbone and Trumbore (2007) for the grasslands and shrublands, where the ^{13}C in aboveground respiration peaked in the first 4 hours after the pulse labeling.

The ^{13}C fixed by shoots may be respired, used in production of new shoot growth, temporarily stored (e.g. in starch), or allocated belowground into roots and from them to soil organisms and belowground respiration (Leake et al., 2006). In belowground respiration there was observed again initially high value of the ^{13}C content. However, we argue that the translocation of C from leaves to roots and into soil respiration occurred later, in the time the next peak in belowground $^{13}\text{CO}_2$ respiration was registered (Fig. 3c). This peak occurred from 16 to 24 hours after the pulse labeling. The same peak was observed also in aboveground respiration, which indicates that we were not able to separate completely above and belowground respiration by subtracting from total respiration the CO_2 evolved from the bare plots. Another possible explanation could be the re-fixation in shoots of the ^{13}C respired from the soil or back flow of the pulse C from belowground, as was observed by Johnson et al. (2002), e.g. from ^{13}C sugar that passed to the roots being combined with ammonium, taken previously up from the soil, to form ^{13}C -enriched amino acids that are then transferred back to the shoots.

Carbone and Trumbore (2007) for a grassland site reported the time lag of less than 4 hours for the belowground grassland respiration, but the number of sampling times in their study was less

frequent and possibly they missed the real peak, which occurs later, as it was shown above. Our results confirm the results of Leake et al. (2006) and Johnson et al. (2002) for grassland, which stated that the rate of loss of C or export of recently fixed C from shoots to roots happens within the first 24 hours after the pulse labeling. As it was mentioned above, Ostle et al. (2003) also have found the peak of ^{13}C enrichment of soil respiration to occur 1 day after the labeling. Other studies performed on trees and grasses stated the time lag of 1-5 days (Warembourg and Estelrich, 2000; Horwarth et al., 1994; Eklab and Hogberg, 2001, Bowling et al., 2002; Carbone et al., 2007; Moyano et al., 2007, 2008). Experiments conducted in laboratory generally report the time lags from minutes to one day. For example, Kuzyakov and Cheng (2001) found the first CO_2 evolution from soil with *Lolium perenne* within the first 4 h after labeling while Cheng et al. (1993) and Xu et al. (2008) found the beginning of emission of labeled CO_2 from winter wheat and rye to occur within the first hour. Gavrichkova and Kuzyakov (2008) showed on lupine and maize, that for different plant species the time lag could differ significantly. The time lag between photosynthetic CO_2 uptake and the ensuing release of C through root respiration for lupine happened within the first 6 hours after pulse labeling and for maize only on the second day, despite the fact that the plants were growing at the same environmental conditions.

The time needed for C translocation from shoots to roots and its following losses through respiration depends on the path length, so then on the size of a plant and on the speed of phloem transport of photoassimilates from the source to the sink, which is driven by changes in hydrostatic pressure (Munch, 1930; Nobel, 2005). The stronger is the sink demand for C, the larger and faster should be the supply of C from the source (Dickson, 1991). A C source is any part of the plant that is producing or releasing sugars. During the plant's growth period, usually during the early spring, storage organs such as the roots could be act as sugar sources, and the plant's many growing areas are sugar sinks that is due to the bidirectional movement in phloem. After the growth period, when the meristems are dormant, the mature leaves are sources, and storage organs are sinks. Developing seed-bearing organs are always acting as sinks.

In June, when the pulse labeling was performed, two main C sinks could be distinguished which compete for the new assimilated C: a production of flowers and seeds and growth of root biomass. The delay in belowground respiration in confront if aboveground one could be attributed to the time preference for the allocation of new C to the organs of reproduction, and only then to the storage pool of roots. Tang et al. (2005) combining continuous data of soil respiration with GPP data have demonstrated that the time lag between C inputs and outputs in oak-grass savannah varies during the growing season, changing from 7h up to 12h in different months, confirming that the relative importance and strength of roots as a C sink is changing during the growing season.

3.4.2. Allocation patterns

Kuzyakov and Domanski (2000) reviewed grassland C budgets based on pulse labeling studies and found that typically for cereals about 30% of total assimilates is transferred into the soil. Of this quantity about half is found in root biomass, the rest of the belowground allocation being respired through root respiration, excreted by root exudation or found in the soil microorganisms or soil organic matter. The relative C translocation of pasture plants into soil was 1.5-2 times higher than that of cereals. Leake et al. (2006) for sub mountain grasslands with lower productivity and soil temperatures and slower turnover rates reported a lower proportion of C allocation to root biomass (12-22% of the shoot pulse ^{13}C). Two distinct pools of C could be recognized from our data: a fast-turning over pool that becomes enriched in a pulse following labeling and a slower turning over pool that remains enriched, but at much lower levels for long after the pulse labeling have been performed. An equal quantity of recently fixed C was allocated to below and above ground respiration during the first day, which represent a pool with a high turnover rates. However, the origin of this C is uncertain with a great probability that it is coming just from the aboveground plant parts (see above). The C with a lower turnover rates on the contrary was allocated preferably to root respiration. Consequently, the aboveground growth and maintenance respiration is fuelled mainly by the assimilates of the current day, while in root respiration the C with higher MRTs values is involved.

The root/shoot ratio reported by Gadghiev et al. (2002) for various grassland ecosystems of Central Asia varies from 7 to 17, stating that grasslands invest heavily in the production of belowground biomass. The root/shoot ration of Amplero (data not shown), estimated in 2007 varied in the course of the year from 10 to 18, confirming that the most part of the net primary production is concentrated belowground. Consequently, the majority of the new coming C is expected to be allocated to belowground also, and would be used then to support the growth of the new roots and the maintenance of existing one. The beginning of June in Amplero, when the pulse labeling was performed is characterized by an intensive accumulation of root biomass (data of 2007, are shown in chapter 2 and 6). Nevertheless, Carbone and Trumbore (2007) showed that the allocation patterns vary slightly during the growing season, with an increased proportion of recently assimilated C allocated to aboveground during the late growing season, which is associated with flowering period, when aboveground biomass reaches its peak. Further experiments with a pulse labeling isotope technique application are needed for the detailed study of the changes in seasonal C allocation patterns.

3.4.3. *Destructive vs. Non-destructive technique*

The time lag between the photosynthetic C uptake and its following evolution through the root respiration was measured applying two different techniques: 1) pulse labeling of plants in $^{13}\text{CO}_2$ atmosphere, which we assume as a non-destructive technique with minimal disturbance of the natural plant growing conditions and 2) by integration of the data of root-derived respiration obtained from partitioning experiment with GPP data from eddy covariance. The last is based on a widely used root exclusion technique and induces a significant disturbance to the soil; we will consider it as a destructive one.

The delay between the photosynthesis and root respiration measured by the destructive technique was 20 hours, the time when the first peak in correlation was estimated, it confirms the results of pulse labeling experiment. But the peak response of root-derived respiration to photosynthesis was observed also on the 28th, 44th and 52^d hour (Fig. 8). A notable decrease in the correlation strength between mentioned hours could be attributed to the plant midday depression, when the stomata conductance is low and photosynthetic activity is decreased due to high radiation, elevated temperatures, accompanied by low soil water content during the most dry months (Farquhar and Sharkey, 1982; Xu, 1997; Du and Yang 1988, 1990; Fu et al., 2006) (Fig.9). Afterwards, the photosynthesis regenerates its initial activity; this is seen in the augment of the correlation strength for the hour 28th and 52^d.

High correlation between the GPP and root-derived respiration was observed up to 6 days after the soil respiration measurements were performed. The longer time lags could be attributed to the similar patterns in GPP for the days, closely located to the day of measurements. In fact, Tang et al. (2005) and Moyano et al. (2008) operating with soil respiration and GPP data have also reported multiple time lags. Moyano et al. (2008) have shown that the increased correlation with GPP on the biweekly bases was well explained by the autocorrelations in weather patterns, with weather fronts passing through the region every two weeks.

Summarizing, besides a significant disturbance of soil by root exclusion technique both methods showed comparable results with the time lag around 20 hours between the photosynthetic C uptake and its following respiration. The fact that such type of partitioning methods are widely used in environmental studies and often are coupled with eddy covariance measurements, makes it promising for the estimation of the speed of the C cycling within and between various ecosystems.

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4. THE EFFECT OF DEFOLIATION MANAGEMENT PRACTISES ON SOIL RESPIRATION OF DIFFERENT ORIGIN AND SOIL BIOCHEMICAL PROPERTIES

Chapter source:

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4.1. Introduction

Land-use changes are considered the most dominant component of global change in terms of impact on terrestrial ecosystems, profoundly altering land cover, vegetation composition and biochemical cycles (Walker et al., 1999). Besides land-use change, land management may have a significant effect on the CO₂ efflux from soil especially in the short-term (Bahn et al., 2006). Land-use change and management practices can affect soil carbon storage by altering the input rates of organic matter and by changing its decomposability (Cambardella and Elliot, 1992; Moscatelli et al., 2007). In grassland ecosystems a very little quantity of organic carbon is stored in the above ground biomass and more than 90% is concentrated in roots and soils (Burke et al., 1997; Parton et al., 1993). Because of the soil significant capacity to store carbon, grasslands associated with a proper management are considered to be potential carbon sequestrators (Post et al., 1982; Degryze et al., 2004; Sun et al., 2004).

Management practices, based on defoliation such as mowing and grazing account for about 20% of the global terrestrial ice-free surface. Historically also Mediterranean grasslands were used for cattle grazing and hay production. Ecosystems under grazing and mowing regimes are usually characterized by increased soil organic carbon content: a large amount of data show that such kind of management, based on defoliation, may substantially influence the below-ground food-web, and thus SOM (soil organic matter) transformation and nutrient cycling (Mikola et al., 2001; Bardgett et al., 1998). Maintenance of SOM and the quality of soil are the key factors in the sustainability of such ecosystems (Conant et al., 2001) and productivity of plant communities (Bending et al., 2000).

However, there is still a dearth of clear information on the effect of defoliation of grassland plants on grassland C cycling, soil biochemical properties and the role of soil organisms in aboveground-belowground feedbacks in grazed and mowed grasslands as different studies show contrasting results (Guitian and Bardgett 2000). Some ecological studies stated that experimental clipping usually reduces CO₂ efflux from soil by 21-49% despite the fact that it increases the soil temperature (Bremer et al., 1998; Wan and Luo, 2003). This decrease was mainly explained by the sensitivity of roots and microbes to the reduction in photosynthetic C supply from aboveground and to the decrease in rhizodeposition process and thus in the amount of easily available C substrates (Craine et al., 1999; Bahn et al., 2006; Zhou et al., 2007). Mawdsley and Bardgett (1997) however conclude that defoliation of *Trifolium repens* increases the rhizosphere microbial biomass and activity, whereas defoliation of *Lolium perenne* had a little effect on soil organisms. Uhlirova et al. (2005) and Zhou (2007) report mowing as a most suitable grassland management as it increased soil microbial biomass and consequently labile carbon pool and enhanced SOM transformation. Soils, under free grazing, on the contrary, tended to loose soil organic matter and reduce labile C availability (Lal, 2002; Zhou, 2007; Stark and Kytoviita, 2006). Bardgett and Leemans (1995) and

Bardgett et al. (1997) showed that sheep grazing positively influenced microbial communities and supported the hypothesis that heavy grazing favours “fast cycles” dominated by labile substrates.

For a complete understanding of the effect of defoliation practices on grassland community ecological and biochemical studies should be combined. Monitoring and partitioning of soil CO₂ efflux *in situ* will allow to estimate the effect of defoliation on each single component of soil CO₂ efflux, including root and microbial respiration in short- and long-term at the field and common soil physical conditions. Soil biochemical analyses permit to explain the observed changes in soil respiration and to forecast the future changes in SOM, which in regard to CO₂-driven greenhouse effect is the only C pool contributing to changes in atmospheric CO₂ concentration (Kuzyakov, 2006).

Microbial biomass (Powlson et al., 1987; Rice et al., 1996) and related metabolic activity (Tracy and Frank, 1998; Bending et al., 2000) can be used as an early predictor of changes in total SOM due to management regime as it is largely responsible for the transformation of SOM and soil nutrient cycling (Rice et al., 1996). In addition any environmental impact that will affect members of a microbial community should be detectable at the community level by a change of a particular total microbial community activity, which can be quantified and visualised in special indexes (Anderson, 2003; Moscatelli et al., 2005). Extracellular enzymes are considered integrative indicators of soil quality; they are relatively simple to determine, have microbial ecological significance, are sensitive to environmental stress and respond rapidly to changes in land management (Dick et al., 1997; Yakovchenko et al., 1996; Turner et al., 2002)

This study reports a two-year investigation performed in a Mediterranean grassland located in central Italy. The aim of the study was to compare the effect of management based on defoliation (grazing and mowing) (MG) to unmanaged plots (UM) on soil respiration of root and microbial origin in short- and long-term and on several biochemical parameters linked to the soil microorganisms pool, its carbon mineralization activity and enzymatic activities.

4.2. Materials and Methods

4.2.1. Research area and experimental design

Site description: The study was conducted in Amplero (AQ) - a Mediterranean grassland site, located in central Italy at 900 m a.s.l. Amplero is a nearly flat to gently south sloping (2-3%) doline bottom with an average annual temperature of 10°C and average annual precipitation of 1365 mm. The site is subjected to a long-term management, which consists in a once-a-year mowing during the peak of the growing season and the rest of the growing season the site is used as a pasture for cattle grazing.

The soil is classified as Haplic Phaeozem (FAO classification) and contains 13% of sand, 33% of silt and 56% of clay, pH_{H_2O} of 6.6, total carbon (C) 3.48 % and total nitrogen (N) 0.28%. The plant cover is mainly represented by the following families: *Caryophyllaceae* (19%), *Faseolaceae* (30%) and *Poaceae* (34%).

Four fenced areas (2.5x2.5 m), which prevent the enclosed plots from cutting and grazing, were established in the territory in the year 2002.



Fig. 1 One of four fences installed in the territory of Amplero (May, 2007)

4.2.2. Soil respiration and partitioning

Soil respiration was measured every two weeks or monthly in managed (MG) and unmanaged (UM) plots during years 2006 and 2007. Measurements were made manually with an infrared gas analyzers (IRGA) operated in the closed path mode. Two closed dynamic systems were involved in the measurements: LI-COR 6400, connected to soil chamber LI-6400 09 (LI-COR Inc., Lincoln, NE, USA) and EGM-4, connected to soil chamber SRC-1 (PP systems, UK). To be able to interpretate the data, a comparison between the two measurement systems was done by operating both systems in the same time.

To avoid root severing PVC soil collars of 11 cm in diameter were inserted only 2.5 cm into the soil and stabilized with two iron legs to prevent its moving when the chamber was placed on it. Soil temperature was measured near each collar at 5 cm depth using either LI-COR 6400 temperature probe either EGM temperature probe. Soil water content ($m^3 m^{-3}$) at depth of 5 cm was measured with a portable system (ThetaProbe ML2x, Delta-T devices Ltd, Cambridge, UK) at three points around each collar of control plots and once inside each collar of partitioning plots.

Partitioning plots were installed both, in MG and UM plots. Inside each fence were placed 2 complete partitioning plots: one in 2006 and one in 2007, the same was done for managed soil outside the fences.

Modified after Leake et al. (2004) and Moyano et al. (2007, 2008) root-exclusion technique was used to partition the CO₂ efflux from soil. A complete partitioning plot is shown in Fig.2

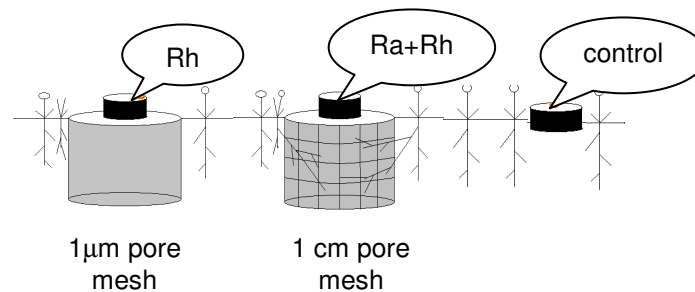


Fig.2 Schematic description of the modified root-exclusion technique. One complete partitioning plot is represented

- Soil cores, 20 cm in diameter and 30 cm deep were sampled; the soil was sieved through 4 mm mesh and all the roots were carefully removed from the soil.
- Half of the sampled soil was placed to the nylon meshes with 1µm pore size and returned to the study site. The CO₂ measured from these meshes was considered as respiration associated with microbial decomposition of SOM – microbial-derived respiration (Rh) after disturbance.
- Another half of the sampled and sieved soil was placed back without any barriers for the roots growing (bags with 1.0cm pore size). The CO₂ efflux, coming from these bags is a sum of microbial and root-derived respiration (Rh+Ra).
- Root-derived respiration was calculated as a difference between these two treatments: ((Rh+Ra)-Rh).
- Total soil respiration (Rs) measured from the undisturbed soil was used as a control and in combination with root-derived respiration was used to calculate the real microbial-derived respiration, excluding the effect of soil sieving: (Rs – Ra).

Summarizing:

1µm = microbial-derived respiration (Rh), after disturbing

1 cm = sum of microbial and root-derived respiration (Rh+Ra)

1cm - 1µm = root-derived respiration (Ra)

No mesh = control for total respiration (Rs)

Control - Ra = microbial derived respiration (Rh), real one

4.2.3. *Soil chemical and biochemical properties*

Soil sampling was performed twice in the year 2006: just after the mowing at the end of June and four months after the mowing in the beginning of October. For each sampling date 16 soil cores were collected: 2 soil samples inside each fenced area, 8 in total (unmanaged, UM), and 2 soil samples outside the fences, 8 in total (mowed and grazed, MG). MG soils were sampled at least 5m away from the fences.

The following analyses were performed: total organic C (TOC), total N (TN), microbial biomass C (MBC), extractable C (C_{ext}), the potential microbial respiration measured during a 28 days incubation, short nitrification assay as a measure of nitrogen mineralization activity (SNA) and eight soil enzymatic activities related to C, N, P and S cycling.

Physical analyses

Soil water content

5 g field-moist soil samples were weighed in ceramic pots. Soils were placed in an oven at 105 °C. After 24 hours soils were removed from the stove and weighed again. Soil water content was referred to the fresh weight.

Water holding capacity

Soil samples were saturated with water in a cylinder. The cylinder was tapped in the bottom by filter paper until the excess water was drawn away by gravity. After 24 h, when equilibrium was reached, the water holding capacity was calculated based on the weight of the water held in the sample vs. the sample dry weight.

Chemical analyses

Total Organic Carbon (TOC) and Total nitrogen (TN)

TOC and TN were determined only in June 2006 in 20 mg of dry soil after HCl addition with a C/N soil analyzer (FlashEA 1112 Series, Thermo Electron Corporation).

Biochemical analyses

Microbial Biomass C

For MBC determination the Fumigation Extraction method (Vance et al., 1987) was used. In brief, two portions of moist soil (20 g oven-dry soil) were weighed, the first one (non fumigated) was immediately extracted with 80 ml of 0.5M K_2SO_4 for 30 min by oscillating shaking at 200 rpm and filtered with filter paper (Whatman n. 42); the second one was fumigated for 24h at 25 °C with ethanol-free $CHCl_3$ and then extracted as described above. Organic C in the fumigated and non fumigated extracts was determined after oxidation with 0.4 N $K_2Cr_2O_7$ at 100°C for 30 min.

Microbial C was calculated as a difference between C content in fumigated and non fumigated extracts divided by a conversion factor (K_{EC} : 0.38). C extracted from non fumigated samples represents the labile pool of K_2SO_4 -extractable C (C_{ext}).

Soil C mineralization

Potential soil respiration rate was calculated following Badalucco et al. (1992): 20 g of sieved soil (60% WHC) were weighed in a beaker and placed in the bottom of a 1L jar. In another beaker 2 ml NaOH 1N were placed inside the jar in order to trap the CO_2 evolved during the incubation period (28 days). The reaction was stopped with 4 ml of $BaCl_2$ 0.75 N to precipitate CO_2 in $BaCO_3$. The excess of NaOH that did not react with the CO_2 was determined by titration with 0.1M HCl after 1, 3, 7, 10, 14, 21 and 28 days.

The C mineralization kinetics up to 28 days was calculated following Riffaldi et al. (1996) using the first order kinetic model:

$$C_m = C_0(1 - e^{-kt})$$

where, C_m is the cumulative value of mineralized carbon during t days, C_0 is the potentially mineralizable carbon, k is the rate constant of labile pool mineralization. The numerical values of the parameters in the equation were obtained by non-linear regression of data on the CO_2 evolution rate.

Microbial indices

The following microbial indexes were calculated:

- Microbial quotient (**q_{mic}**) – it is calculated as the ratio of microbial biomass to total organic C: μg of Biomass C μg total organic carbon⁻¹ (Anderson and Domsch, 1989). q_{mic} is an index of the availability of C substrates to soil microbes and is also considered an early predictor of SOM accumulation.
- Metabolic quotient (**qCO_2**) – it is calculated as the quantity of C respired per unit of microbial biomass μg C- CO_2 basal h^{-1} μg Biomass C⁻¹ (Dilly and Munch, 1998). The mean values of the hourly CO_2 evolved after the 10th day of incubation were used as the basal respiration value because, after that period, the soil reached a relatively constant hourly CO_2 production rate. The metabolic quotient informs on the microbial efficiency in utilizing the available carbon resources.
- The potential initial mineralization rate (**C_0k**) - it is an index derived from the kinetic model $C_m = C_0(1 - e^{-kt})$ and can be used as an indicator of the degree of availability or of differences between the mineralized organic compounds (Riffaldi et al., 1996).

Soil enzymatic activity

Criteria for choosing enzyme assays were based on their sensitivity to soil management, importance in nutrient cycling and SOM decomposition.

Enzyme activity was measured according to the method of Marx et al. (2001) and Vepsäläinen et al. (2001), partially modified, and based on the use of fluorogenic methylumbelliferyl (MUF)- and (AMC)- substrates. The soil was analysed for β -cellobiohydrolase (exo-1,4- β -glucanase, EC 3.2.1.91), N-acetyl- β -glucosaminidase (EC 3.2.1.30), β -glucosidase (EC 3.2.1.21), α -glucosidase (EC 3.2.1.20), acid phosphatase (EC 3.1.3.2), β -xylosidase (EC 3.2.2.27), arylsulphatase (EC 3.1.6.1) and leucine-aminopeptidase (EC 3.4.11.1). The respective substrates were 4-MUF- β -D-cellobioside, 4-MUF- N-acetyl- β -glucosaminide, 4- MUF - β -D-glucoside, 4-MUF - α -D-glucoside, 4- MUF -phosphate, 4- MUF -7- β -D-xyloside, 4- MUF -sulphate and L-leucine-7- amino-4-methylcoumarin. 2.0g of fresh soil were homogenised in 100ml of 0.5M acetate buffer, pH 5.5, using an Ultra Turrax IKA for 3 minutes at 9600 rev/min. Aliquots of 100 μ l of diluted soil were pipetted in a 96 multiwell plate with three replicates. Each substrate was added in each well in aliquots of 100 μ l for a final concentration of 500 μ M; then the microplates were incubated at 30 °C for 3 hours, with fluorescence readings occurring every 30 minutes. For the calibration curve 0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1 nmoles of MUF or AMC, for leucine-aminopeptidase, were added to the same aliquots of soil suspension to take into account the quenching effect on fluorescence intensity (Freeman et al., 1995). Fluorescence readings (excitation 360 nm; emission 450 nm) were performed using a Fluoroskan Ascent (Thermo electron) fluorometer.

The synthetic enzymatic index (SEI) was calculated following Dumontet et al. (1998) and using specific enzyme activities releasing the same reaction product (MUF).

Soil N mineralization (Short Nitrification Assay)

Potential nitrification was measured after inhibition of N-NO₂⁻ oxidation with sodium chlorate (10mM) according to the short nitrification assay (SNA) reported by Hopkins et al. (1988).

