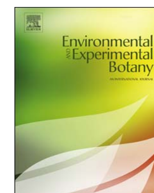
Environmental and Experimental Botany 141 (2017) 170–180

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot



Does long-term warming affect C and N allocation in a Mediterranean shrubland ecosystem? Evidence from a ^{13}C and ^{15}N labeling field study

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ARTICLE INFO

Keywords:
Climate change
Temperature increase
Respiration
Photosynthesis
Cistus
C cycle
N cycle

ABSTRACT

In the Mediterranean basin the effects of climate warming on ecosystem functioning will strongly depend on the warming intensity directly but also on its effects on evapotranspiration and nutrient cycling. Climate manipulation experiments under field conditions are a source of unique empirical evidence regarding climate-related modifications of biotic processes.

A field night-time warming experiment, simulating the predicted near-future increase in ambient temperatures (+0.3 up to 1 °C), was established in a Mediterranean shrub community located in Porto Conte (Italy) in 2001. After 11 years of continuous treatment, we labeled the dominant shrub *Cistus monspeliensis* with $^{13}CO_2$ and studied the dynamics of the label allocation between aboveground and belowground pools and fluxes in warmed and ambient plots within 2 weeks of the chasing period. The interactions between C and N metabolism were assessed by parallel labeling of soil with $K^{15}NO_3$.

Most of the assimilated ^{13}C was respired by *Cistus* shoots (28–51%) within two weeks. *Cistus* under warming respired more ^{13}C label and tended to allocate less ^{13}C to leaves, branches and roots. The higher C and N content in microbial biomass in warming plots, combined with the higher N content in plant tissues and soil, evidenced a greater N mobilization in soil and a better nutrient status of the plants as compared to the ambient treatment. Acceleration of N cycling is probably responsible for higher respiratory C losses, but combined with the reduction in the number of frost days, should also positively affect plant photosynthetic performance.

We conclude that, although *Cistus* plants are already growing in conditions close to their thermal optimum, long-term warming will positively affect the performance of this species, mainly by reducing the nutrient constraints. This positive effect will highly depend on the frequency and amount of rain events and their interactions with soil N content.

1. Introduction

Greenhouse gas emissions continue unabated, and rapid changes in the global climate are predicted. During the 20th century the global temperatures have risen by 0.6 °C. A further increase of 0.3–0.7 °C is forecast for the period 2016–2035 (IPCC et al., 2014). Parallel to the temperature rise, an intensification of the hydrological cycle and increase in the frequency and severity of climate extremes are expected (Jentsch et al., 2011; Frank et al., 2015).

The Mediterranean basin is one of the most biologically rich and climatically complex regions on Earth due to its unique location (Blondel, 2010). Climate change here is expected to follow trends different from the global average: summer warming will be more intense in the Mediterranean area and it will be accompanied by a further decrease in summer precipitation, significantly intensifying summer droughts (Christensen et al., 2007; IPCC et al., 2014). Therefore, the success of biodiversity conservation, carbon (C) sequestration and related ecosystem functions will depend on the ability of Mediterranean

Abbreviations: A, ambient treatments; W, warming treatment; EC, extractable carbon; EN, extractable N; *Cistus*, *Cistus monspeliensis*

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http://dx.doi.org/10.1016/j.envexpbot.2017.07.013
Received 22 February 2017; Received in revised form 22 June 2017; Accepted 19 July 2017
Available online 23 July 2017
0098-8472/ © 2017 Published by Elsevier B.V.

ecosystems to acclimate to increased temperatures and water limitations.

A considerable part of Mediterranean area is covered by shrublands (INFC, 2005). Arid and semi-arid shrublands, however, are not limited to the Mediterranean area but cover up to 35% of the world's terrestrial surface (Asner et al., 2003). Despite their potential impact on the global C cycle, shrubland ecosystems are rarely investigated and in less detail compared to other terrestrial ecosystems (Lavorel et al., 1998; Gorissen et al., 2004; Sardans et al., 2008).

For ecosystem C balance, an important consequence of rising temperatures is the forecasted reduction of net primary production (NPP) induced by increased C losses through autotrophic respiration and soil organic matter decomposition (Melillo et al., 2002; Bekku et al., 2003; Luo, 2007). Nonetheless, an acclimation of soil C losses with the subsequent return to pre-heating values, as well as the absent effect of moderate warming on CO_2 fluxes, are frequently reported (Kirschbaum 2004; Eliasson et al., 2005; de Dato et al., 2010). Moreover, the consequences of C gains are also controversial and often depend on the climatic region (Liang et al., 2013). On one hand, if a plant species is growing below its thermal optimum, then an increase in assimilation rate and growth is expected (Grogan and Chapin, 2000; Marchand et al., 2005). On the other hand, for species growing close to their thermal optimum, like in Mediterranean, temperature increase will likely decrease photosynthetic performance and growth of plants (Larcher, 2000; Rehfeldt et al., 2002; Körner, 2003; Llorens et al., 2004). However, the phenology and interaction with hydrological and nutrient cycles should be also considered (Peñuelas et al., 2007): many Mediterranean woody species are characterized by semi-deciduous and dimorphic phenology, avoiding periods of intensive droughts partially shedding leaves in summer and concentrating a proper growth and activity in autumn, winter and spring months when temperature is still the main limiting factor for biological processes (Aronne and De Micco, 2001; Milla et al., 2004; Palacio et al., 2007; de Dato et al., 2013). Indirect effect of temperature on plant performance in this period is executed through modifications in nitrogen (N) cycle. Higher availability of NH_4^+ and NO_3^-, due to the greater N mineralization in response to soil warming, has been frequently reported (Melillo et al., 2002; Wang et al., 2006; Sardans et al., 2008a). An increase in plant N uptake capacity was also signed (An et al., 2005). Microbial enzyme activity could, however, be hindered by *i*) lowering the soil water content with rising temperatures and *ii*) the canceling effect (Davidson et al., 2006; Razavi et al., 2015). Changes in N availability might affect both respiration and plant photosynthetic performance (Lewis et al., 2004; An et al., 2005). This, in turn, will affect the capacity of the ecosystems to sequester C (Luo et al., 2004), especially in the Mediterranean area where N is often limited (Villar-Salvador et al., 2004; Fernández et al., 2006; Sardans 2008a,b).

Overall, the consequences of warming to plant and ecosystem productivity in arid environments remain controversial, with increases (Huxman et al., 1998; Li et al., 2014), decreases (Peñuelas et al., 2007) or no change (Sardans et al., 2008b) being reported.

Long-term field manipulation experiments, which simulate expected near-future changes in air temperatures and water regimes, represent a unique opportunity to investigate the responses of various ecosystem processes to changing climate. In this study we investigate how long-term exposure of a shrub vegetation characterized by dimorphic phenology to increased temperatures affects the C allocation between pools and fluxes in the plant-soil-atmosphere continuum.

We took advantage of an experimental facility located in the Mediterranean shrubland of Porto Conte (Sardinia, Italy). Here, the temperature was continuously manipulated for 11 years by means of a passive night-time warming technique (Beier et al., 2004; Bruhn et al., 2013). This produced an increase in the average air and soil temperature ranging from 0.3 to 1 °C, reflecting predicted near-future global heating patterns, where major increases are registered in night rather than in daytime temperatures (Alward et al., 1999). We labeled ambient and warmed plots with *Cistus monspeliensis* shrub