Briefly, 2g of soil (60% WHC) were taken from each soil sample and were extracted with (NH₄)₂SO₄ solution during 24h shaking. Extracts were centrifuged at 3800 rev per 20 min, then 1ml from each sample was transferred to 50 ml flasks together with diazotizing and coupling reagents. Colorimetric readings were performed on the spectrophotometer (UV mini 1240, UV-vis spectrophotometer, Shimadzu). To construct a calibration curve 0, 0.25, 0.5, 1, 2, 3, and 4 ml were

taken from 1 $\mu\text{g N-NO}_2 \text{ ml}^{-1}$ solution, transferred to 50 ml jars and then treated similarly to soil extracts.

4.3. Results

4.3.1. Soil respiration and partitioning

Seasonal variation during two years of measurements of total, root- and microbial-derived soil respiration in MG and UM plots is shown in figure 3. Fluxes of respiration from soil varied significantly in the course of the year with minimum values of less than 1 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ registered for root-derived respiration to upper limit of 8 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ observed for microbial component. No clear difference was observed between MG and UM plots in respiration rates from various sources in 2007. In 2006, the respiration was mainly higher in unmanaged soils.

The cumulative values of root- and microbial-derived respiration were calculated by summing the products of soil respiration and the days between the samplings. Our measurements collected between 10:00 and 13:00 hours and being 90% of the observed diurnal averages, were assumed to be representative. No differences in cumulative respiration of various origin between MG and UM plots were observed (Fig.4) for neither of years. However, microbial and root-derived respiration were generally higher in 2006 in unmanaged plots, in 2007 managed soil experienced higher respiration rates before the defoliation practices, after which the increment in cumulative value was slowed down and resulted in no difference between the treatments. This effect was observed only for microbial component of soil respiration.

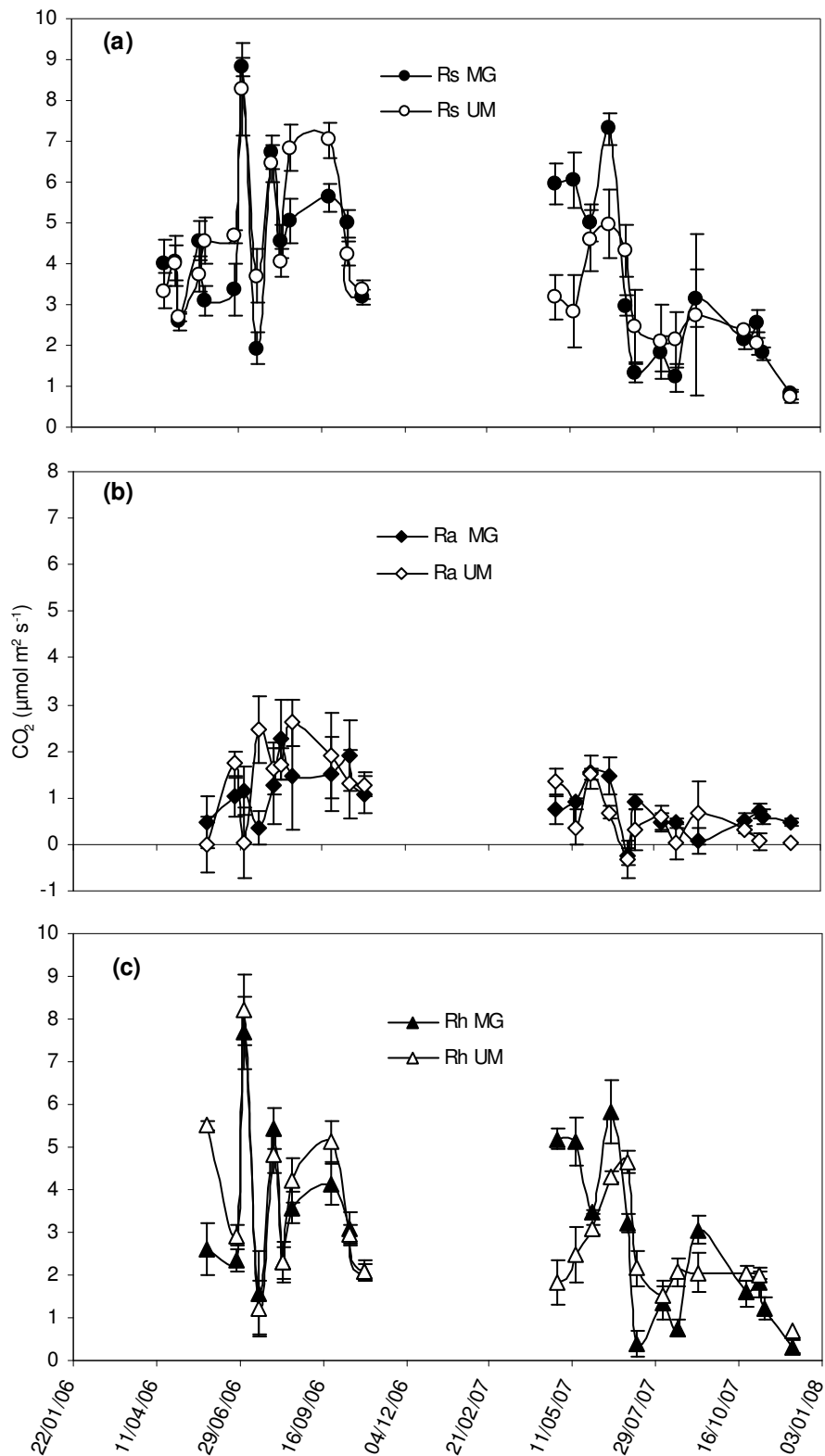


Fig. 3 Total (a), root-(b) and microbial- (c) derived respiration measured in 2006 and 2007 in managed (MG) and unmanaged (UM) plots ($\pm$ SE).

Defoliation practice has influenced significantly soil physical properties in managed plots (Fig.5). Compared to control, soil temperature was much higher in managed soil, in some days the difference between treatments amounted to 5°C. Soil water content (SWC) was also modified positively by defoliation, with elevated values in managed soil, indicating different transpiration

rates between clipped and unclipped plants. Dense herb cover in UM plots have also influenced SWC by decreasing the quantity of water reaching the soil surface after the rain events. All these aspects should be taken into account while interpreting the data of soil respiration from soils under different management.

No difference were observed in belowground biomass between treatments (Fig.6)

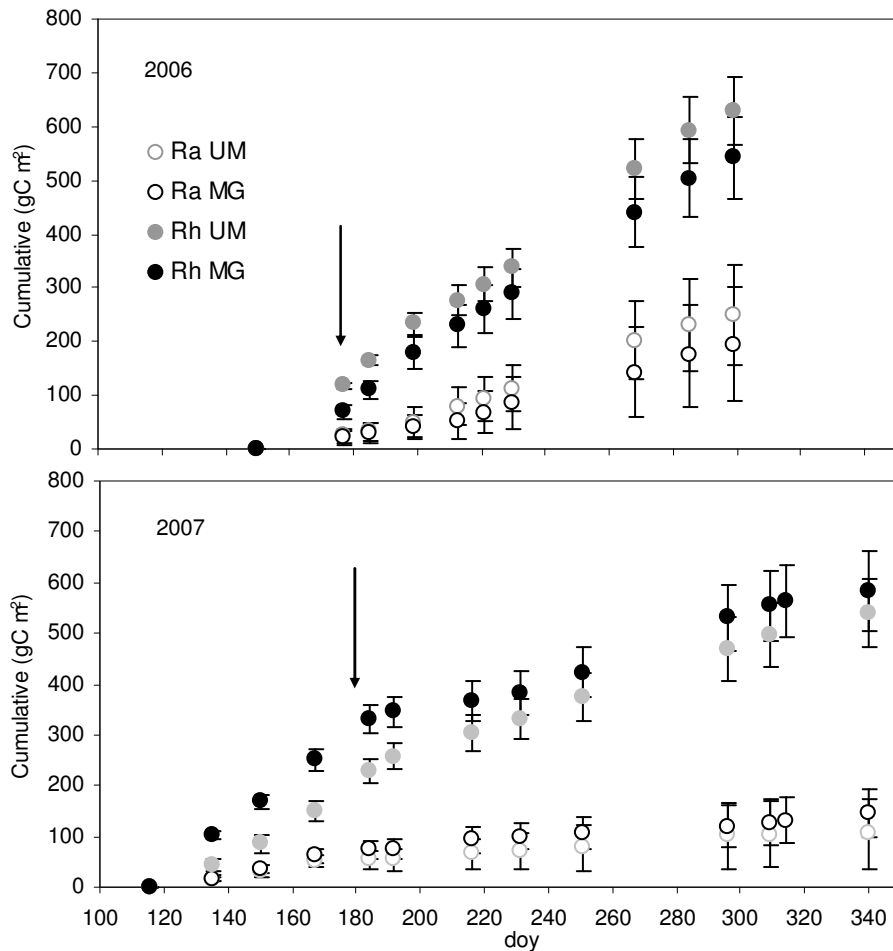


Fig.4 Cumulative CO₂ efflux of root and microbial origin estimated in 2006 and 2007 ($\pm$ SE). Arrows indicate the day when moving was performed.

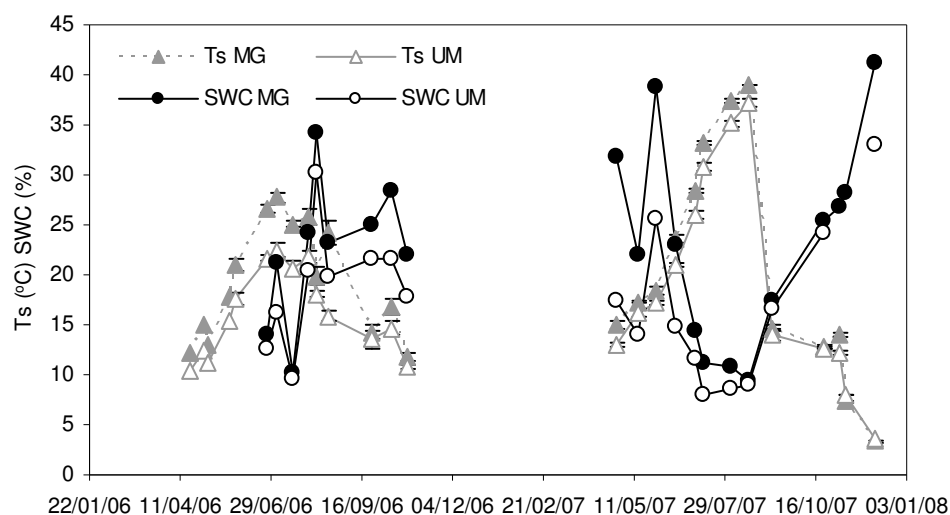


Fig.5 Soil temperature (Ts) and Soil water content (SWC) at 5 cm depth in managed (MG) and unmanaged (UM) plots ($\pm$ SE).

Temperature sensitivity of soil respiration was also influenced by defoliation practice. The effect was especially pronounced for root-derived respiration (Fig.7 and Table 1). Defoliation constrained temperature response of R_a under favourable soil moisture. The data obtained after the mowing were out of the general trend indicating that other factors are responsible for changes in root respiration, which is more likely a supply from aboveground of photoassimilates, the effect of which is often masked by temperature.

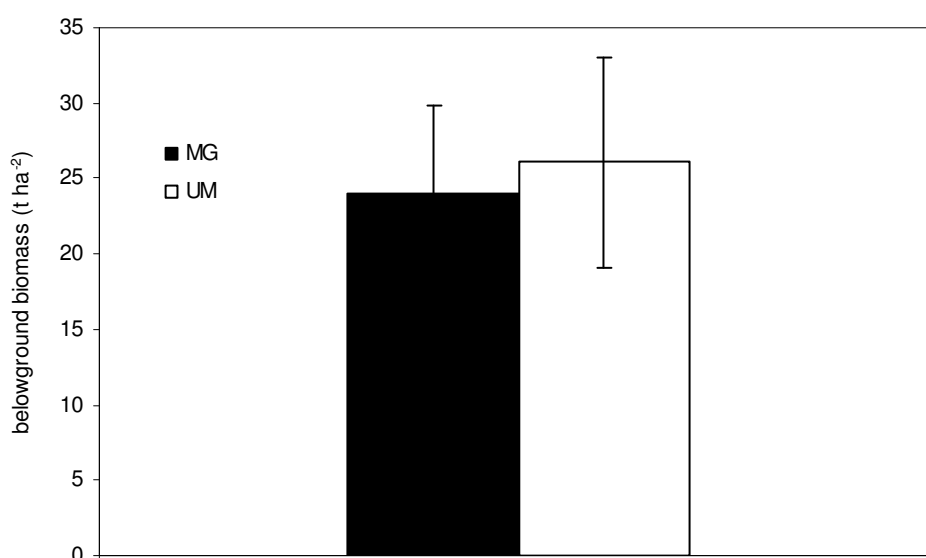


Fig.6. Belowground biomass in managed (MG) and unmanaged plots (UM), measured in July 2006.

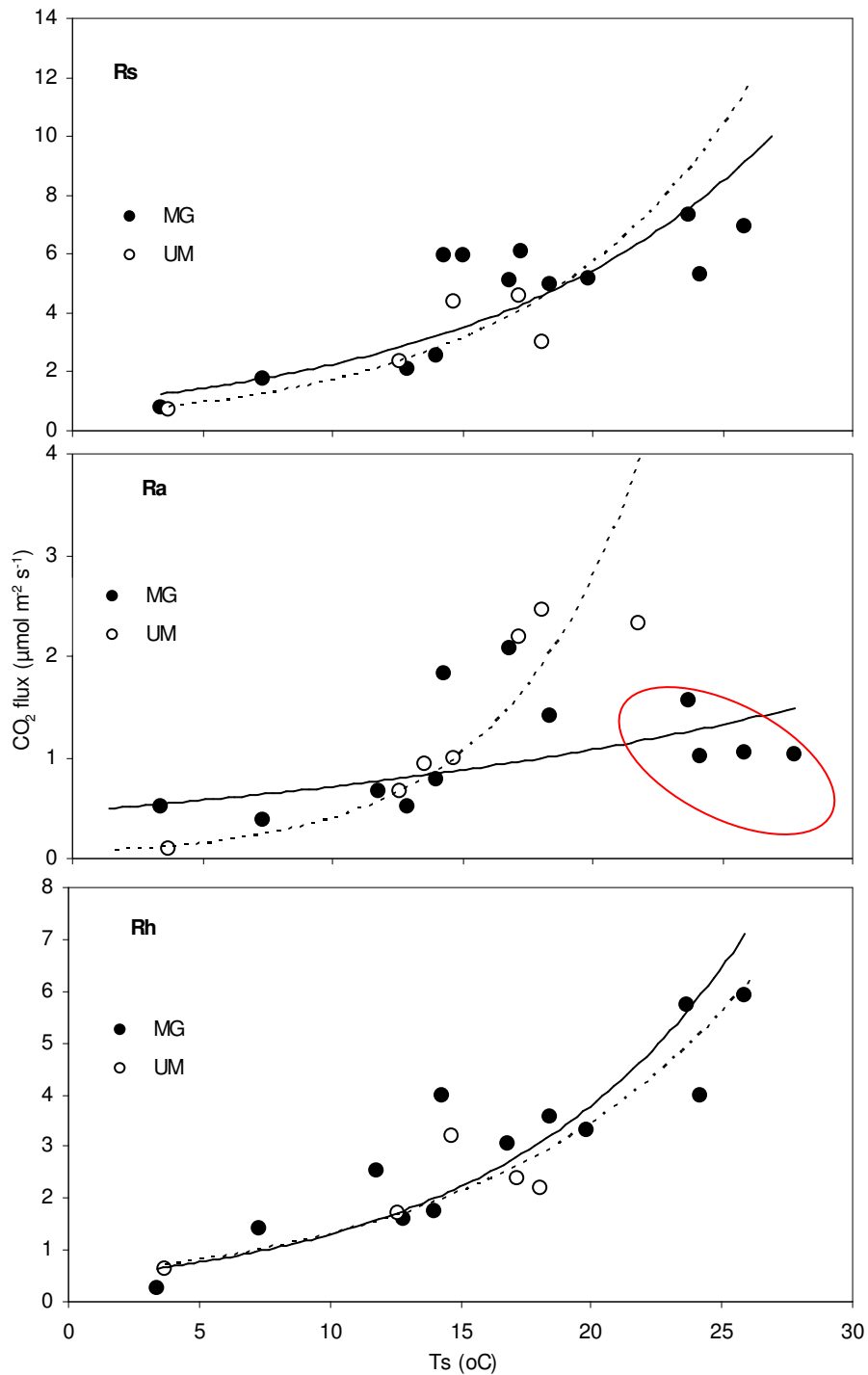


Fig. 7 Total (Rs), root (Ra)- and microbial (Rh)- derived respiration vs. changes in soil temperature (Ts) at 5 cm depth in managed (MG) and unmanaged (UM) plots measured in 2006 and 2007. Data at SWC<20% were excluded from the analyses.

	Q_{10}	
	MG	UM
Rs	2.42	3.31
Ra	1.55	6.98
Rh	2.91	2.69

Table 1. Q_{10} values for total, microbial and root-derived respiration in managed (MG) and unmanaged (UM) plots for 2006 and 2007.

Microbial respiration was also affected by treatment. To account for the above reported differences in soil physical conditions (moisture and temperature), total and microbial-derived respiration in MG and UM plots were calculated at a common temperature and SWC (Fig.8). For these purposes respiration of managed plots was calculated at the same temperature of non managed soil at the non limited SWC and during the drought period, when water content exerts a significant influence on soil respiration, at the same SWC as for non managed soils.

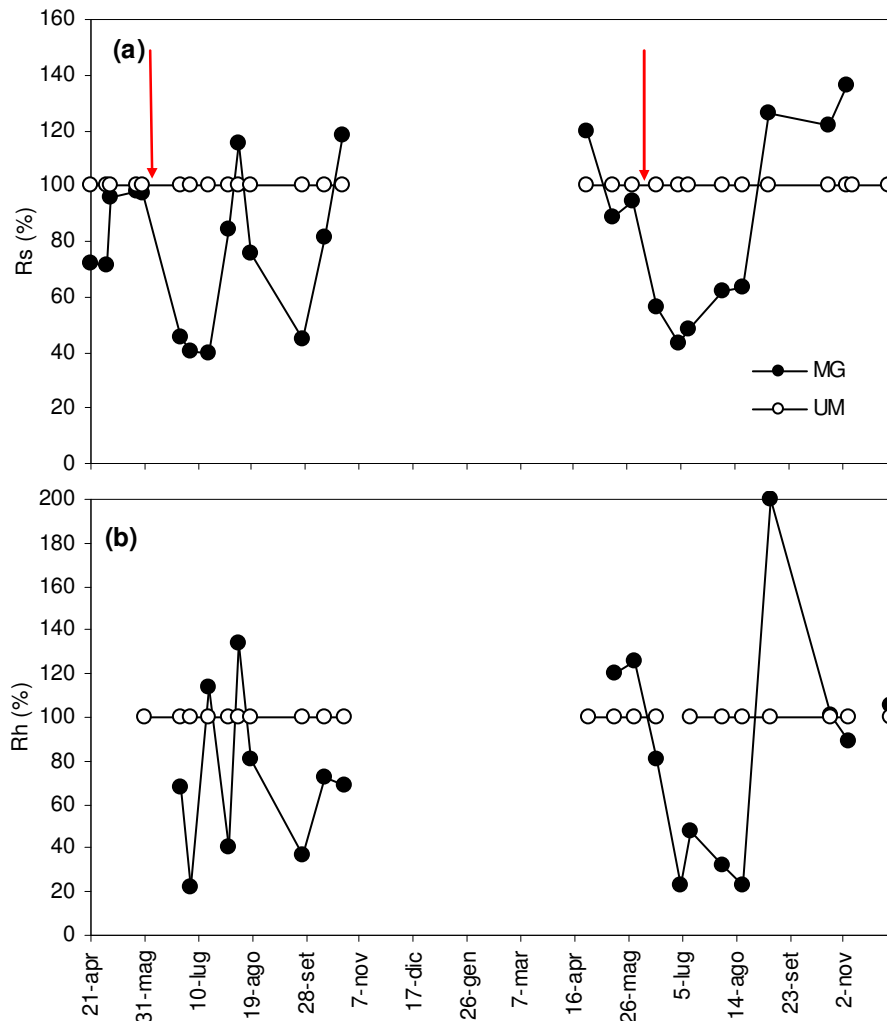


Fig. 8 a) Total (Rs) and b) microbial (Rh) derived respiration of managed in % from unmanaged plots corrected to a common Ts and SWC in 2006 and 2007. Arrows indicate the time of mowing.

Temperature and SWC response were derived from a regression using all soil respiration measurements of the managed plots over the course of the experiment. When corrected for a temperature and moisture effect, defoliation practices reduced soil respiration of microbial origin and total CO₂ efflux from soil by ca. 20%.

Microbial-derived respiration after normalization to soil temperature at a reference value of 15°C demonstrated a different response to changes in SWC in MG and UM plots (Fig.9), indicating possible functional changes between two microbial communities.

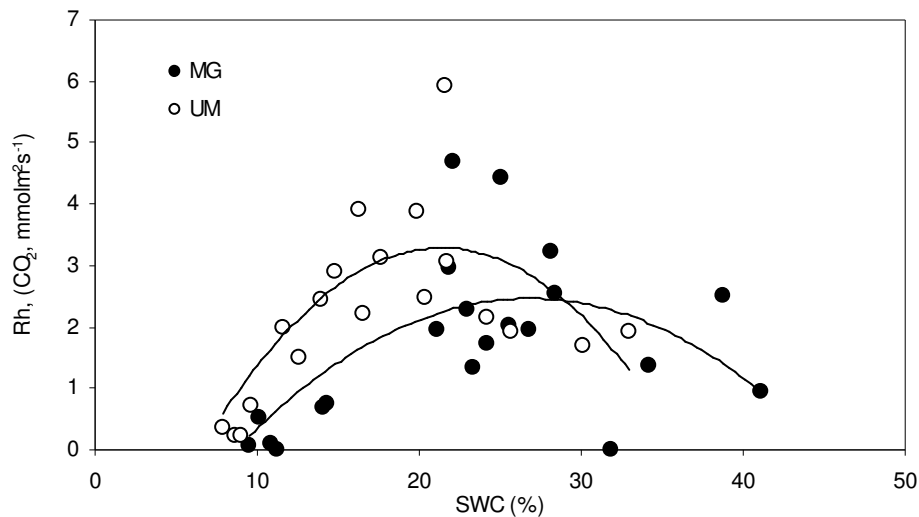


Fig. 9 Normalized microbial-derived respiration vs. soil moisture measured in 2006 and 2007.

4.3.2. Soil biochemical properties

To get deeper insight into the possible effects of management on soil microbial pool and activity some soil chemical and biochemical analyses were performed.

TOC and TN did not vary significantly between MG and UM plots ($3.7\% \pm 0.16\%$ and $0.3\% \pm 0.01\%$ in UM soils and $3.5\% \pm 0.15\%$ and $0.3\% \pm 0.01\%$ in MG soils for TOC and TN respectively).

Extractable C (C_{ext}), representing a source of labile C, shows a significant difference between MG and UM soils in the month of June with higher values for the MG (+24%, $p < 0.001$); no significant changes were observed between the two sampling dates (Fig.10a).

A positive trend could be seen in MBC just after the mowing practice; though, the difference between treatments was not significant. This parameter was however significantly influenced by sampling time showing the highest values in October (Fig.10b).

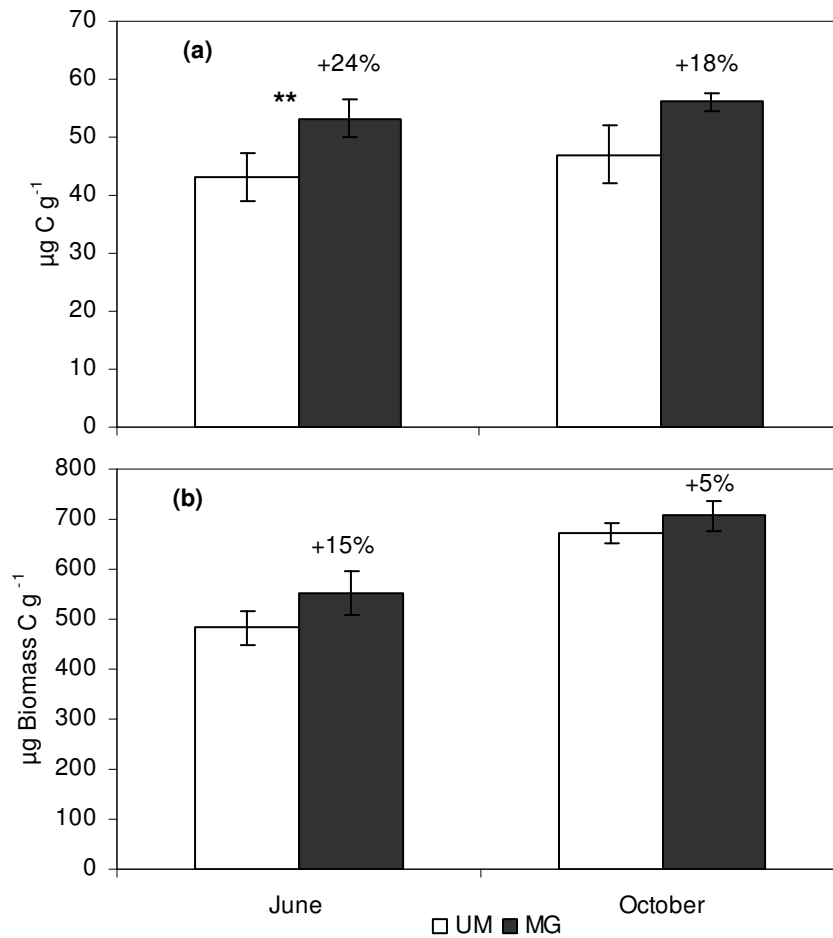


Fig 10. a) K₂SO₄-extractable C (C_{ext}) and b) Microbial biomass C (MBC) measured in managed (MG) and unmanaged (UM) soils in June and October. The magnitude of management effect in confront with control is indicated in %. **p* < 0.001, ***p* < 0.01, **p* < 0.05.**

Carbon mineralization kinetics

A time-course of organic C mineralization in the soil was analysed by fitting the experimental values with the first order equation $C_m = C_0 \times (1 - e^{-kt})$. The cumulative mineralized C presented a curvilinear relationship with time over the 28-day incubation period in June and October as shown in Fig.11. All treatments showed a similar pattern, with a larger initial release of CO₂ followed by a slower linear increase throughout the remaining period of incubation.

CO₂ production after 24 hours (MR_{24h}) was modified by management treatment with lower values for MG soils (-20%, *p*<0.005), sampling time also caused a significant increase of the initial flush of CO₂ in October sampling (Fig 12a). The total amount of C mineralized at the end of incubation period (*C_m*) decreased significantly in MG soils in June (-19%, *p*<0.05) (Fig. 12b). Significant differences were also found due to sampling time.

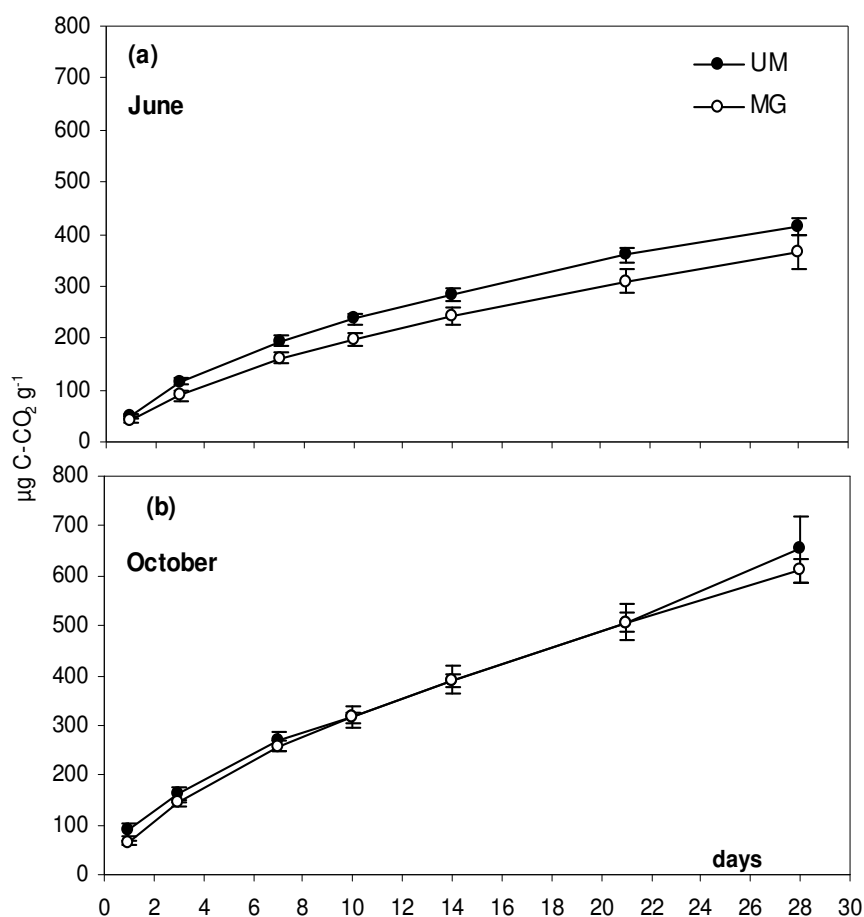


Fig. 11 Cumulative microbial respiration measured over 28 days of incubation in mowed and grazed (MG) and unmanaged (UM) soils in June (a) and October (b).

Microbial indexes

The q_{mic} was significantly affected by sampling time increasing from June to October and by management practice, with higher values for MG soil in October, a positive but not significant trend was observed also in June (Fig.13a).

The qCO_2 , (the microbial community respiration per biomass unit) was significantly decreased by the management practices in June, a negative but not significant trend was observed also in October; no significant changes were observed due to sampling time (Fig.13b).

The C_0k (potential initial mineralization rate) significantly varied due to the season ($p < 0.0001$) and to the management activity ($p = 0.02$) (Fig.13c), confirming the different quality of decomposing substrate in control and treated soil.

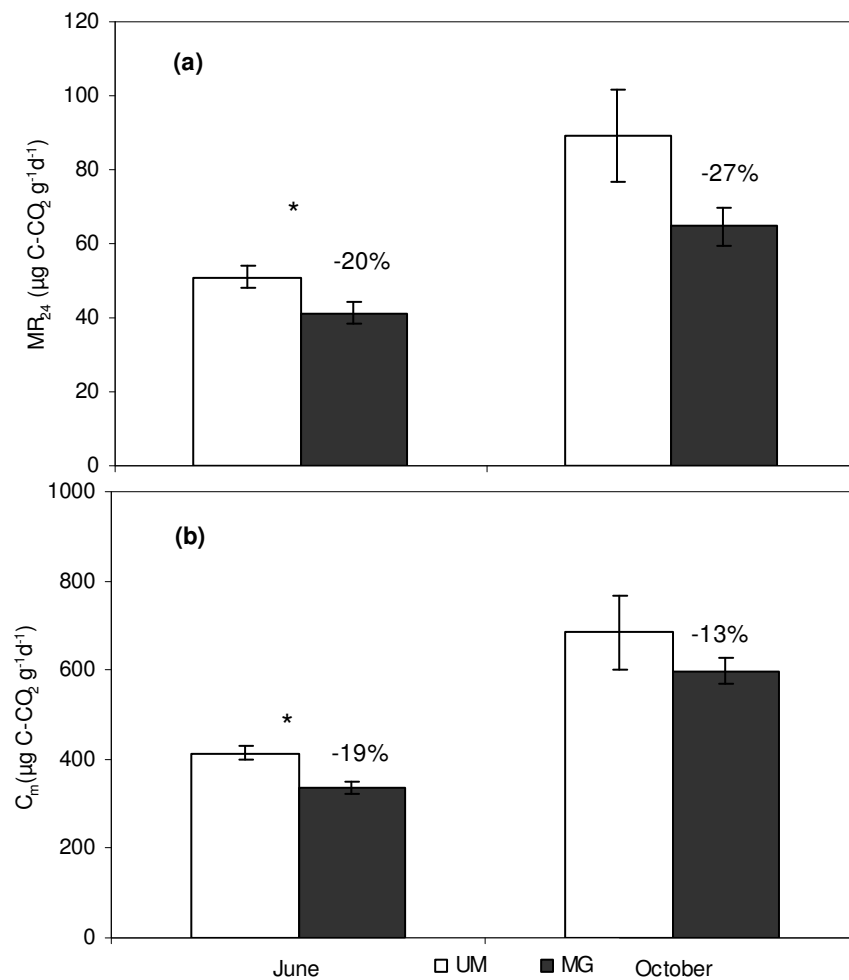


Fig. 12. a) Microbial respiration measured after 24h of incubation (MR_{24}) b) C mineralized at the end of incubation (C_m) The magnitude of management effect in confront with control is indicated in %. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Soil enzymatic activity

Almost all measured soil enzymes, associated with microbial decomposition activity and involved in the cycling of C, P and S have diminished their activity in MG soils in June with respect to UM soils (Fig.14 and Table2). The only enzyme, which experienced a positive influence under the management regime in June, was leucine-aminopeptidase, involved in the N mineralisation process, indicating an enhanced demand for N in these soils. In October no difference in soil enzymatic activities between treatments for most enzymes was registrated. The same trends were observed for specific enzymatic activity (data not shown).

To confirm the above hypothesis the potential nitrification activity was also measured according to the short nitrification assay (SNA). Confirming the data of leucine amino-peptidase activity an enhanced N mineralization activity was observed in the managed soils just after the mowing in June, while in October no difference was registrated between treatments (Fig. 15). Furthermore a significant correlation between the N mineralization process and leucine-aminopeptidase activity was observed in June (Fig.16a).

After the calculation of synthetic enzymatic index (SEI), a linear positive relationship was observed with $q\text{CO}_2$ in MG and UM plots (Fig.16b).

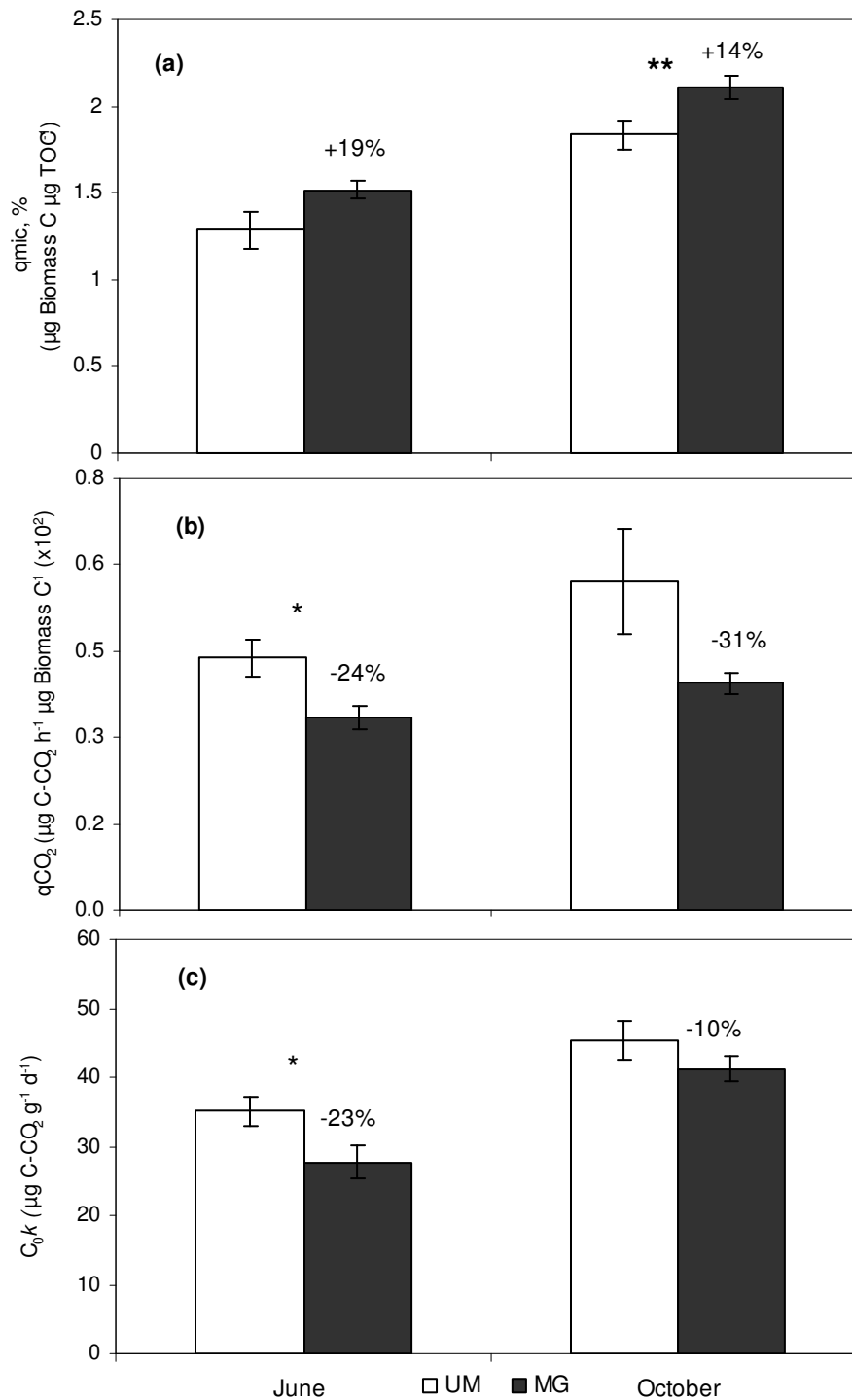


Fig 13. a) Metabolic quotient ($q\text{CO}_2$); b) microbial quotient ($q\text{mic}$) and c) initial potential mineralization rate (C_0k) in managed (MG) and unmanaged (UM) plots. The magnitude of management effect in confront with control is indicated in %. * $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.**

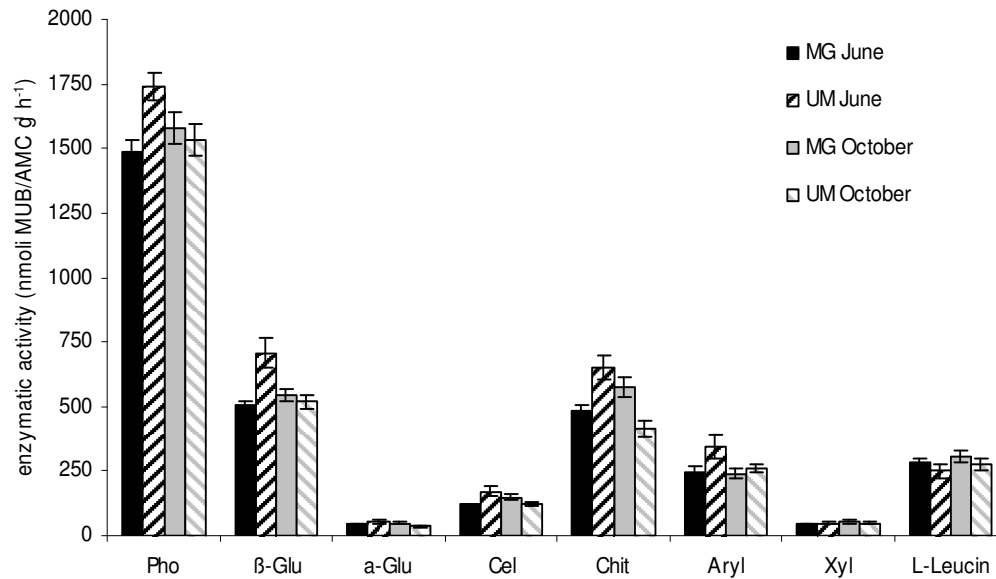


Fig. 14: Enzymatic activity in June and October 2006 in managed (MG) and unmanaged (UM) plots. Pho= acid phosphatase; β-Glu= β-glucosidase; α-Glu= α -glucosidase; Cel= β-cellobiohydrolase; Chit= N-acetyl-β-glucosaminidase; Aryl=arylsulphatase; Xyl=β- xylosidase; L-Leucin= and leucine-aminopeptidase

Effect of management and season on soil enzymatic activity								
	Pho	β-Glu	α -Glu	Cel	Chit	Aryl	Xyl	L-Leucin
% Effect June	-14.37**	-28.30**	-19.45*	-29.91*	-25.49**	-28.47	-7.72**	12.61
% Effect October	2.83	5.62	27.13	19.89	37.95	-7.67	13.96**	11.73
% Seas.variation MG	5.88	7.57	12.55*	24.43	18.47	-3.17	22.32*	9.72
% Seas.variation UM	-11.84*	-26.97**	-28.68*	-27.25*	-36.01**	-24.99	-0.95	10.58

Table 2: Effect of management regime (MG and UM) and season (June and October) on the soil enzymatic activity. Numbers indicate effect of treatment in % from control or effect of time in % from first sampling. * $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.**

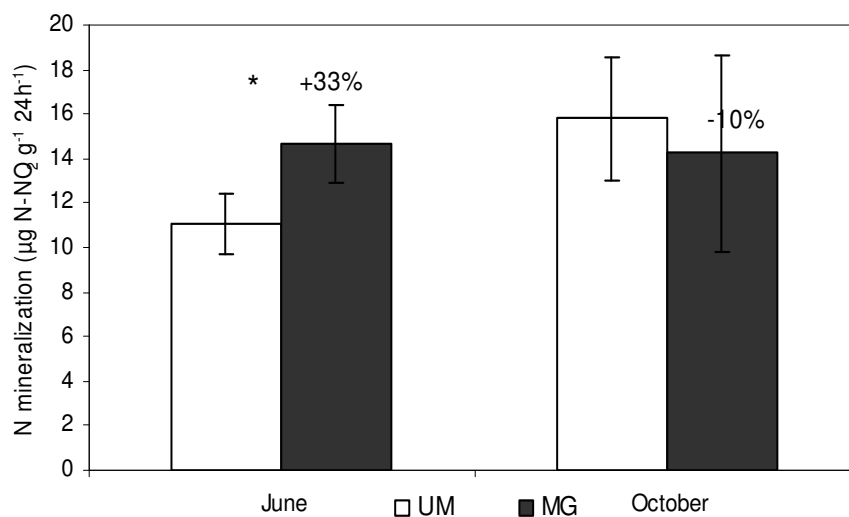


Fig. 15 Potential N mineralization activity in June and October 2006 in managed (MG) and unmanaged (UM) plots. The magnitude of management effect with respect to control is indicated in %. * $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.**

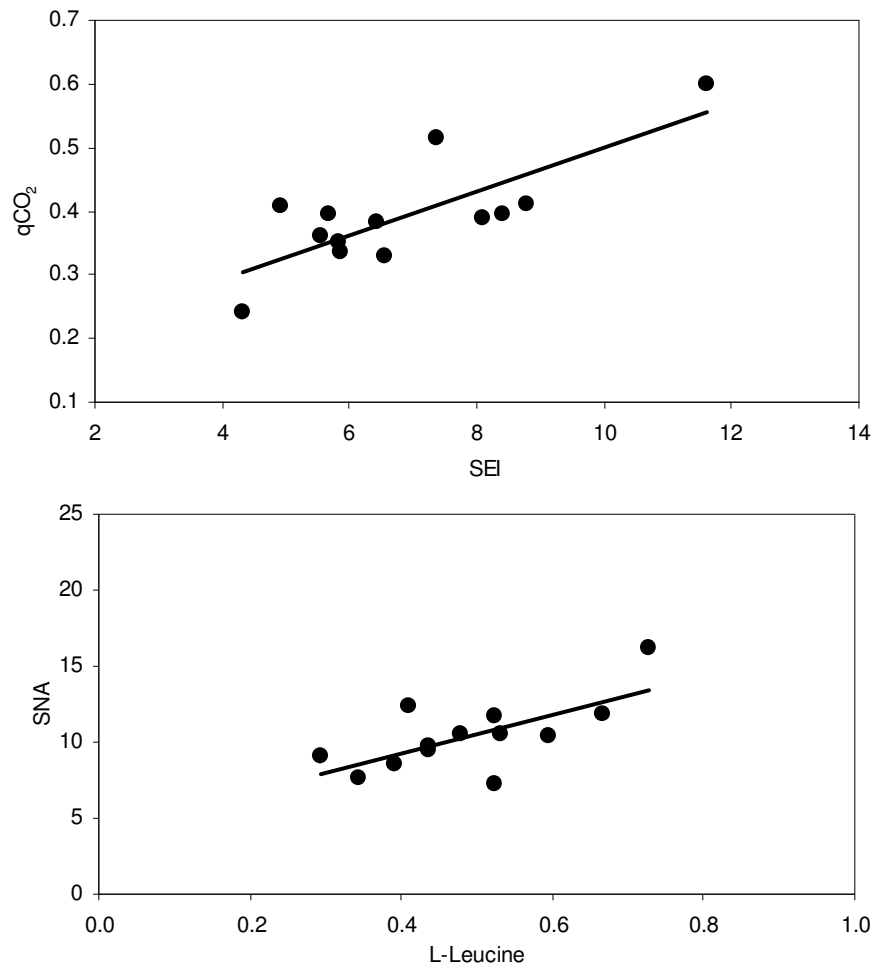


Fig. 16: a) Relationship between synthetic enzymatic index (SEI) and metabolic quotient (qCO_2) in June; $R^2=0.60$, $p<0.001$ b) Relationship between leucine-aminopeptidase activity and nitrogen mineralization process (SNA) in June $R^2=0.43$, $p<0.001$.

4.4. Discussion

4.4.1. Soil respiration and defoliation

Many studies reported that the removal of aboveground biomass by clipping, trenching, shading or girdling reduces the supply of current photoassimilates to roots and mycorrhizal fungi, usually resulting in a decrease in soil CO_2 efflux by 19-49% at a short-term period (several days to month) (Bremer et al., 1998; Boone et al., 1998; Craine et al., 1999; Hogberg et al., 2001; Zhou et al., 2006; Bahn et al., 2006). In our study, at common soil temperature and humidity, defoliation practice reduced total and microbial-derived respiration by ca. 20% (Fig. 7). A reduction of microbial respiration after clipping was previously suggested for grassland communities (Craine et al., 1999; Zhang et al., 2005; Zhou et al., 2007 and Banh et al., 2006). The main explanation given for this effect was a depletion of fresh C availability from rhizodeposition. Our results confirm the decrease in microbial respiration but we argue that the reason for this was exactly the opposite. Defoliation of plants provoked an enhanced rhizodeposition from roots, which resulted in general

relax of the microbial community in terms of C gaining and C mineralization activity as other easily available C substrates were present in the soil. This was confirmed by the results of Cext (a labile source of soluble C) measured just after the mowing in June and four months after the mowing in October (see below).

Numerous studies have observed increases in soil CO₂ efflux in response to warming (Peterjohn et al., 1994; Rustad et al., 2001; Melillo et al., 2002; Niinisto et al., 2004; Zhou et al., 2007). The warming-induced response of soil respiration may be regulated by acclimatization of respiration (Luo et al., 2001), phenological and physiological adjustments of plants and microbes (Melillo et al., 2002), extension of growing season (Dunne et al., 2003; Wan et al., 2005, Zhou et al., 2007), changes in net N mineralization (Wan et al., 2005) and stimulation of C₄ plant productivity (Wan et al., 2005). Our study showed that warming of the defoliated soil significantly increased respiration of microbial origin, resulting in an increase of the total CO₂ efflux from soil. These increase likely resulted from enhanced oxidation of soil C compounds in warmed plots for microbial-derived respiration and possibly for the microbial component of rhizomicrobial respiration (root-derived one). Warming of the managed soil cancelled the effect of defoliation and resulted in the absence of difference in the cumulative soil respiration and its components between treatments.

It was not possible to account for the dynamics in root-derived respiration at common physical environmental conditions in MG and UM plots as this component of soil CO₂ efflux was not related to changes in soil temperature (Fig. 6). It is clear that temperature sensitivity of root-derived respiration at the non-limited SWC was modified by treatment. In UM plots an obvious exponential increase of root-derived respiration with a high Q₁₀ values was registered, whereas in plots subjected to defoliation practice the Q₁₀ was reduced considerably ($p < 0.05$) and the dependence on changes in soil temperature was lost. We didn't observe any difference in belowground biomass between MG and UM treatments (Fig.6), so the only factor which could influence and change patterns of root-derived respiration is the supply of current photoassimilates. The dependence of root-derived respiration on photosynthesis is often masked by temperature, as the aboveground biomass usually peaks at the high temperatures and favorable soil water conditions which was observed in the UM soils. Management, based on defoliation unmask this effect, showing a clear uncoupling with temperature under mowing and grazing. Some studies (Hogbert et al., 2001; Bahn et al., 2006; Zhou et al., 2007) reported no changes in root respiration under experimental clipping and girdling, suggesting that carbohydrates stored in tree and grass organs may buffer the effect of reduced assimilate supply and sustain root metabolism for some days or even weeks. Our measurements were taken with bimonthly frequency, which means that just after

an annual mowing the measurement was taken only once, so we are not able to account for a short term effect of defoliation.

Summarizing, our result show that factors affecting canopy C status and carbohydrate supply to roots influence both root and microbial soil respiration components being microbial respiration affected both in short and long term and root-derived respiration affected clearly on the long-term bases. The effects were however buffered by increased soil temperature and soil water content under defoliation.

4.4.2. *Microbial activity and defoliation*

Our results prove a significant effect of grazing and mowing on microbial biomass community and its metabolic activity. The ratio of biomass C to soil organic C (q_{mic}) reflects the contribution of microbial biomass to soil organic carbon (Anderson & Domsch, 1989). It also indicates the substrate availability to the soil microflora or, in reverse, the fraction of recalcitrant organic matter into the soil (Brookes, 1995). After a change, the soil microbial biomass responds more quickly to the change than does the amount of organic matter in the soil, which is relatively slower. Thus, q_{mic} ratio will increase for a certain time if the input of organic matter to a soil is increased, and decreases if the input is decreased (Anderson and Domsch, 1989). The elevated value of q_{mic} observed in this study in MG soils, indicates a major availability of C substrates as confirmed also by the increase of easily available C source (C_{ext}) which is considered an active organic fraction playing an important role in the substrate availability for microbes (Dumonent et al., 2001). Similar results were found by Uhlirova et al. (2005) reporting a significantly higher amount of potassium sulphate-extractable C in mowed grassland soils. In fact the positive effect of defoliation on microbial biomass is often explained as due to an increase of root exudation, resulting in a larger supply of easily available C and energy sources to microbial communities, enhancing their biomass and nutrient cycling (Bardgett and Leemans, 1995; Bardgett et al. 1998; Guitian and Bardgett 2000; Kuzyakov et al., 2002; Kuzyakov and Domanski 2000; Rice et al., 1996; Zhou et al., 2006; Hamilton III et al., 2008). Compounds that are exuded by roots of defoliated grasses are comprised of organic acids, sugars and amino acids (Bokhari, 1977; Whipps, 1990; Marschner, 1995) which stimulate rhizospheric processes and also the availability of nutrients to the defoliated plants. Moreover microbial population in grazed grasslands is sustained by inputs of labile C from dung deposition and increased root turnover/rhizodeposition beneath grazed plants (Tracy and Frank, 1998).

Significant differences were observed in the metabolic activity of microorganisms between MG and UM plots: the respiration rates were significantly decreased by mowing and grazing either in the short term (MR_{24h}) or in the long term (C_m). Also the initial potential mineralization rate

(C_0k) was negatively affected by the treatment indicating a different quality or different sources of decomposing substrates with respect to UM soils (Riffaldi et al., 1996).

All these evidences support the hypothesis that microorganisms decreased their mineralization activity in MG soils as other easily available energy sources were present.

It is widely documented that the soil metabolic quotient (qCO_2) is elevated when the soil microbial biomass is operating inefficiently and is diverting a high proportion of C to maintenance requirements than biosynthesis (Anderson and Domsch, 1985).

In our results qCO_2 and soil enzymatic activity were significantly lower in MG plots and suggested that the increase of easily available C in the rhizosphere of defoliated plants, resulting from a short-term flux of photoassimilate C below-ground, decreased the maintenance energy requirements and shifted the energy from maintenance to biosynthesis processes as the increase of q_{mic} also demonstrated (Bardgett et al., 1998; Holland et al., 1996; Dilly, 2005). According to Schjonning et al. (2002) the decrease in qCO_2 and specific enzyme activity indicates: a) a more efficient microbial community and b) a better use of the available organic substrates. Higher C use efficiency under defoliation was also reported by Guitian and Bardgett (2000) and Uhlirova et al. (2005).

In our study almost all enzymes, involved in the cycling of S, P and C have demonstrated to be valid indicators of changes under management practices and have diminished its activity in managed soil. Only β -cellobiohydrolase and arylsulphatase didn't show a significant decrease in MG plots, even so the negative trend for these enzymes was also observed. More likely that enhanced root exudation, leading to a larger supply of easily available C and energy sources to microbial communities resulted in general suppression of decomposition activities just after the mowing procedure. A significant relationship between SEI and qCO_2 (Fig.14b) could be used as an extra prove of the bacterial community "relax" in terms of C gaining in that period. N-acetyl- β -glucosaminidase and arylsulphatase are also considered as indirect indicators of the presence of the fungal biomass because sulphate esters (substrates of arylsulphatase) and chitin are major components in fungal cells (Bandick and Dick, 1999). These could suggest a presence of a more efficient microbial community in the managed plots.

The enhanced N mineralization activity just after the mowing and increase in the activity of leucine amino-peptidase (involved in the N cycling) suggest that in grassland community the defoliation practise stimulated the flow of C to roots and into the soil, increased the size of microbial community, which in turn stimulated potential net N mineralization rates. Herbivores for example often enhance soil N cycling (McNaughton et al., 1997) which can result in the improved N availability to plants. In particular key microbially mediated processes involved in soil N cycling (nitrification, denitrification and N_2 -fixation) that largely control the balance the forms of soil

mineral N available to plants and N conservation at the ecosystem level can be affected. This could finally result in the positive feedbacks for plants in term of the quantity of available N in the soil and shoot N content. In fact, it was demonstrated also by Hamilton III et al. (2008) and Frank (2008) that herbivore-induced C exudation increased the availability of plant-limiting nutrients in these systems, which may be important mechanisms promoting growth of grazed plants in grasslands that experience high chronic rates of herbivory or other defoliation practices. Similarly the effect of mowing on N fluxes and N retention in grasslands have been reported by Marron and Jeffries, 2005. No difference was observed in total N between MG and UM plots, however we expect that there were some differences in the mineral N content between treatments.

The different season of sampling produced significant variations of the microbial parameters with more elevated values in October than in June probably because of the high soil water content in the autumn favouring the microbial size and metabolism. Different seasonal patterns of microbial parameters in grasslands are also reported by Uhlirova et al. (2005) and Tracy and Frank (1998). On the other hand the differences due to the management practices were mostly evident in June sampling. This could be due to the fact that soil sampling was performed after few days from the annual mowing and thus soil microorganism metabolism was still influenced by the availability of soluble C sources released by roots (Guitian and Burdgett, 2000), suggesting also a fast response of plants to defoliation which results in the enhanced rhizodeposition process and further equal rapid changes in microbial activity. In fact, Henry et al. (2008) have shown on *Plantago arenaria*, grown in soil, that within 1.5 days defoliated plant rhizosphere soil had decreased soluble C and increased microbial biomass and within 8.5 days there were no significant difference in either parameter. Other studies, investigating the belowground responses to aboveground defoliation, have shown that the response can vary depending on plant species identity (Guitian and Bardgett 2000; Hokka et al., 2004; Mikola et al., 2005), on the timing of defoliation during the plant development (Ilmarinen et al., 2005) and on other biotic and abiotic factors. These could explain the absence of significant differences between the treatments in October, when the plants were still experiencing a grazing pressure; however the effect was not randomly distributed in the field as in comparison with mowing in June, the growing stage and environmental conditions were also different.

4.4.3. Conclusions

Grassland management, based on plant defoliation (mowing and grazing) appears to be a suitable management practice, influencing the below-ground food-web, and thus SOM transformation and nutrient cycling. In our study it increased the quantity of easily available C sources for microbial biomass, decreasing C mineralization rates and enhancing C use efficiency.

These factors are very important for SOM maintenance and sustainability in grassland ecosystems and, although, we did not find significant changes in TOC content, the considerable increase of the microbial quotient could indicate a future positive trend of SOM accumulation; q_{mic} has in fact been widely used as an indicator of future changes in organic matter status due to alterations in land use and management, cropping systems and tillage practices (Sparling, 1997). The flush of labile C in the rhizosphere of defoliated plants stimulated the growth and activity of microbial community associated with N mineralization processes and thus availability N to plants. This could result in the higher quality of the leaf tissue, which eventually benefits grazers.

Two methodological approaches applied for estimation of C mineralization activity under different management practices: *in situ* measurements of microbial-derived respiration at normalized Ts and SWC and potential microbial respiration obtained in laboratory confirmed the results of each other.

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5. FOCUSING ON ROOT-DERIVED RESPIRATION: C COSTS OF NITRATE REDUCTION AS ESTIMATED BY ¹⁴CO₂ LABELING OF LUPINE AND CORN

Chapter source:

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5.1. Introduction

Nitrogen (N) alongside carbon (C) is the element needed in greatest abundance by lower and higher plants. Due to losses by leaching and microbial consumption, N is often a limiting factor for plants, especially in soils with temperate and humid climates. Therefore, plants have developed mechanisms to cope with the low N supply. These include very sensitive uptake systems and possibility to grow on various N sources. The main N sources include ammonium (NH_4^+) and nitrate (NO_3^-) (Tischner, 2000) however, in some environments amino acids are considered as significant organic N source (Nasholm et al., 2000; Xu et al., 2006).

Utilization of N in the form of either nitrate (NO_3^-) or ammonium ions (NH_4^+) may affect the carbohydrate metabolism and energy economy of the plant (Blacquièrè, 1987). NH_4^+ is fixed by GS/GOGAT pathway into amino acids (glutamine/glutamate), this incorporation occurs immediately after N uptake in roots and no significant amount of ammonium have ever been discovered in the xylem sap (Tischner, 2000). In the case of NO_3^- as N-source is well established that it is taken up by roots and its reduction occur in both roots and shoots of higher plants (Beevers, 1980). Reduction of NO_3^- to NH_4^+ , catalyzed by nitrate and nitrite reductase enzymes, together with subsequent assimilation of NH_4^+ is among the most energy-intensive processes in plants and in some cases is accompanied by additional respiration.

However, there are still active debates on the effect of the N source on root respiration, as attempts to explain it experimentally have led to arguable results supporting different hypotheses. Some authors suggest that, when compared to NO_3^- nutrition, NH_4^+ nutrition stimulates the rate of root respiration, attributing this increase to the stimulation of alternative pathway activity (Barneix et al. 1984; Blacquièrè 1987, Lasa et al., 2002). There are two pathways involved in respiration: the phosphorylating cytochrome and the nonphosphorylating alternative pathway. The physiological role of the latter is not clear but several authors suggest that this alternative pathway could avoid the overreduction of the electron transport chain and the subsequent production of reactive oxygen species (Purvis and Shewfelt 1993). Thus, this pathway could allow oxidation of TCA cycle reductant, maintaining TCA cycle carbon flow for provision of biosynthetic intermediates for NH_4^+ ion assimilation.

On the other hand, NO_3^- coming to the plant before assimilation have to be firstly reduced to NH_4^+ , and this process, together with assimilation, is among the most energy-intensive processes in plants, in some cases followed by an additional CO_2 evolution (Atkins et al., 1979; Aslam and Huffacker, 1982; Ninomyia and Sato, 1984; Warner and Kleinhofs, 1992; Blacquièrè, 1987; Tischner, 2000). The process proceeds in two steps: conversion of NO_3^- to NO_2^- and the following conversion of NO_2^- to NH_4^+ . In illuminated leaves, both processes are coupled to photosynthetic electron transport. However, in roots and during darkness, reducing equivalents are generated by

oxidation of carbohydrates with subsequent evolution of CO₂ (Aslam and Huffacker, 1982; Ninomyia and Sato, 1984).

Depending on the species, the site of NO₃⁻ reduction could be located in shoots or roots (Andrews, 1986; Oaks and Hirel, 1985; Pate and Layzell, 1990; Schilling et al., 2006; Silveira et al., 2001; Vuylsteker et al., 1997). By this property, plants are divided into three groups: species reducing NO₃⁻ predominantly in roots, species reducing NO₃⁻ predominantly in shoots, and those that do both. The C costs for reduction of NO₃⁻ to NH₄⁺ depend on the site of nitrate reduction in plants.

Nevertheless, the contribution of roots and shoots to whole plant nitrate reduction remains also uncertain. The location of nitrate reduction site seems to be even not species- and cultivars-dependant (Schilling et al., 2006; Silveria et al., 2001), but could vary within a single plant. Previous studies confirm that environmental conditions, plant growing stage and the quantity of N supply could influence and change the reduction site location within a plant, although the major part of these findings were done in nutrient solution studies, which do not reflect the real plant uptake rates, root and microbial respiration under true soil conditions. It was found that NO₃⁻ translocation from the root to the shoot depends on the N concentration in soil solution. At low NO₃⁻ concentration the reduction occurs mainly in roots, at higher concentrations storage and then transport is normally adjusted (Atkins et al. 1979, 1980; Oscarson & Larsson 1986; Agrell, et al., 1997). The nitrate uptake and reductase capacity is not equally distributed along a root axis and not identical in roots of different age and ontogeny (Lazof et al., 1994; Cruz et al., 1995; Di Laurenzio et al., 1996). Siebrecht, et al. (1995) and Pan et al. (1985) located a high uptake rate together with a highest reductase activity in the root apical region. Older root parts were more active in root uptake, but nitrate reductase was low, indicating a possible NO₃⁻ translocation from these root parts to the shoots. Gojon et al. (1986) have found that roots participate actively in the reduction of incoming nitrates during induction process, shortly after the fertilization, after that the root contribution decrease sufficiently.

Based on these results, obtained mainly from the experiments in nutrient solutions, we hypothesized that the form of N nutrition may have a significant effect on the amount of CO₂ released by root respiration under true soil conditions. Additionally, we expected that the effect of the form of N nutrition may change between the species with different location of nitrate reduction site and during the growth of a single plant.

Summarizing the above findings and uncertainties, the aims of the study were: a) to evaluate the effect of N form (NO₃⁻ vs. NH₄⁺) on CO₂ efflux from soil and root respiratory losses for species with different location of nitrate reduction site b) to verify if the relative contribution of roots to the whole plant nitrate reduction process remains stable during various growing stages of a single

specie, c) an additional aim was to investigate these processes under soil conditions and not in nutrient solution.

We selected corn and lupine since the two species have different sites of nitrate reduction: *Zea mays* reduces half of the NO_3^- in shoots and half in roots and *Lupinus albus* reduces the major part of the NO_3^- in roots (Pate, 1973). Plants were placed in $^{14}\text{CO}_2$ atmosphere shortly after N addition to separate the effects of N form on recently assimilated ^{14}C from the respired C, assimilated days earlier. Additionally, we used soil with and without plants as control for possible effects of N form on CO_2 from microbial decomposition of SOM. Corn was labeled twice: on the sixth and eighth leaf collar stage.

The difference between total CO_2 efflux from the plant-soil system and microbial respiration from bare soil incubated at the same conditions was compared with the results of the principal method of labeling for root-derived CO_2 quantification.

5.2. Materials and methods

5.2.1. Soil

The soil, a loamy Haplic Luvisol, was taken from the top 10 cm (Ap horizon) of the Karlshof long-term field experimental station of the University of Hohenheim. Soil samples were air dried, mixed and passed through 5 mm sieve. The soil contained 1.5% C_{tot} and 0.14% N_{tot} , 25 $\mu\text{g g}^{-1}$ N_{min} , 94 $\mu\text{g g}^{-1}$ available K, 16 $\mu\text{g g}^{-1}$ available P, with 2.9% sand, 74.5% silt and 22.6% clay; pH 6.5.

5.2.2. Plants and growth conditions

50 ml polyvinyl chloride (PVC) pots were filled with 50 g of soil each and were used for the plants growing. Twelve pots remained unplanted to measure microbial respiration from bare soil.

Seeds of corn (*Zea mays* L.) and lupine (*Lupinus albus* L.) were germinated in Petri dishes on moist filter paper for 2 days. Germinated seedlings were transplanted to the PVC pots, with one seedling per pot, and were grown under controlled laboratory conditions with a 12h/12h day/night period at a constant day and night temperature of $25 \pm 0.5^\circ\text{C}$, photosynthetically active radiation (PAR) intensity was approximately $800 \mu\text{mol m}^{-2}\text{s}^{-1}$ at the top of the plant canopy. A constant day/night temperature was chosen to avoid the effects of changing temperature on CO_2 fluxes. During the experiment, soil water content in each pot was maintained gravimetrically at about 60% of its holding capacity by checking its weight daily. Before the labeling, the weakest plants were eliminated and only thirty six plants (24 of corn and 12 of lupine), similar in development and height were chosen for the following treatments. Pots with bare soil were incubated at the same conditions.

5.2.3. ^{14}C labeling and ^{15}N application

Corn plants were divided into two groups with twelve plants in each. The first group of plants was labeled with $^{14}\text{CO}_2$ on the sixth leaf collar stage (V6 corn), the second group was labeled on the eighth leaf collar stage (V8 corn). Preliminary studies showed that about 2 weeks after germination, corn starts to increase its biomass linearly. So, the uptake of water and nutrients, inclusive N, on the mentioned stages is nearly linear and therefore, the results can be extrapolated for longer periods. Twelve plants of lupine were labeled on the 36th day after germination, the eleventh leaf collar stage (V6).

One day before the respective labeling, the top of each pot was sealed with a silicone paste (NG 3170 from Thauer & Co., Dresden, Germany). The seal was tested for air leaks. Pumping the air through the soil column before labeling flushed out the CO_2 accumulated in the soil during the plant's growth.

Three N treatments were applied four hours before each ^{14}C labeling: (a) a control treatment without any added N, (b) an ammonium treatment, with ^{15}N as $(^{15}\text{NH}_4)_2\text{SO}_4$ and (c) a nitrate treatment, with ^{15}N as K^{15}NO_3 . Four plants of each specie and growing stage were exposed to each N treatment. Dicyandiamide (DCD) at 20 mg kg^{-1} soil was applied in solution with ^{15}N fertilizer to all treatments in order to achieve an effective nitrification inhibition throughout the soil column (in the ammonium treatment) and to balance the side effects of the inhibitor (in the nitrate and control treatments). The amount of ^{15}N applied to a pot was calculated to produce an average concentration of $60 \text{ mg of N kg}^{-1}$ soil for each N species added. Four unplanted pots were fertilized with half amount of nitrate or ammonium to estimate the effect of N fertilization on respiration of soil microorganisms.

The ^{14}C labeling process has been described in detail by Kuzyakov et al. (1999, 2001) and Domanski et al. (2001). Briefly, sealed pots with plants were put in a plexiglas chamber, $^{14}\text{CO}_2$ was introduced to the chamber by adding 1 mL of 5 M H_2SO_4 to a $\text{Na}_2^{14}\text{CO}_3$ (1.5 MBq) solution. This allowed complete evolution of $^{14}\text{CO}_2$ into the chamber atmosphere. After a 2h-labeling period, trapping of CO_2 from the chamber through 10 mL of 1 M NaOH solution was started to remove the remaining unassimilated $^{14}\text{CO}_2$. When the chamber was opened, pots with the plants were connected by tubes to an output of membrane pumps: air was pumped through every single pot from bottom to top. Another tube was connecting each pot to a CO_2 trapping tube, filled with 3 mL of 1 M sodium hydroxide (NaOH) solution. The output of the trapping tube was connected to the input of the membrane pump. Therefore, the air containing CO_2 evolved from the soil respiration was circulating in a closed system: from the plant-soil system to the trapping solution to the membrane pump and back to the plant-soil system.

5.2.4. *Sampling and analyses*

NaOH in the trapping tubes was changed twice a day, in the morning and in the evening, for six days after the labeling, aiming to collect the CO₂ evolved in the rhizosphere during day- and night-periods separately. NaOH traps were analyzed for total trapped CO₂ and for ¹⁴C activity (only for planted pots).

The ¹⁴C activity was measured in 1 mL aliquots of NaOH with 2 mL of the scintillation cocktail EcoLite⁺ (ICN) after the decay of chemiluminescence by a liquid scintillation counter (MicroBeta, TriLux). Total assimilated ¹⁴CO₂ was determined as a difference between the ¹⁴CO₂ added to the labeling chamber and the ¹⁴CO₂ recovered from the solution with the remaining unassimilated ¹⁴CO₂.

To estimate total CO₂ efflux from the soil with and without plants, CO₂ trapped in NaOH solution was precipitated with a 0.5 M barium chloride (BaCl₂) solution and then NaOH was titrated with 0.1 M hydrochloric acid (HCl) against phenolphthalein indicator (Zibilske, 1994).

On the sixth day after each labeling, all the plants were harvested: each shoot was cut at the base, the lid of the pot was opened, and each root-soil column was pulled out of the pot. Roots were carefully washed with deionized water to remove soil particles. Shoots and roots were dried at 70 °C, weighed and grounded with ball mill (Fa Retsch) for analysis of C_{tot}, N_{tot}, and ¹⁵N content. Three g of soil were taken from each soil sample, dried at 70 °C and grounded for the same purposes. Total C and total N and the isotope ratio ¹⁵N/¹⁴N in plant and soil samples were determined using Carlo Erba NA 1500 gas chromatograph (Carlo Erba Instruments, Milano, Italy) coupled on isotope ratio mass spectrometer (Delta plus IRMS 251, Finnigan Mat, Bremen, Germany).

5.2.5. *Statistics*

The experiment was conducted with four replicates. All replicates were analyzed for ¹⁴C, ¹⁵N, C_{tot}- and N_{tot}-contents in shoots and roots. ¹⁴C data are presented as the percentage of ¹⁴C assimilated during exposure of plants to the pulse labeling. All data were analyzed with SYSTAT 11.0 (SPSS Inc.). Effects of different N treatment (no N, NH₄⁺-N and NO₃⁻-N), plant growing stage and sampling time (day and night) were tested using two-way analysis of variance (ANOVA). We have calculated the least significant difference (LSD 0.05) in a post hoc Newman–Keuls test to identify significant differences between treatments.

5.3. Results

5.3.1. Aboveground and belowground plant biomass

No significant differences were observed in aboveground biomass of *Zea mays* and *Lupinus albus* between N treatments. An average increase of AG biomass with time between the first and second labeling of corn plants amounted for 30% (Fig. 1).

Different N fertilizers significantly affected ($p < 0.01$) root biomass of V6 corn with a lowest value being 0.25 g pot^{-1} for NH_4^+ -N fertilized plants and the highest being 0.39 g pot^{-1} for the control (Fig. 1). On the contrary, there was no difference in root biomass between N treatments for V8 plants. Positive effect of plant age on the root biomass was observed for plants fertilized with N. In control without N fertilization no effect of time on the root biomass was registered. No significant differences in root biomass between treatments ($p = 0.1$) was observed for lupine plants.

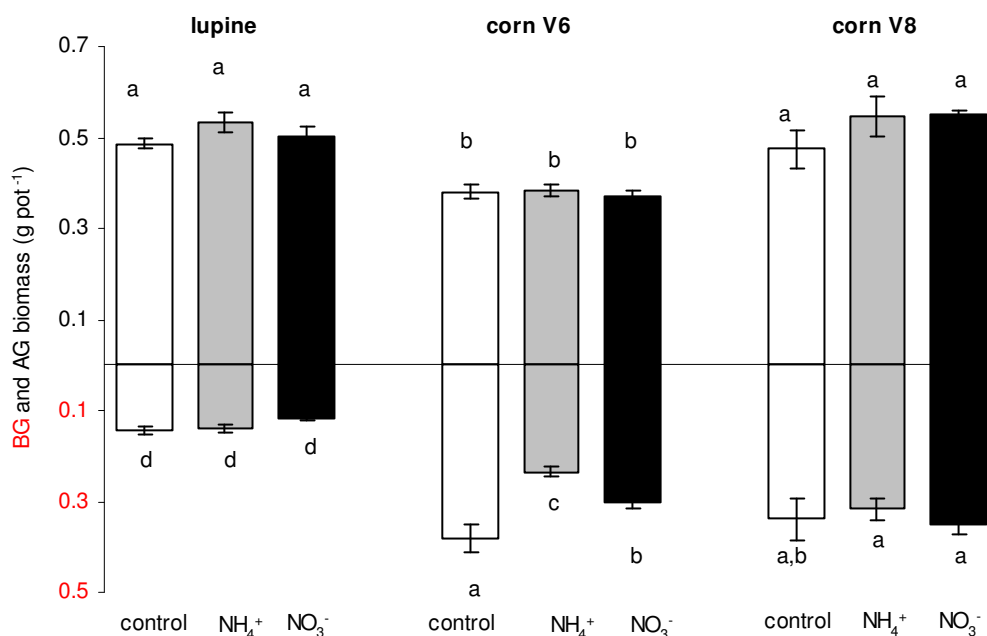


Fig. 1 Aboveground (AG, above zero) and belowground (BG, below zero. In red) biomass of *Lupinus albus* and V6 and V8 *Zea mays* under various N fertilization types ($\pm$ SE). Letters indicate the significance of the differences at $p = 0.05$ between the treatments.

5.3.2. Dynamics of ¹⁴CO₂ efflux from a soil compartment with *Lupinus albus* and *Zea mays*

***Lupinus albus*:** The maximum of isotopically enriched respiration was registered within the first six hours after the start of the labeling (Fig. 2, up). Soon after, the emission rate declined from the maximum levels of 3.4% for control, 5.8% for NH_4^+ -N, and 7.3% for NO_3^- -N of total assimilated $^{14}\text{C d}^{-1}$ to 0.9% C d^{-1} on the third day.

Rhizosphere respiration of recently assimilated C (^{14}C) from the soil in all N treatments showed clear diurnal dynamics. The diurnal dynamics of recently assimilated ^{14}C in respired CO_2 were strongly pronounced for non-fertilized plants. This is especially obvious after calculation of

the differences between N and control treatments. The maximum difference between control and soil with added N was found during the night periods and minimum values were found during the day (Fig. 1, up).

The difference between plants fertilized by NO_3^- -N and NH_4^+ -N in the quantity of ^{14}C respired, was highest during the first two days after the labeling. After two days already no significant differences between N treatments were measured (Fig. 1, up).

Cumulative ^{14}C respiration of roots and rhizosphere microorganisms during six days after the labeling reached 6.8% of assimilated ^{14}C in soil without N fertilization, 8.3% for the NH_4^+ -N treatment, and 9.3% for the NO_3^- -N treatment, and was significantly different ($p < 0.001$) between all N treatments.

The ^{14}C losses from the soil were recalculated per unit of root biomass (Fig. 3) measured six days after the labeling. The maximum of $^{14}\text{CO}_2$ emission was chosen for the comparison between the N treatments as it relates closer to the root respiration than the cumulative $^{14}\text{CO}_2$ efflux, which has a significant part of rhizomicrobial respiration (see below). Although there were no significant differences in root biomass between treatments ($p = 0.1$) (Fig. 1), strong effects of N fertilization on recently assimilated C in CO_2 were observed. Taking the control treatment without N as a 100% reference, the respiration losses of ^{14}C from the plant-soil system with lupine after six days amounted to 182% under NH_4^+ -N and 268% under NO_3^- -N ($p < 0.001$).

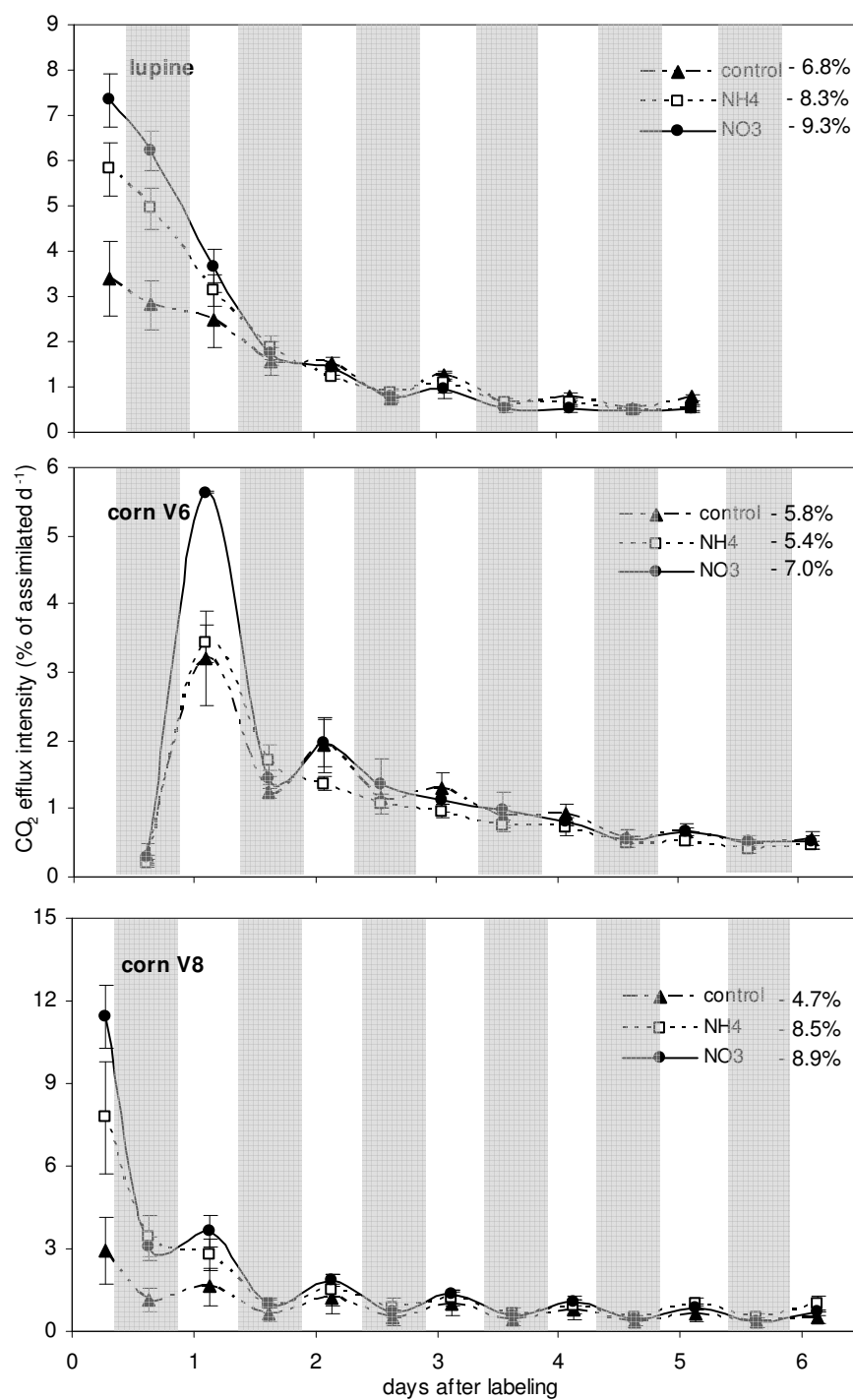


Fig. 2 Effect of N fertilization type on dynamics of $^{14}\text{CO}_2$ efflux rate from the soil ($\pm$ SE) planted with *Lupinus albus* (top) and *Zea mays*, labeled on the V6 (middle) and V8 (bottom) stage. Night periods are shown in grey, day – in white.

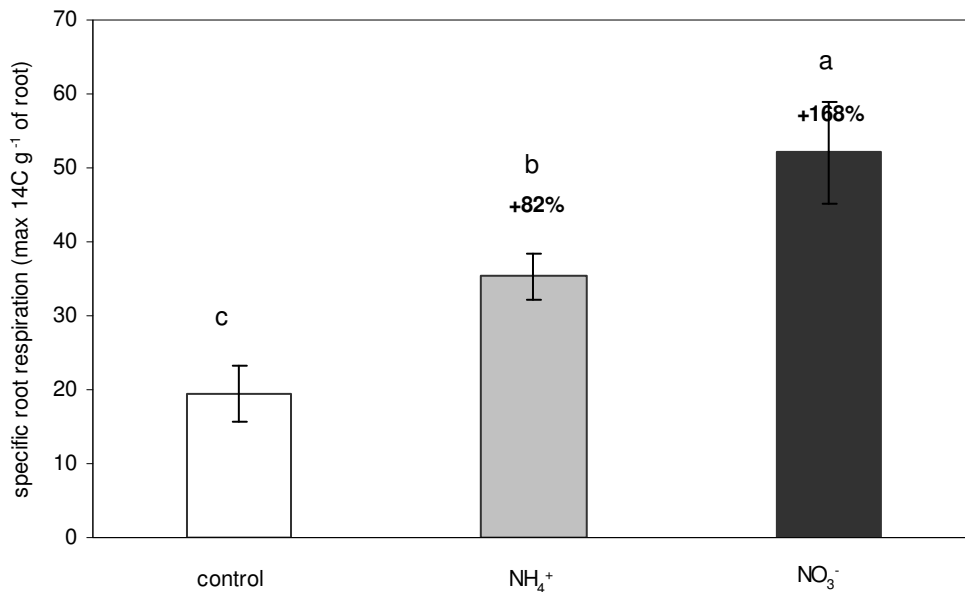


Fig.3 Intensity of $^{14}\text{CO}_2$ efflux (maximum efflux) respired from root-soil system with *Lupinus albus* per unit of root biomass ($\pm$ SE). Percentages indicate the increase of $^{14}\text{CO}_2$ efflux after NH_4^+ or NO_3^- fertilization as related to the $^{14}\text{CO}_2$ from control without N. Letters indicate the significance of the differences at $p=0.05$ between the treatments.

Zea Mays V6: For all three N treatments, the peak of $^{14}\text{CO}_2$ evolution from root-soil compartment was observed on the second day between 26 and 30 hours after $^{14}\text{CO}_2$ pulse labeling (Fig. 2, middle). The $^{14}\text{CO}_2$ emission rate declined rapidly from the maximum levels of $3.3\% \text{ d}^{-1}$ for NH_4^+ -N and control, and of $5.7\% \text{ d}^{-1}$ for NO_3^- -N of total assimilated ^{14}C , to the value of about $1\% \text{ d}^{-1}$ on the fourth day. A clear diurnal dynamic in rhizosphere respiration of recently assimilated C (^{14}C) was noticed (Fig. 2, middle).

The difference in $^{14}\text{CO}_2$ respired from soil with plants fertilized by NO_3^- -N and NH_4^+ -N was highest during the first days after the labeling. No difference was observed in the peak values of $^{14}\text{CO}_2$ from soils under NH_4^+ and control treatments ($p=0.77$).

Cumulative $^{14}\text{CO}_2$ respired by roots and rhizosphere microorganisms during the six days of the experiment reached 5.8% for the control, and 5.4% and 7% for the NH_4^+ -N and NO_3^- -N treatments respectively (Fig. 2, middle). The difference between the two types of applied N was significant ($p<0.001$), but no difference was observed between NH_4^+ -N and the control ($p=0.70$).

To consider possible effects of variation in root biomass, the maximal intensity of $^{14}\text{CO}_2$ losses from the soil were recalculated per unit of root biomass measured six days after the labeling (Fig. 4a). The maximum of $^{14}\text{CO}_2$ emission was used for the comparison between the N treatments. A strong effect of N fertilization on recently assimilated $^{14}\text{CO}_2$ was found ($p<0.001$). Significant difference was observed also between two types of N applied. Taking the control treatment without

N as a 100% reference, the respiration losses of ^{14}C from the plant-soil system with corn labeled on the V6 stage amounted to 175% under $\text{NH}_4^+\text{-N}$ and 220% under $\text{NO}_3^-\text{-N}$.

Zea Mays V8: In contrast with V6 stage, the maximum of $^{14}\text{CO}_2$ efflux after the second labeling was registered within the first day, 8 hours after the start of the labeling. The peak of $^{14}\text{CO}_2$ efflux appeared before the first sampling (Fig. 2, bottom). Soon after, the emission rate declined from the maximum levels of 3% d^{-1} for control, 7.8% d^{-1} for $\text{NH}_4^+\text{-N}$, and 11.9% d^{-1} for $\text{NO}_3^-\text{-N}$ of total assimilated ^{14}C to 1.2 % C d^{-1} on the third day.

$^{14}\text{CO}_2$ efflux from the soil in all N treatments showed clear diurnal dynamics with an increase of the $^{14}\text{CO}_2$ respiration during the day and decrease at night time.

Similarly to V6 corn, the difference in the quantity of ^{14}C respired from plants under $\text{NO}_3^-\text{-N}$ and $\text{NH}_4^+\text{-N}$ was highest during the first two days after the labeling. Cumulative ^{14}C evolved from roots and rhizosphere microorganisms during six days after the labeling amounted for 4.7% of ^{14}C input in soil without N fertilization, 8.5% for the $\text{NH}_4^+\text{-N}$ treatment, and 8.9% for the $\text{NO}_3^-\text{-N}$ treatment (Fig. 2b).

The ratio between maximum of $^{14}\text{CO}_2$ efflux and root biomass demonstrated no difference between two types of N applied (Fig. 4a). Losses of ^{14}C from the plant-soil system with V8 corn, taking control as a 100% reference reached 281% under the $\text{NH}_4^+\text{-N}$ treatment and 373% under the $\text{NO}_3^-\text{-N}$ treatment ($p < 0.01$). V8 growth stage increased the quantity of respired ^{14}C in confront with the younger plants (Fig. 4a).

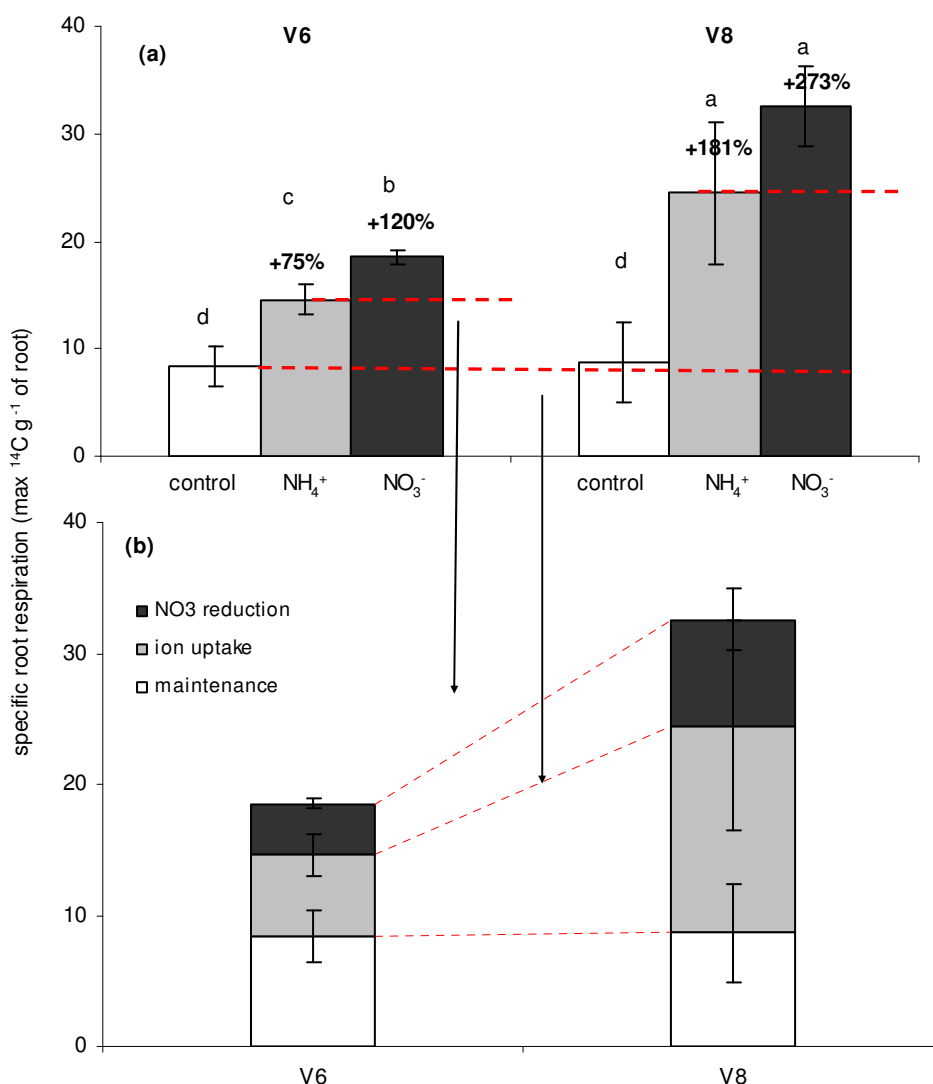


Fig. 4 a) Intensity of $^{14}\text{CO}_2$ efflux (maximum efflux) respired from root-soil system with V6 and V8 *Zea mays* per unit of root biomass. Percentages indicate the increase of $^{14}\text{CO}_2$ efflux after NH_4^+ or NO_3^- fertilization as related to the $^{14}\text{CO}_2$ from control without N; **b)** Increase in respiration rates (maximum efflux per unit of root biomass) associated with maintenance, ion uptake and NO_3^- reduction on two growing stages of corn. Letters indicate the significance of the differences at $p=0.05$ between the treatments.

5.3.3. ^{15}N uptake by plants

***Lupinus albus*:** Significantly more ^{15}N was recovered from shoots and roots of lupine plants under NH_4^+-N ($p<0.001$) (Fig. 5). The major part of the ^{15}N remained in roots (58% for NH_4^+-N and 64% for NO_3^--N).

***Zea mays*:** Corn took up twice as much ^{15}N as the lupine. On both growth stages significantly more ^{15}N was recovered from shoots and roots of plants fertilized with NH_4^+-N (Fig. 5). V8 stage significantly decreased the quantity of ^{15}N recovered in shoots and roots in confront with V6 plants. Total quantity of ^{15}N incorporated in shoots and roots at V6 was $0.71 \text{ mmol g}^{-1} \text{ dw}$ under NH_4^+-N and 0.45 under NO_3^--N . At growth stage V8 only $0.43 \text{ mmol g}^{-1} \text{ dw}$ of ^{15}N was recovered under NH_4^+ treatment and $0.34 \text{ mmol g}^{-1} \text{ dw}$ under NO_3^- .

The distribution of N between shoots and roots also varied depending on the growth stage: under $\text{NH}_4^+\text{-N}$ 72% of the ^{15}N was recovered in shoots of V6 corn, on V8 stage only 58% of the found ^{15}N had a shoot origin. Under $\text{NO}_3^-\text{-N}$ the difference between two growing stages in distribution of absorbed ^{15}N was not so clear: 67% and 62% of recovered ^{15}N respectively for V6 and V8 plants had a shoot origin (Fig. 5).

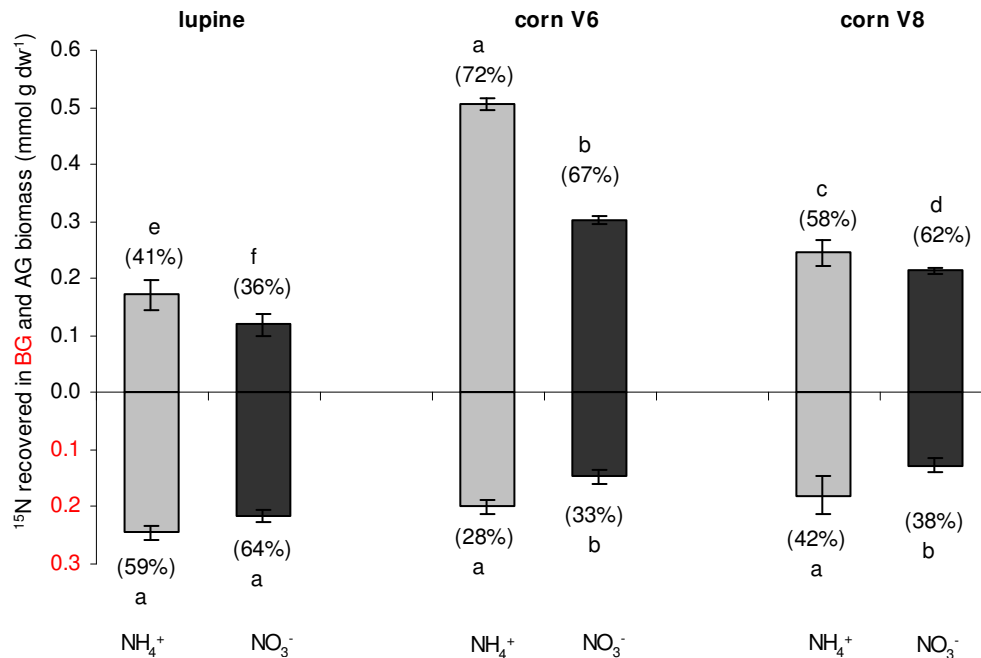


Fig. 5. ^{15}N amount recovered from shoot (above zero) and roots (below zero. In red) of *Lupinus albus* and *Zea mays* on V6 and V8 growing stage. Percentages indicate ^{15}N distribution between shoots and roots of plants. Letters indicate the significance of the differences at $p=0.05$ between the treatments.

5.3.4. Total CO_2 efflux from planted soil with *Lupinus albus* and *Zea mays* (V6)

The difference between total CO_2 efflux from the root-soil system and microbial respiration from bare soil incubated at the same conditions was used to calculate the CO_2 respired by roots and associated rhizosphere microorganisms (root-derived respiration) and to compare with the results from ^{14}C labeling for root-derived CO_2 .

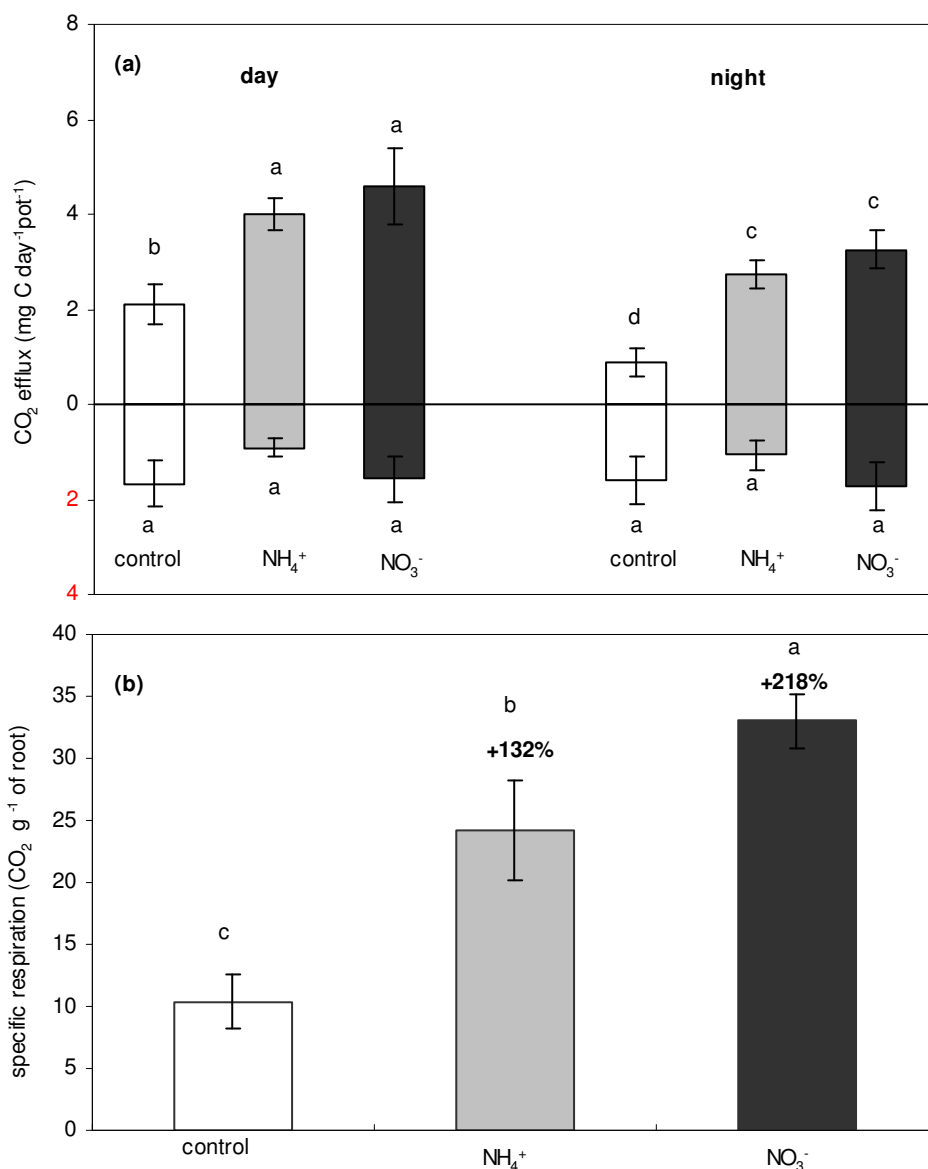


Fig. 6 a) values above zero: root-derived CO₂ from root-soil system with *Lupinus albus* (as a difference between total and microbial respiration from bulk soil), values below zero (in red): microbial CO₂ efflux from the bare by three N treatments: averages for day and night periods **b)** Root-derived respiration (as a difference between total and microbial respiration) respired from root-soil system with *Lupinus albus* per unit of root biomass. Percentages indicate the increase of CO₂ efflux after NH₄⁺ or NO₃⁻ fertilization as related to the CO₂ from control without N. Letters indicate the significance of the differences at p=0.05 between the treatments.

Lupinus albus: Total and root-derived CO₂ efflux from the soil in all planted treatments showed a clear diurnal dynamic (Fig 6a). Average total CO₂ respired from the plant-soil system was lowest for the control (3.13 mg C d⁻¹ pot⁻¹), and amounted to 4.36 mg C d⁻¹ pot⁻¹ for NH₄⁺-N and 5.58 mg C d⁻¹ pot⁻¹ for NO₃⁻-N. The difference in total CO₂ respired from soils with different types of N applied was significant during the whole measurement period (p<0.05). On the average, the largest root-derived respiration during the day and during the night was observed under NO₃⁻-N even if the difference with NH₄⁺-N was not significant (Fig. 6a).

CO₂ efflux from unplanted soil had no diurnal changes and the difference between N treatments was not significant (p=0.74) (Fig. 6a).

The value of root-derived CO₂, calculated as a difference between total CO₂ efflux from soil with roots and microbial respiration from bare soil, was recalculated per units of root biomass and presented as a percent of the control (Fig. 6b): root-derived CO₂ from the plant-soil system with lupine was found to be 233% for the NH₄⁺-N treatment and 318% for the NO₃⁻-N treatment (p<0,001).

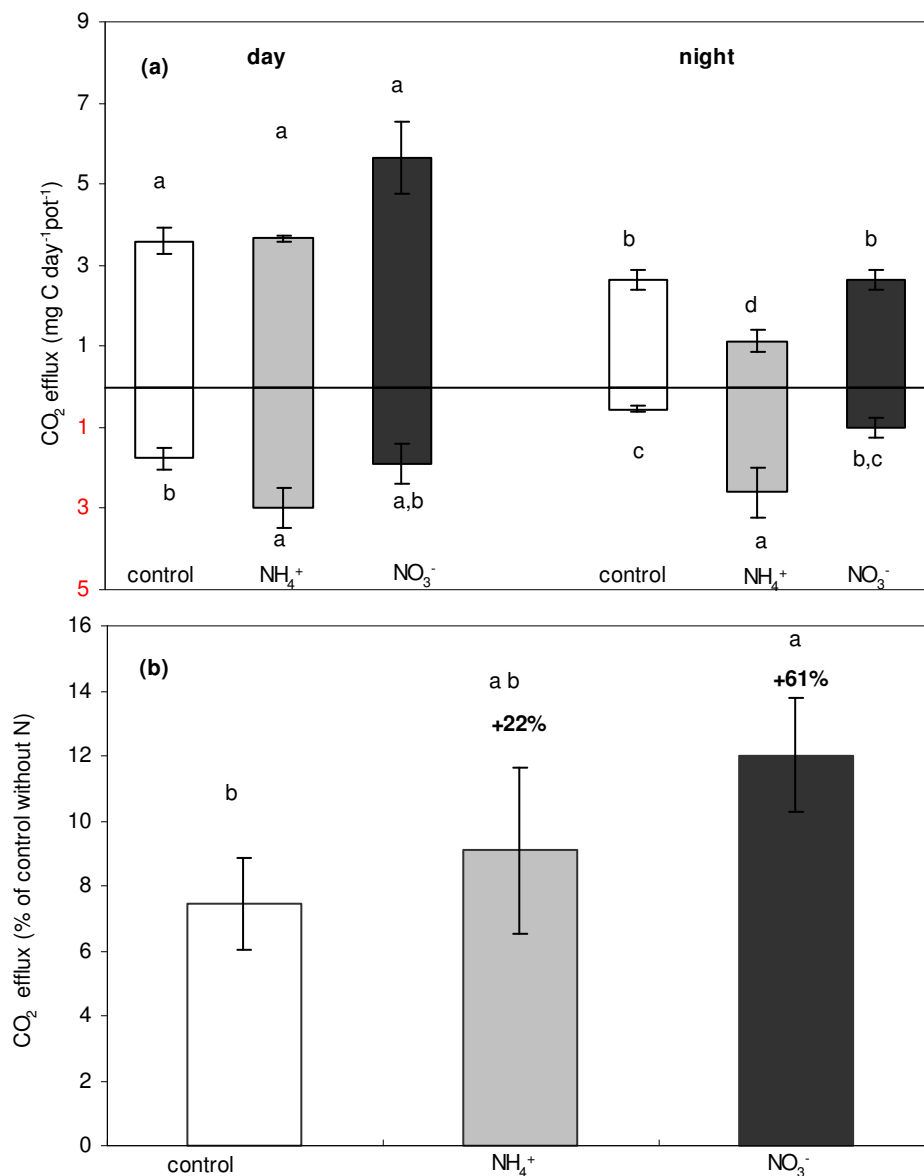


Fig.7 a) values above zero: root-derived CO₂ from root-soil system with *Zea mays* (V6) (as a difference between total and microbial respiration from bulk soil), values below zero (in red): microbial CO₂ efflux from the bare soil by three N treatments: averages for day and night periods **b)** Root-derived respiration (as a difference between total and microbial respiration) respired *Zea mays* (V6) per unit of root biomass. Percentages indicate the increase of CO₂ efflux after NH₄⁺ or NO₃⁻ fertilization as related to the CO₂ from control without N. Letters indicate the significance of the differences at p=0.05 between the treatments.

Zea mays: A clear diurnal dynamic of total and root-derived CO₂ from the soil for all N treatments was observed also for the plant-soil system with maize (Fig. 7a). Average total CO₂ respired from the plant-soil system was lowest for the control (3.94 mg C d⁻¹ pot⁻¹), while under NH₄⁺-N, the efflux rate was 4.79 mg C d⁻¹ pot⁻¹ and under NO₃⁻-N, it was 5.31 mg C d⁻¹ pot⁻¹. However, the difference between the two N treatments was not significant (p>0.05). The largest average root-derived respiration in the day and in the night was observed under nitrate N (p<0.05) (Fig.7a).

Relative to the control, the losses of CO₂ from the plant-soil system were 122% under the NH₄⁺-N treatment and 164% under the NO₃⁻-N treatment (Fig. 7b). However the difference between the two N treatments was not found to be significant.

5.4. Discussion

5.4.1. Root-derived CO₂ – comparison of two methods

Two methods for estimating root-derived CO₂ efflux were used in this study: 1) pulse labeling in ¹⁴C atmosphere with subsequent tracing of recently assimilated ¹⁴CO₂ from soil and 2) comparison between the CO₂ efflux from soil with plants and that from bare soil, the difference being accepted here as equal to the contribution of plant roots to the total CO₂ efflux.

In our experiment, both methods showed very similar results, with plants grown under NO₃⁻-N respiring more C than those grown under NH₄⁺-N: for lupine the method based on ¹⁴C gave an 47% increase in respiration for NO₃⁻-N relative to NH₄⁺-N while the second method gave an 37% increase (Fig. 2, up and Fig. 6b). For V6 corn, both methods were also similar in magnitude: the ¹⁴C method giving a 27% difference between the two N fertilizer types and the second method giving 33% (Fig. 2, middle and 7b)

The suitability of ¹⁴C or ¹³C labeling and the following tracing of recently assimilated C in order to quantify root-originated CO₂ has been confirmed by many studies (Rygiewicz and Andersen, 1994, Kuzyakov et al., 2001; Kuzyakov, 2006, Wang et al., 2005; Carbone and Trumbore, 2007). Labeling of plants is one of few approaches which potentially permit estimation of root-originated respiration minimizing the soil disturbance.

The second approach, comparing planted and unplanted soil, is a cheap and simple one, but it gives only a crude estimate of root-derived CO₂ and SOM-derived CO₂ from planted soil (Kuzyakov, 2006). Possible errors in the method come from the fact that it does not consider possible interactions between growing roots and SOM decomposition (Cheng et al., 2003), the so-called rhizosphere priming effects (Kuzyakov, 2002). The cycling of nutrients, the water regime, and temperature balance in the planted soil are also different from that in the unplanted soil (Fisher

and Gosz, 1986; Ross et al., 2001; Paterson, 2003; Cheng and Kuzyakov, 2005). Additionally, it does not allow separating the rhizomicrobial respiration, associated with microbial decomposition of rhizodeposits and dead roots from the root respiration, which can be estimated using pulse labeling in the form of the first CO₂ evolved after the pulse, assuming the temporal difference between the CO₂ evolved from different sources.

In our study, we did not use the absolute values of ¹⁴CO₂ and unlabeled CO₂, but instead related them to the changes in root-derived CO₂ induced by the change in the form of N fertilization. Therefore, despite their mentioned differences, both approaches for estimating the root-derived CO₂ showed similar results.

5.4.2. ¹⁴C-CO₂ efflux from soil

We have found the maximum ¹⁴CO₂ efflux from soil within 26-30 hours after the labeling for V6 corn and within the first 6 hours for V8 corn and lupine. This difference in the time lag between C assimilation and its following respiration through the rooting system in two growth stages could influence the quantity of ¹⁴C, which was involved to sustain the NO₃⁻-N reduction process. That's why it is important to specify what factors were responsible for the observed difference in the lags between C assimilation and root respiration.

Many studies confirm that assimilation of CO₂ and the downward transport of C in plants, as well as the utilization of assimilated C by root respiration, are very rapid processes (Cheng et al., 1993; Gregory and Atwell, 1991; Kuzyakov et al., 1999, 2001, 2002; Nguyen et al., 1999; Swinnen et al., 1994). The time lag between photosynthetic CO₂ uptake and the ensuing release of C through root respiration varies among studies from minutes to days. For example, Kuzyakov et al. (2001) found the first CO₂ evolution from soil with *Lolium perenne* within the first four hours after labeling while Cheng et al. (1993) found the beginning of emission of CO₂ from winter wheat and rye to occur within the first 30 minutes. Field studies usually report lags higher than found in the laboratory; Tang et al. (2005) found evidence for time lags from 7-12 hours up to 5-6 days, Horwath et al. (1994) reported a lag of 2-3 days for tree-soil systems.

Physical factors which could influence the soil CO₂ production and diffusion rate (Tang et al., 2003; Carbone & Trumbore 2007) were equal for lupine and on both growing stages of corn: plant growing conditions (soil water content, temperature, PAR) were uniform and the effect of the soil air vertical flow through the soil column could be negligible as we continuously pumped the air from the bottom to the top of the pot. So, we can conclude that species – specific and growth-stage-specific difference in the transport rates of assimilates are responsible for the observed difference in lags between C assimilation and utilization of this C by roots for respiration. The difference in path length can not explain such changes in the time lag as the shoots height was similar among all the

plants. The growth stage could control the metabolic orientation of plants, influencing source (photosynthetically active leaves, which supply a new C) - sink (developing organs of plants, which compete for the new C) interactions (Farrar & Jones, 2000; Carbone & Trumbore, 2007). The flow of C to sinks depends on the strength of the sink, the sink size, and the growth rate (Dickson, 1991; Farrar & Jones, 2000).

The earlier evolution of CO₂ from the soil corresponded to the later growing stage of plants of corn and lupine. The major energetic costs belowground are the growth of new roots and maintenance of existing one (Dobrowolski et al., 1990). We have observed a significant difference in the root biomass between two growing stages of corn. Intensively growing roots and higher belowground biomass may accelerate downward transport of assimilates to them in the case of V8 corn, on the contrary developing shoot cells could have preference over roots in the competition for recently assimilated C in the case of younger corn plants. The same could be true for lupine plants, however, the difference in the N acquisition strategy between lupine and maize makes the comparison between these species particularly complex. As an example other studies showed that symbiosis of lupine with rhizobacteria increases the C sink and so may accelerate downward transport of assimilates compared to non legume plants like maize (Layzell, et al., 1979; Minchin et al., 1981), however the nodulation of lupine was not quantified here.

Since, we can't specify the intensity of the N uptake process in different time of the chase period we assume that N was taken up uniformly during the first days after pulse labeling, when the maximum of the ¹⁴C efflux occurred for both growing stages of corn.

Diurnal changes in ¹⁴CO₂ and total efflux from planted soil were observed for both species and all N treatments (Fig. 2). In our experiment, plants were grown at a single constant temperature during both day and night. As CO₂ efflux from unplanted soil was independent of a day/night changes (Fig. 6a and 7a), the daytime increase in ¹⁴C evolution is attributed to the assimilation of C by photosynthesis and the ensuing rapid translocation to roots, with an associated signal in the root-derived CO₂. This observation was confirmed also by Kuzyakov and Cheng (2001).

5.4.3. *NH₄⁺ versus NO₃⁻ supply – effect on the root respiration*

Some studies confirmed that in non-green root cells and in darkness, the process of nitrate reduction is supplied by reducing equivalents from the degradation of carbohydrates with an additional CO₂ production (Aslam & Huffacker, 1982; Ninomyia & Sato, 1984), whereas the same process performed in leaves during the day is coupled directly with photosynthetic electron transport (Aslam & Huffacker, 1982; Atkins et al., 1979; Warner & Kleinhofs, 1992) without extra losses of C as CO₂. Following these observations, we expected to observe differences in the

quantity of recently assimilated $^{14}\text{CO}_2$ respired by *Lupinus albus* and *Zea mays* under NO_3^- -N or NH_4^+ -N nutrition.

For both plant species and on both growth stages of corn, plants grown under NO_3^- -N respired significantly more $^{14}\text{CO}_2$ compared to NH_4^+ -N nutrition. As no effect of N form was observed on the respiration from unplanted soil we can conclude that the form of N (NO_3^- or NH_4^+) affected mainly root-derived respiration and not SOM-derived one. But, a major question in this work is whether the differences in respiration reflect actual root respiration rather than exudation and microbial respiration in the rhizosphere. It's clear that N form affects respired C, but both plants and microbes have to reduce NO_3^- to NH_4^+ before assimilating it, and the C costs might be similar, making it difficult to simply conclude that any extra C respired has a root-origin. Thus, while we cannot establish the answer to the question definitively, we believe that the time course of respiration, as viewed in the light of previous studies, strongly suggests the observed differences are due to changes in root respiration rather than microbial one. After Kuzyakov et al. (1999) and Kuzyakov&Domanski (2002) the $^{14}\text{CO}_2$ efflux after pulse labeling originating from different sources appears at different time after the labeling: $^{14}\text{CO}_2$ from root respiration occurs earlier than $^{14}\text{CO}_2$ from microbial respiration by decomposition of root exudates because the latter consists of a chain of successive processes: exudation from the root, intake by microorganisms, and only then respiration by microorganisms. It was shown on *Lolium perenne* that the actual root respiration affects the $^{14}\text{CO}_2$ efflux curve only during the first 24 hours after pulse labeling and the maximum effect of exudation on rhizomicrobial respiration predominates in the $^{14}\text{CO}_2$ efflux only after about one – two days after the pulse labeling (Kuzyakov et al., 2001; Kuzyakov&Domanski, 2002). In our study, for both plant species, N treatment affected $^{14}\text{CO}_2$ efflux most during periods when the dominant source of $^{14}\text{CO}_2$ was likely root respiration. Therefore, the results are consistent with N form having a significant effect on respiratory costs of plants associated with N assimilation.

Additionally, the results of the ^{15}N analyses in shoots and roots demonstrated that lupine and corn at both development stages took up more ^{15}N under NH_4^+ -N than under NO_3^- -N, making the difference between two types of N applied in the quantity of the respired C even more clear.

Summarizing the above findings, plant nutrition with nitrates increases the autotrophic component of soil CO_2 efflux compared to nutrition with ammonia. We suggest, interpreting the data of CO_2 efflux from soil and particularly separating estimation of individual CO_2 sources which contribute to the total soil CO_2 efflux to take into consideration the form of mineral N in soil. So, in well aerated, coarse and medium textured soil (sandy and loamy soils) any N form will be quickly converted to nitrate. The uptake of nitrate by roots will be followed by an additional evolution of the CO_2 through root respiration due to extra energy requirements for nitrate reduction and assimilation. Urea and ammonium in such soils are supposed to be rapidly converted into

nitrate. At temperatures of 15 - 20°C the conversion of ammonia to nitrate takes less than 1-2 weeks, so irrespective of the type of N fertilizer applied, plants absorb by far most of their N as nitrate. The same plant species will respire much less CO₂ growing on fine textured soils or water saturated soils. The most of mineral N will be taken up in the form of ammonium because under anaerobic conditions the soil bacteria are unable to perform the nitrification. In this case the CO₂ efflux originated from root respiration will be smaller.

5.4.4. Carbon costs of nitrate reduction – comparison between species and different N supplies

We chose two species with different sites of nitrate reduction. According to Pate (1973), *Lupinus albus* reduces the major part of incoming nitrate in roots. On the contrary, *Zea mays* reduce only half of the nitrate in roots and the other half translocates to the shoots for reduction. This difference in the reduction site could lead to differences in the quantity of CO₂ respired per unit of N absorbed, given that in non-green root cells and in darkness, the process of nitrate reduction is supplied by reducing equivalents from the degradation of carbohydrates with an additional CO₂ production (Aslam and Huffacker, 1982; Ninomyia and Sato, 1984), whereas the same process performed in leaves during the day is coupled directly with photosynthetic electron transport (Aslam and Huffacker, 1982; Atkins et al., 1979; Warner and Kleinhofs, 1992) without additional CO₂ evolution.

Between species comparisons demonstrated a significant difference in the amount of CO₂ evolved under NO₃⁻-N and NH₄⁺-N supply: the increase in respired ¹⁴CO₂ for NO₃⁻-N relative to that for NH₄⁺-N was 47%, 27% and 32% for lupine, V6 and V8 corn, respectively. Microbes also pay the cost for assimilation of NO₃⁻-N and might cause an increase in ¹⁴C respired as was mentioned before. But, the effect would be the same across plant species and so we consider the observed variation in respired ¹⁴C to be determined by plant physiology. A higher difference between the two N fertilizers in the case of lupine could be explained as lupine is referred to the plants reducing the major quantity of nitrates in roots, resulting in an enhanced demand for reducing equivalents of the carbohydrates degradation-origin with a subsequent evolution of CO₂. The result achieved by ¹⁴C pulse labeling was supported also by an independent method based on the measurements of unlabeled total CO₂ respired from planted and unplanted soil, although between species variation was not so pronounced. The relative difference in the respired ¹⁴CO₂ between the two types of N applied amounted for 37% for lupine and 33% for corn on V6 stage (Fig. 6b and 7b).

5.4.5. *Effect of growing stage on root respiration*

Two growing stages of corn differed in ^{15}N content, its distribution between shoots and roots and the $^{14}\text{CO}_2$ respiration rates also varied between stages and different N treatments.

The $^{14}\text{CO}_2$ evolved per unit of root mass was greater on V8 stage for both types of N applied. The absence of increment in root biomass and in peak of respired $^{14}\text{CO}_2$ for control treatment in time (Fig. 1; Fig. 2 middle and bottom) indicate that the main respiratory costs were associated with maintenance of the existing plant material, rather than growing of the new plant structures. After the fertilization of corn with N, respiration associated with ion uptake was appended to these basic maintenance costs. In fact, the transport of NO_3^- , NH_4^+ , K^+ , H_2PO_4^- , SO_4^{2-} and Cl^- into root cells is facilitated by carrier proteins or electrochemical gradients produced by pumping protons out of the cells, and this transport is driven by energy in the form of ATP, which is generated through respiration (Leonard, 1984; Mengel & Kirkby, 1982). This form of respiration is proportioned to the one observed under NH_4^+ nutrition of plants (Fig. 4a and b). The third form of respiration could be distinguished after fertilizing of plants with NO_3^- – respiration associated with reduction of NO_3^- to NH_4^+ , which as was shown above could account for a significant part of total respiratory losses (Fig. 4a and b). Respiratory costs associated with ion uptake were greater on the V8 stage, although the quantity of N ions absorbed and recovered from plants were less than on the V6 stage. The possible explanation for this observation could be a general depletion of the ion concentration in the soil solution with time, so that on the later growing stage a plant invest more for its uptake from the soil in confront with a younger one which is still subjected to a sufficient nutrient supply.

The main difference between two growing stages fell mainly on respiration associated with ion uptake and nitrate reduction (Fig. 4a and b). On V6 stage a 1.3 times significant increase in respired recently assimilated $^{14}\text{CO}_2$ was observed under NO_3^- -N over NH_4^+ -N; one week after the difference between two types of N applied was not significant (Fig. 4), indicating that root contribution to whole plant nitrate reduction may be especially important during the early phases of plant growth, following by a decrease in nitrate reduction in roots with time.

Pan et al. (1985) and MacKown et al. (1983) showed that root morphological characteristics may play an important role by influencing the location of the nitrate reduction site. Like that, Pan et al. (1985) have found a positive relationship between the percent of nitrates reduced in roots and the number of lateral roots per unit mass, indicating that that corn root tips maintain higher level of nitrate reductase activity than more mature roots section. Hence, nitrate partitioning may differ along the root axis, and younger regions of the root may reduce a higher proportion of incoming nitrates than older one. The difference in the root biomass between V6 and V8 corn in our

experiment could be responsible for the observed difference in the quantity of nitrates reduced in roots.

It's worth to note that some studies (Ashley et al., 1975; Breteler et al., 1980; Talouizte, et al., 1984; Gojon et al., 1986) have found that root contribution to the whole plant nitrate reduction may be important in the early phase of nitrate utilization, during so called induction process which appears just after the fertilizer addition and could last up to a couple of days. In our experiment the maximum difference in $^{14}\text{CO}_2$ respiration between two types of N fell exactly on this phase - the first hours after ^{15}N addition and uptake. Generally, after the termination of the induction period, the contribution of roots to the nitrate reduction process decreases due to several possible reasons: 1) the nitrate reductase level in roots could be depressed by the supply of amino acids from the shoots; 2) decrease in the nitrate uptake after induction phase could be responsible for a limitation of the nitrate supply to root nitrate reductase, particularly because translocation, which could compete with reduction in roots (Rufty et al., 1981) 3) Both nitrate and ammonium assimilation during the first period could depress the carbohydrate content of the roots and curtailed further nitrate uptake and reduction which appear to depend on it (Fiedler et al., 1975; Jackson et al., 1976). The above findings indicate that the observed difference in the quantity of respired CO_2 between two types of N could change after concluding of the initial phase of N uptake. Such possibility could be verified in the future experiments applying $^{14}\text{CO}_2$ pulse labeling technique.

5.4.6. *Conclusions*

Various factors could influence the contribution of vegetation to soil CO_2 efflux from soil. Difference in plant species composition, growth stage, and environmental factors such as intensity of photosynthetically active radiation and temperature are well known one. This study brings out another factor, which could influence radically the contribution of autotrophic component of soil respiration to the total CO_2 efflux. Pulse labeling of plants in $^{14}\text{CO}_2$ atmosphere allowed evaluating the effect of N form (nitrate or ammonia) on recently assimilated CO_2 efflux from rhizosphere. Nitrate affected negatively the carbohydrate metabolism and energy economy of corn and lupine: in respect to ammonium, nitrate nutrition increased root-derived CO_2 efflux up to 47%. However, comparison of $^{14}\text{CO}_2$ efflux from soil at two plant development stages showed that root contribution to the whole plant nitrate reduction process is not stable during a plant ontogenesis. Contribution of roots could be more important during the early phases of plant growth, following by a decrease in nitrate reduction in roots with time. These, consequently reduces C costs associated with nitrate reduction for more mature plants. All these should be taken into account while modelling and interpreting the data of CO_2 efflux from soil, particularly separating estimation of individual CO_2 sources which contribute to the total soil CO_2 efflux.

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**6. CONTRIBUTION OF ROOT RESPIRATION TO CO₂
EMISSION FROM SOIL IN GRASSLANDS:
COMPARISON OF PARTITIONING METHODS**

6.1 . Introduction

In studies of the carbon (C) cycle of terrestrial ecosystems, soils have received considerable interest because of their critical role in the long-term storage of C sequestered from the atmosphere. The C balance of soils depends on the balance of C input and release, which show different temporal patterns and respond differently to environmental drivers.

Soil respiration is the result of the production of CO₂ in soils from a combination of several belowground processes (Ryan and Law 2005; Trumbore, 2006; Kuzyakov, 2006), which integrates growth and maintenance respiration of autotrophic roots and associated rhizosphere organisms, heterotrophic bacteria and fungi active in the organic and mineral soil horizons, and soil faunal activity (Edwards et al. 1970).

Heterotrophic processes control soil C storage and nutrient dynamics, while autotrophic component reflect plant activity and supply of organic compounds to roots from canopy (Hogberg et al., 2001). The dynamic of different components of soil respiration is controlled by different biotic and abiotic factors, such as temperature, water availability, photosynthetic activity, or plant phenological development. Whereas the activity of soil heterotrophic organisms is proportionate to the decomposition of soil C, which is tightly related to changes in soil temperature, CO₂ lost from root and rhizosphere activity is tied to the consumption of organic compounds supplied from above ground organs of plants (Horwath et al. 1994). The temperature sensitivity of root respiration have been reported to differ from that of heterotrophic decomposition (Boone et al., 1998; Pregitzer et al., 2000; Epron et al., 2001; Zhou et al., 2007) exhibiting various Q_{10} values. Thus, the potential change in soil CO₂ efflux associated with global warming will largely depend on the relative contribution of root and microbial-derived respiration tot total CO₂ efflux.

To date, a variety of methods both, under laboratory conditions or *in situ*, have been used to separate individual components of the overall soil CO₂ efflux and to calculate their contribution to total soil respiration. Partitioning of soil respiration allows researchers to measure the contribution of each respiration source to total fluxes and to account for the individual response of each source to environmental factors. However, the basis assumptions and results obtained by these methods vary significantly among the studies. Hanson et al. (2000) reviewing partitioning methods and results obtained from different biomes and ecosystems, indicated a mean contribution from autotrophic sources of 48% and 60% for forest and nonforest ecosystems, with a range of 10–90% for contribution from different studies. The variation is considerable because of the diversity of ecosystems, and potential biases associated with application of different techniques and of various time scales.

The results reported by different partitioning techniques give only a limited insight into specific settings, because they act over a range of spatial scales, from the isolation of individual

plant organs (roots) or soil components (e.g. surface litter), to input from aboveground plant parts at the stand level (trenching or forest girdling), as well as over a wide range of temporal scales (from hours or days in pulse label experiments to several years in long-term field studies). As most techniques used to separate components of soil respiration are associated with disturbance of the soil system, it is inevitable that a bias is introduced by the choice of technique to achieve the flux separation. In addition to the physical disturbance, there are a range of assumptions or corrections that are not implemented in a uniform way, complicating further a direct comparison of results obtained with different techniques. It remains thus unclear if the observed variation in results is method-dependant due to the fact that each partitioning approach integrates the biases associated with the proper limitations and shortcomings, or reflects varying experimental conditions, like soil type, plants cover, equipment, environmental conditions etc. Comprehensive reviews of these methods are given by Hanson et al. (2000), Kuzyakov and Larionova (2005), Kuzyakov (2006), and Subke (2006). No intercomparison of results obtained from different methods applied in the same study site using a unique measurements equipment have been published yet despite a wealth of studies reporting soil CO₂ efflux partitioning from almost all of the world's terrestrial biomes.

The term “root-derived” (R_a) will be used in this study to describe the sum of actual root (R_r) and CO₂ evolved by microbial decomposition of exudates, secretion as well as root residues such as sloughed root cells, root hairs and dead roots. The term microbial-derived respiration (R_h) integrates the CO₂ coming from the decomposition of SOM in root-free soil. Microbial-derived together with rhizomicrobial respiration sources represent the whole microbial pool (R_m) of soil respiration.

The objectives of the study were:

- To compare estimates of root/microbial contribution to soil respiration obtained by three widely used partitioning methods *in situ*;
- To evaluate possible methodological shortcomings associated with an accuracy of estimation of single components of soil respiration.

Were chosen three widely used and perspective partitioning techniques: 1) mesh exclusion technique, a modification of widely used root exclusion method, which was chosen as a reference method in 2007-2008 (Used in: Fisher and Gosz, 1986; Bowden et al., 1993; Buchmann, 2000; Ross et al., 2001; Lee et al., 2003; Moyano et al 2007; Moyano et al., 2008). It has experienced some important modifications which make it less invasive and more friendly to the surrounding soil conditions in confront with standard invasive root exclusion techniques; 2) Combined method: soil induced respiration (SIR) and component integration, applied in 2007 (Used in: Pannikov et al., 1991; Larionova et al., 2006; Yevdokimov et al. (not published). The method was chosen to the fact that it permits to separate more accurately root from microbial component of soil respiration; 3)

Regression analyses technique, which was applied 2008 (Used in: Kucera and Kirkham, 1971; Gupta and Singh, 1981; Behera et al., 1990; Hill et al., 2004; Rodeghiero and Cescatti 2005; Wang et al., 2007). The method doesn't imply any soil disturbance but up to now was used only in forest ecosystems and croplands, which makes it interesting to be performed in grasslands.

6.2. Materials and Methods

6.2.1. Mesh- exclusion technique

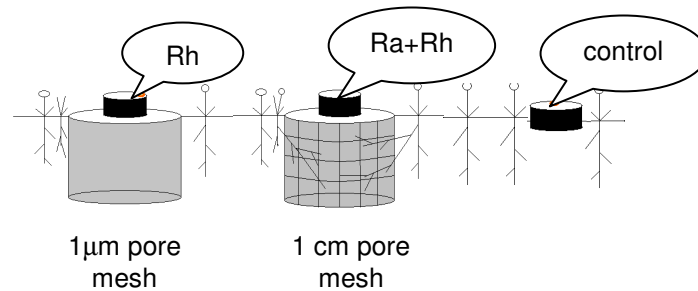


Fig.1 Schematic view of a complete partitioning plot with 1 µm and 1 cm pore meshes and a control collar placed on the undisturbed soil.

Partitioning technique, based on the utilization of the nylon mesh bags was modified after Moyano et al. (2007; 2008), making it more adaptable for the particularities of grassland ecosystems. The novelty of this method is the possibility of using small pore sizes which allow the movement of bacteria, rhizodeposits and other substances through the mesh while excluding roots, maintain by that environmental conditions similar to the exterior soil.

In details partitioning plots preparation and establishment is discussed in chapter 2. Briefly, by calculating differences in respiration between 1 µm, 1 cm and control (undisturbed soil) treatments, a partitioning of soil respiration into its root-derived and microbial-derived components was done as follows:

- 1 µm = *microbial-derived respiration (Rh), disturbed*
- 1 cm = *microbial plus root-derived respiration (Ra+Rh)*
- 1cm – 1µm = *root-derived respiration (Ra)*
- control = *total soil respiration (Rs)*
- control – Ra= *microbial-derived respiration (Rr), undisturbed*

Consequently, the method allowed to separate root respiration together with the respiration of the associated microorganisms (rhizomicrobial respiration) from SOM-derived respiration.

Ten complete partitioning plots, each consisted of 3 collars were installed at Amplerö. Measurements of soil respiration components were performed by closed dynamic system EGM-4, connected soil chamber SRC-1 (PP-System, UK) on bimonthly basis, both in 2007 and 2008.

6.2.2. Combined method: SIR+component integration

The idea of the combined method is that, after addition of glucose (dry or as water solution, 3 mg glucose g⁻¹ soil), the heterotrophic respiration (here, including microbial and rhizomicrobial respiration R_{mic}) which is limited by easily available C substrate increases with the enlargement factor k_{mic} , while the actual root respiration (R_a) remains at the same level (Panikov et al., 1991; Larionova et al., 2004). The method seemed promising because of its potential possibility for separation of root and rhizomicrobial respiration.

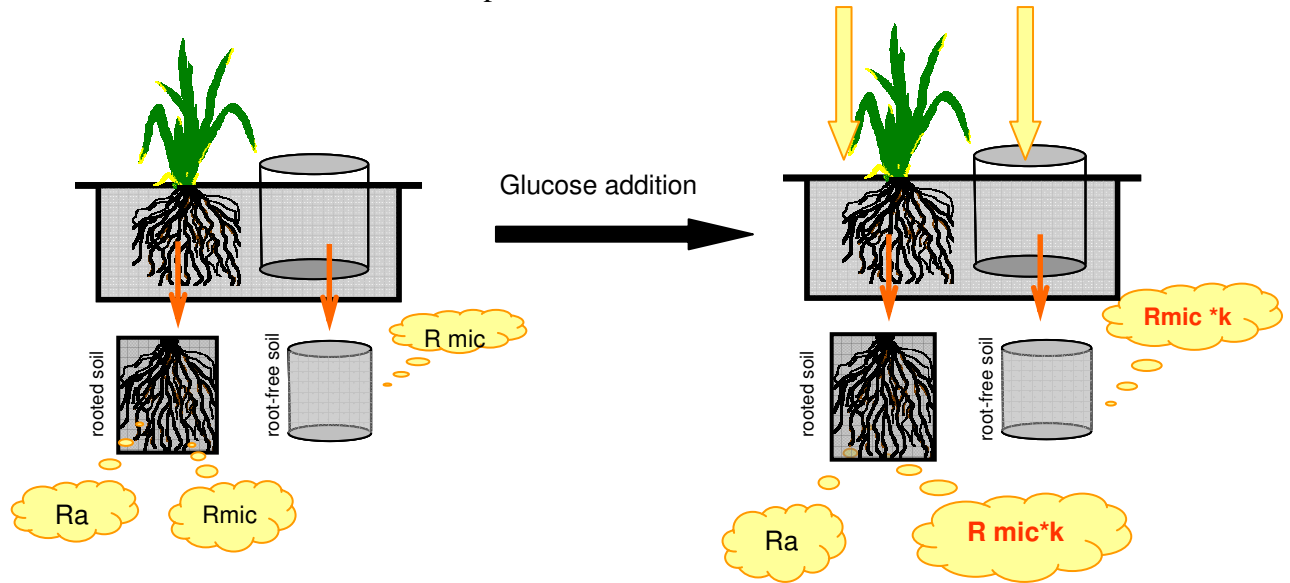


Fig.2 Schematic view of combined method: rooted and root free soil before and after the glucose addition.

Root respiration and respiration of microorganisms were calculated by solving the system equations:

$$R_1 = R_a + R_{mic};$$

$$R_2 = R_a + (k * R_{mic}) \quad (\text{eqn.1})$$

where, R_1 and R_2 are the intensity of the CO₂ emission prior to and after the glucose introduction respectively, and R_{mic} is the respiration of the microorganisms. A soil sample with carefully removed roots installed in the study site one week before the experiment was used for determination of the coefficient of soil respiration increase after the glucose introduction (k). Plant litter was however left in the sample.

$$k = R_{mic_2} / R_{mic_1} \quad (\text{eqn.2})$$

where R_{mic_1} and R_{mic_2} are the intensity of the CO₂ emission, respectively prior to and after the addition of glucose to the soil sample without roots. Soil respiration response was measured every hour from time 0, up to 5 hours after glucose addition. To account for the effect of soil moistening another set of plots was treated with addition of only water.

The respiratory response with glucose treatment was determined three times during the year 2007: in June, July and September. Measurements of soil respiration by root-exclusion technique were done in the same days, simultaneously with combined technique.

6.2.3. Regression analyses technique

Regression approach was firstly suggested by Kucera and Kirkham (1971) and is based on the assumed linear relationship between root biomass and amount of CO₂ respired by roots and rhizosphere microorganisms. The amount of CO₂ derived from SOM decomposition correspond to the y-intercept of the regression line between root biomass (independent variable) and total CO₂ efflux from soil (dependant variable).

$$R_s = a \times B_{Gb} + k_h; R_h = k_h \text{ and } R_a = R_s - R_h \quad (\text{eqn. 3})$$

where R_s -total soil respiration; B_{Gb} – belowground biomass, k_h – y-intercept, which is assumed to be a measure of SOM-derived respiration.

The method is comparatively simple and was applied in numerous studies mostly on forest ecosystems (Gupta and Singh, 1981; Behera et al., 1990; Hill et al., 2004; Rodeghiero and Cescatti 2006; Wang et al., 2007).

The measurement procedure was the following: in the beginning of 2008 twelve pairs of soil collars were installed into the soil not far from the partitioning plots of root-exclusion technique. Soil respiration was measured on bimonthly bases from all the collars. In the beginning of June, during the peak of root biomass (from the data of 2007), twelve collars were removed and soil cores of 8cm diameter and 30 cm depth were sampled under the soil respiration measurement plots for further analyses on the belowground biomass. The rest of the collars were leaved in soil and the measurements of soil respiration were continued.

6.3. Results

6.3.1. 2007: Mesh- exclusion vs. combined SIR

The results of partitioning of soil respiration by mesh-exclusion technique are shown in Fig. 3. The contribution of root-derived respiration to total CO₂ efflux from soil varied in the range of 2% - 79%, with an average value of 28%.

Once a month, in June, July and September simultaneously with measurements by mesh-exclusion technique but on the separate plots the application of the glucose solution and water was performed. An example of the response of total and microbial respiration to the addition of glucose and water, and the final results of the partitioning are shown in figure.4. Effect of moistening on total CO₂ efflux from soil was pronounced during the first hour after the treatment, after that the respiration level decreased to the initial value. The effect of the glucose solution was more evident;

the respiration response of microbes was increasing sharply and stabilized only 2.5 hours after the treatment.

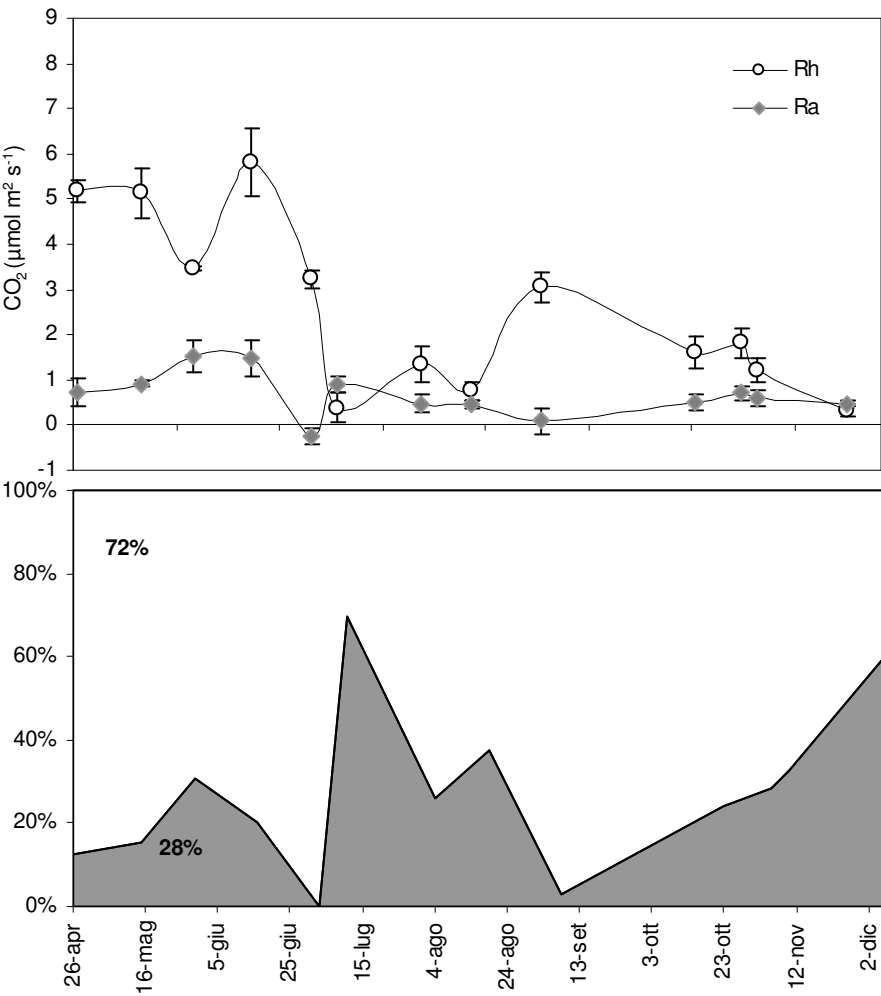


Fig. 3 a) Root- and microbial-derived respiration obtained by root-exclusion technique. b) Contribution of root and microbial respiration to total CO₂ efflux from soil.

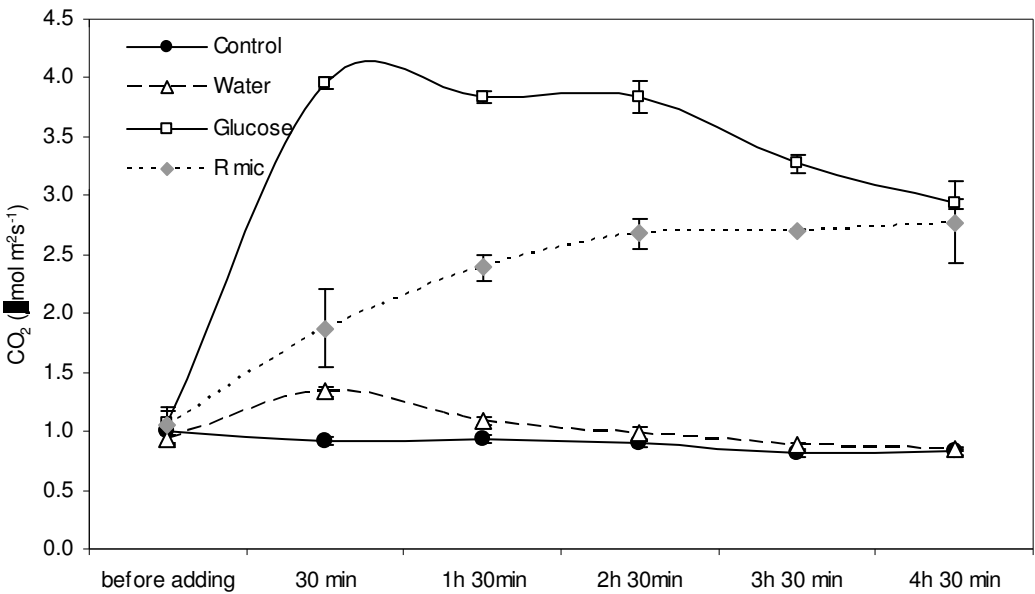


Fig. 4 Example of the effect of glucose (3 mg g⁻¹ soil) and water addition on total and microbial respiration in June.

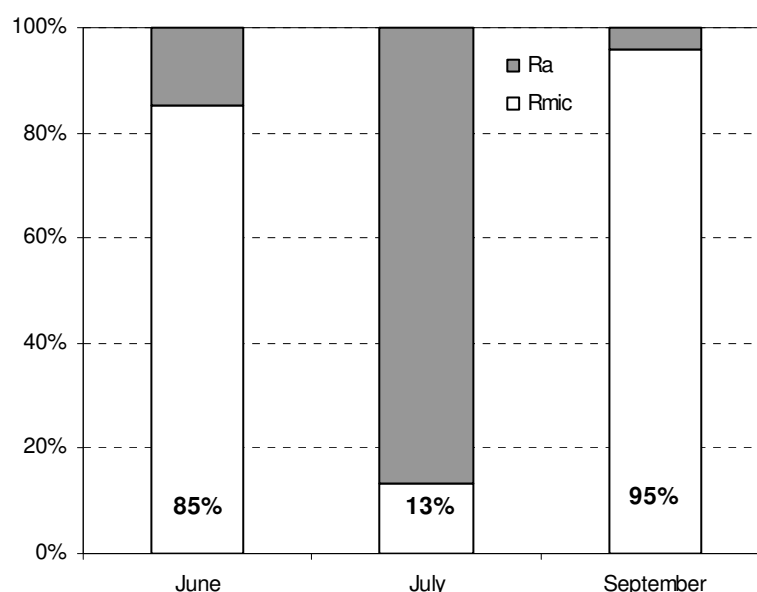


Fig. 5 Results of partitioning obtained by combined soil induced respiration method.

The final result showed a significant variation in the contribution of root and microbial respiration during the growing season. In September the soil CO₂ efflux was represented mostly by microbial respiration, while in July the major part was respired by roots.

The results of two partitioning techniques, performed in 2007 were pooled together (Fig.6). Soil respiration components showed similar patterns. In June no difference were observed in root and microbial respiration between two techniques. In July the estimated values of microbial-derived respiration were equal, but the root component was three-folds higher in Combined SIR method. In September on the contrary, no difference was observed in the assessment of root-derived respiration but increased microbial component given by Combined SIR. However, the general seasonal dynamic of both root- and microbial- derived respiration was quite similar between both techniques.

To check the presence of the lateral flow of C in the root-free soil of the mesh bags, the surrounding soil was labeled in a ¹³CO₂ atmosphere. For these purposes a special chambers for trapping of the respired CO₂, which fit the size of the meshes were constructed. The details of the labeling procedure are described in Chapter 3. The trapping of the CO₂ was performed twice: 1 and 4 hours after the label introduction. The results are shown in figure 7. The background signature of the CO₂ respired from the mesh bags was in the magnitude of -20‰, already one hour after the ¹³C signature rose up to -10‰ with the following decrease on the fourth hour of the chase period. Its worth to note that the labeling zone was covering the soil only from one side of the meshes, which means that the observed values could be underestimated.

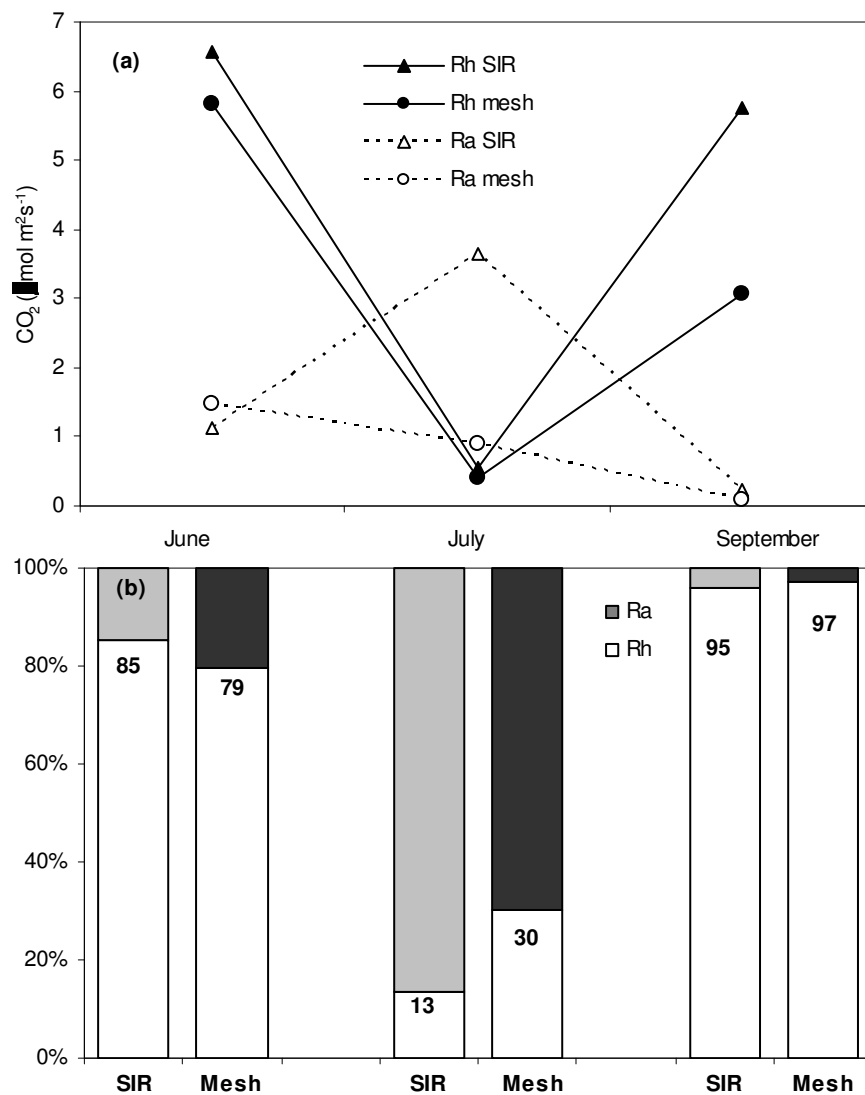


Fig. 6 Partitioning obtained by mesh-exclusion technique vs. combined soil induced respiration. Results are shown in a) CO₂ efflux, μmol m⁻² s⁻¹ b) % of each component from total CO₂ efflux

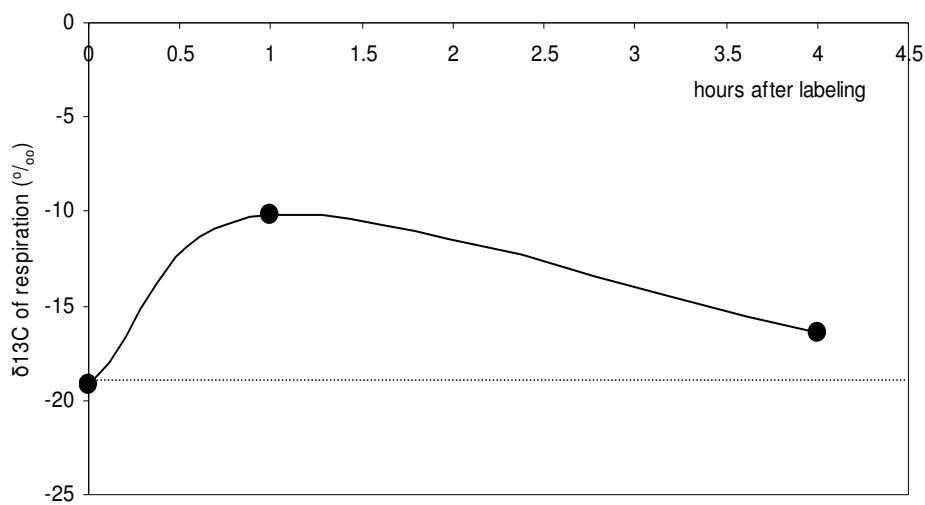


Fig. 7 Raw isotopic value of the ¹³CO₂ respired from the mesh bags during four hours after the labeling.

6.3.2. 2008: Mesh- exclusion vs. regression technique

The results of root-derived respiration estimation implying mesh exclusion and regression techniques are shown in figure 8. Data obtained by regression method were generally significantly higher. In fact, an average contribution of roots to total CO₂ efflux amounted to 63% for the regression method and only 31% for mesh exclusion (Fig. 9).

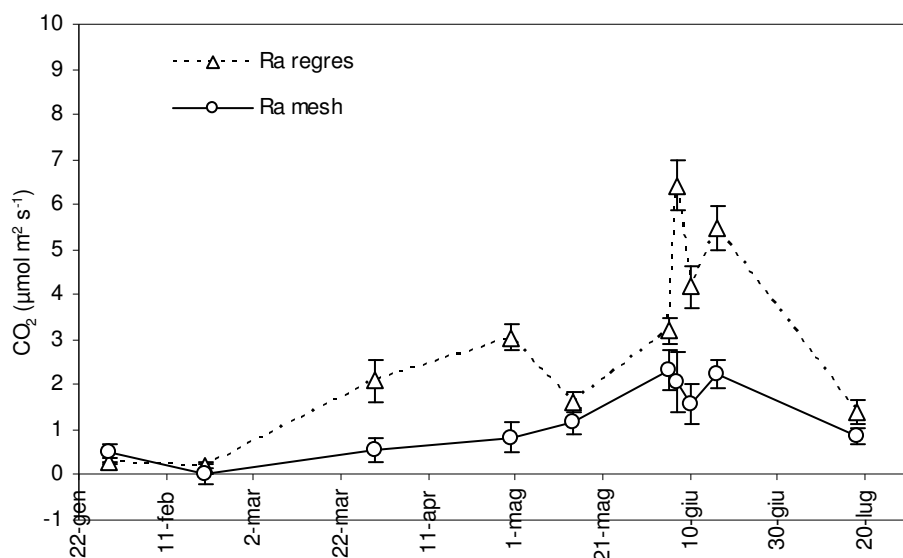


Fig. 8 Root-derived respiration obtained by mesh exclusion and regression techniques in 2008.

General trend of seasonal variation in contribution of root-derived respiration to total CO₂ efflux were however similar in both methods, especially for the period after the biomass sampling was done. Regression curves, obtained after plotting of the total CO₂ efflux vs. changes in belowground biomass were however characterized by low values of R², varying from 0.1-0.6 and often the obtained correlations didn't result significant (Table 1). For the period prior to the biomass sampling, the y-intercept of the regression line was even negative; a 0 value of microbial respiration was accepted in these cases.

Date	R ²	p	meaning
29-gen	0.01	0.82	n.a.
20-feb	0.37	0.19	n.a.
30-mar	0.67	0.01	*
30-apr	0.20	0.05	*
14-mag	0.13	0.05	*
5-giu	0.14	0.47	n.a.
7-giu	0.19	0.38	n.a.
10-giu	0.10	0.57	n.a.
16-giu	0.13	0.40	n.a.
18-lug	0.03	0.70	n.a.

Table 1. Correlation and regression coefficients obtained from the liner relationship between soil CO₂ efflux and belowground biomass.

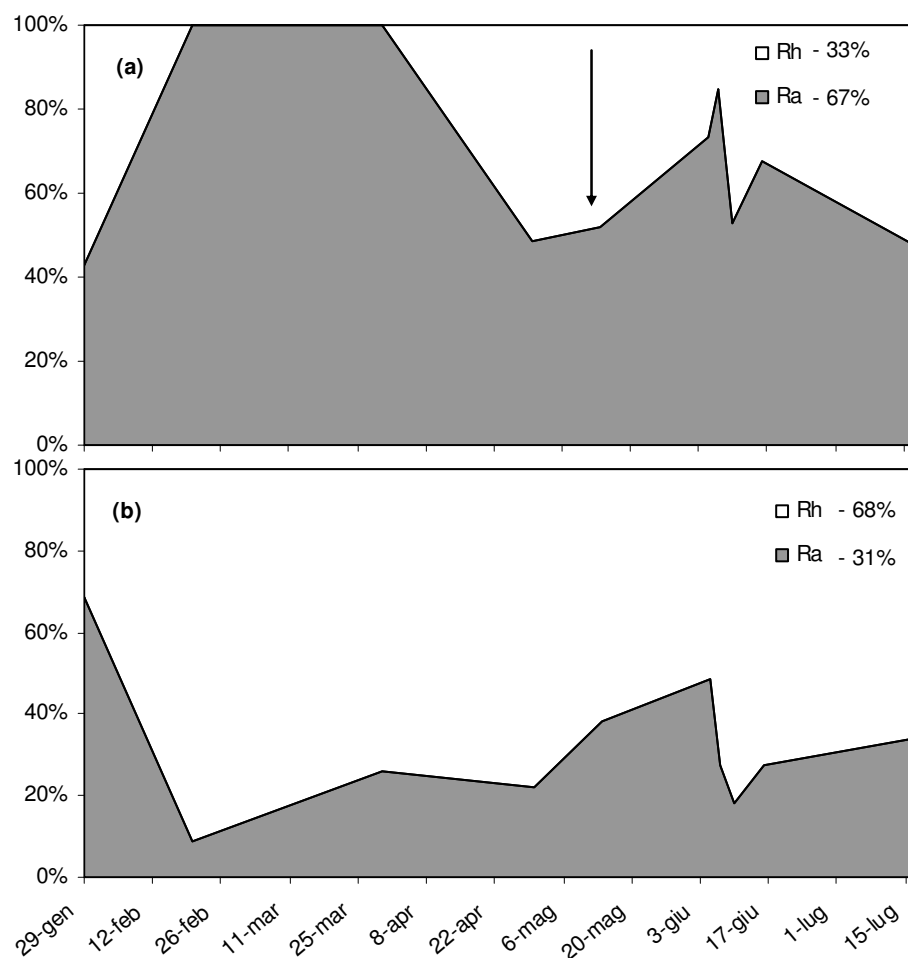


Fig. 9 Contribution of root and microbial respiration to total CO₂ efflux from soil a) by regression and b) root-exclusion technique. An arrow indicates a time of soil sampling for belowground biomass for the regression technique.

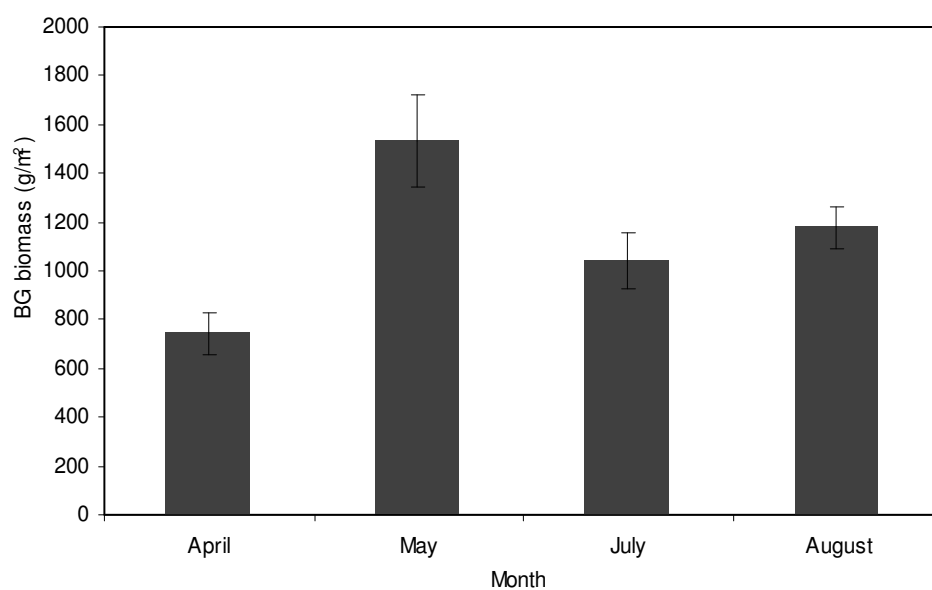


Fig. 10 Seasonal changes of belowground (BG) biomass at Amplero.

6.4. Discussion

6.4.1. Mesh-exclusion

Mesh-exclusion technique is a type of widely used root exclusion methods. Such type of partitioning methods potentially changes soil nutrient and water status and in combination with a lack of competition with roots and mycorrhiza could alter microbial respiration of SOM and litter in the root free soil. Because of the absence of root exudates in the soil core it is not possible also to account for any priming effect, which is usually appears to be under the natural soil conditions (Kuzyakov, 2002; Kuzyakov and Bol, 2005; Kuzyakov, 2006). The modification of the method and introduction of the nylon mesh bags with various apertures seems promising, allowing the flow of water and dissolved substances through the mesh, thus minimizing the disturbance of natural soil environment.

However, there are some particular shortcoming and limitation associated with the utilized methodology, which result in a possible overestimation of microbial-derived respiration and underestimation of root-derived one.

Namely, the shortcomings of the method are: disturbance of the soil structure by sieving and lateral diffusion of CO₂ (Jassal and Black, 2006; Moyano et al., 2008) to the mesh bags from the surrounding soil and inability to separate root respiration from rhizomicrobial one.

We tried to minimise the effect of the soil disturbance on microbial activity and respiration fluxes by installing an additional mesh of 1cm, and obtaining the value of root-derived respiration as a difference between two meshes with the equally disturbed soil. However, here we make an assumption that there is no difference in the in-growth patterns of roots between non disturbed soil and soil which was previously sieved. Microbial-derived respiration was calculated further from the difference between control (non disturbed soil) plots and root respiration.

Presence of the CO₂ from the surrounding soil in the mesh bags by its lateral diffusion through the soil pores was confirmed by the pulse labeling of the adjacent soil in ¹³CO₂ atmosphere and its tracing in the ¹³CO₂ respired from the mesh bags. The magnitude of the influence of the lateral CO₂ on the respiration rate is however difficult to estimate. Moyano et al. (2008) reported a value of ca. 10%, obtained by inserting a PVC tubes over the mesh soil cores. As the procedure is associated with additional disturbance of the measurement plots we preferred to avoid it. Presence of laterally diffused CO₂ results in a systematic overestimation of the microbial-derived respiration. Here we make a second assumption: the influence of the lateral flow of CO₂ is constant during the growing season, and doesn't modify the seasonal trend in contribution of soil respiration components to the total one. We also argue that an introduction of the additional 1 cm mesh core with the same influence of the incoming CO₂ from the surrounding soil could potentially minimize the error in estimation of root-derived respiration.

The estimate of root and microbial respiration is subjected to additional errors, as the fluxes are not estimated directly but are calculated from the other measurements, each subjected to its own errors. We suggest establishing a large amount of partitioning plots to minimize the errors in calculation of soil respiration components.

6.4.2. *Combined SIR*

Substrate induced respiration (SIR) is based on the response of microbial respiration to the addition of glucose and the absence of response for root respiration (Panikov et al., 1991). Combination of methods of substrate induced respiration and component integration into one allowed moving the experiment from the laboratory to the field. The difference in the response and comparison of CO₂ efflux from soil with and without roots before and after glucose addition, in the concentration much lower than the concentrations of the soluble carbohydrates in the root tissue, allow calculating root and microbial respiration components. After the introduction of glucose the respiration of microorganisms that was limited in the soil with C substrates increases several times while the respiration of the living roots remains the same. In confront with the other methods used in this study, combined SIR allows more exact separation of actual root respiration, from all the other respiration sources by accounting a rhizomicrobial component as a part of microbial respiration.

The applied glucose concentration (3mg/g of soil) is much lower than the concentration of soluble carbohydrates in the roots observed for meadow grasses, which ranges from 5 to 50 mg/g of root depending on the plant species and the growing period (Naumov, 1988), so the uptake of glucose by roots couldn't have a significant effect on the respiration of the living roots.

Microbial respiration in the experiment was influenced significantly by the glucose introduction. However, Larionova et al. (2006) showed that the coefficient of the respiration increase (k) differs, depending on the source of microbial respiration: microbial decomposition of dead roots, falloff, detritus or soil organic matter. The coefficient of decomposition of plant residues (detritus, dead roots, litter) was as rule lower than the k in the soil. Experiments on roots showed that the k increases two times already on the second day after the root destruction; however it remains stable on different stages of decomposition (Larionova et al., 2006). This is the one of the complications of the combined SIR technique: to determine exactly the k in the soil with the plant residuals it is necessary to remove only the living root from the soil monolith. We have tried to leave the litter and coarse dead plant residues in the soil sample used for k determination, however, it was not possible to separate all dead and live root visually. This implies a certain underestimation of the coefficient and further overestimation of microbial respiration. An alternative for future studies could be the determination of the respiration response prior and after the glucose

introduction in a small soil sample without plant residues and then the final k could be calculated using a proportion between the soil weight and the weight of the plant residues in the monolith (Larionova et al., 2006):

$$k = (R_{2s}m_s + R_{2rs}m_{rs}) / (R_{1s}m_s + R_{1rs}m_{rs})$$

where, R_{1s} and R_{2s} are the respiration response of the soil prior and after the glucose addition respectively; R_{1rs} and R_{2rs} are the respiration response as a result of decomposition of plant residues prior and after the glucose addition and m_s and m_{rs} are the masses of soil and plant residues in the monolith. This however, couldn't be done in the field and implies a k determination in the laboratory.

Another shortcoming of the method is a possible boosting of root respiration after the addition of glucose in water solution, which could be especially true under insufficient soil moisture conditions. This could result in further overestimation of microbial component of soil respiration. The glucose solution was always added in the way not to exceed the 60% of the soil water capacity, we were also measuring the effect of only water on total soil respiration rates. As could be seen from the figure 4, the main effect of water occurred during the first hour after the start of the treatment with the further stabilization of the respiration rate at the initial level. The effect was longer and more pronounced in July, under the limited natural soil content (data not shown), however the soil respiration have decreased and stabilized after 5 hours of experiment, when the glucose treatment on the contrary just reached its peak.

6.4.3. Regression analyses technique

Although regression technique was nominated in the recent review of partitioning methods (Kuz'yakov, 2006) as the most universal one due to its low cost, applicability to a wide range of ecosystems and the absence of the soil disturbance, its application in this study on a grassland community showed contrasting results.

The main problem we faced with regression analyses was the absence of significant correlation between the root biomass and soil CO₂ efflux. The correlation was weak for the biomass sampling day and the measurement days adjacent to the day of the biomass sampling, no correlation was observed further. Despite this fact, the method managed to detect some particular changes in the contribution of root and microbial respiration, which were observed also by the mesh exclusion technique (ex.: Fig. 9, peaks in root respiration in July). This could indicate that the augment of the measurement points may improve the correlation strength.

The particularity of the grassland ecosystems is that the most of the belowground biomass is represented by the fine roots. Larionova et al.(2006) showed that fine root biomass is characterized by a greater variation in root specific activity, ranging from 0.2-1.2 mg C-CO₂/g root while the

activity of the coarse roots, which account for the greatest part of belowground biomass in forest ecosystems is more stable and vary from 0.01 to 0.05 mg C-CO₂/g root. Other studies have also demonstrated that specific root respiration and its Q_{10} decrease with increasing root diameter (Ryan et al. 1996; Pregitzer et al. 1998; Bahn et al. 2006), decreasing specific root length (Tjoelker et al. 2005) or the ratio of long to fine roots (Kutsch et al. 2001). Such effects may be partly related to a decrease in N concentration (Ryan et al. 1996; Pregitzer et al. 1998; Bahn et al. 2006) and an increase in tissue and plant age (Palta and Nobel 1989; George et al. 2003; Volder et al. 2004).

In the course of the growing season belowground biomass at Amplero experienced significant changes (Fig.10). In spring, during the peak of the biomass growth, it has almost doubled in only one month. This means that one biomass sampling is not enough to account for the seasonal variation of different soil respiration sources; multiple sampling is needed for the reliable results.

Regression technique is however an indirect method of respiration components estimation, and allow only an approximate quantification of root contribution to soil respiration. The theoretical basis of the regression technique is that the autotrophic component of soil respiration is proportional to the belowground biomass and in the absence of roots (i.e. $x=0$) the soil respiration is represented only by microbial component. However, recent studies have shown that root respiration is considered to be fuelled by non structural C, rather than structural C which is fixed in the cell wall of roots. Among the non structural C sources are starch, organic acids and soluble sugars as fructose, glucose and sucrose. Starch is formed as a transitory reserve when the supply of soluble sugars exceeds the actual demand within the plant (Hansen and Beck, 1994). Soluble sugars and organic acids are important substrates for root respiration. For example respiratory utilization of recently assimilated photosynthates such as sucrose in roots provides the essential energy for nutrient uptake, growth and maintenance as well as symbiotic processes and defences (Veen et al., 1980; Farrar, 1985; Bouma et al., 1996; George et al., 2003; Martinez et al., 2002; Xu et al., 2008). Moreover it has been shown that exogenously added sugars rapidly increase respiration rates of roots and shoots (Saglio et al., 1980; Azcon-Bieto et al., 1983; Journet et al., 1986). The above findings suggest that the non structural C content in roots could provide a better estimate of root respiration than bulk root biomass alone, since structural C fixed in the cell walls comprising the major root C pool cannot be utilized by roots and is only slowly utilized by rhizospheric microbes (Xu et al. 2008). Another limitation of the method, is that it doesn't consider a spatial variation of microbial respiration, whereas in some ecosystems (ex. the forest soils) due to the large spatial heterogeneity the microbial respiration may vary in space, limiting the reliability of the method. Rodeghiero and Cescatti (2006) for such cases proposed to introduce into the model additional

component, expressing a total soil respiration as a sum of root and microbial-derived sources, linearly dependent on root density and soil C content respectively.

6.4.4. General comparison of three partitioning methods

After the discussion of all the particularities and shortcoming of the partitioning techniques applied in this study it is possible to compare the results obtained by the methods.

The main positive observation is that mesh exclusion and combined SIR techniques, so different in its theoretical and methodological basis, showed quite similar results both in the magnitude of soil respiration fluxes almost for all the measurement days and in its seasonal variation patterns. Actually we expected to observe a higher microbial-derived respiration with combined SIR, as it allows an additional separation of root and rhizomicrobial respiration components. The difference between microbial respirations, obtained from two techniques could be used as an estimate of the rhizomicrobial component of soil respiration. However, having the different sources of the mistake, both methods result in the systematic overestimation of microbial respiration component. The magnitude of this error is uncertain, which limits the reliability of such calculation of rhizomicrobial respiration.

The results obtained by mesh exclusion and regression analyses technique in 2008 differed significantly both in the magnitude of the respiration fluxes and in the seasonal patterns of relative contribution of single components to total CO₂ efflux. Regression technique, given the most uncertain results, during the whole period of measurements was overestimating the root-derived respiration in confront with mesh exclusion method, sometimes calculating the root contribution as a 100% to total CO₂ efflux from soil. To overcome all the uncertainties, which were mainly associated with the particularities of the grassland communities the method requires further development and standardization.

Summarizing, the comparison of methodologies in soil CO₂ efflux partitioning techniques showed generally good agreement, there are numerous assumptions masked within these results, and we have tried to indicate where potential biases arise and corrections are needed.

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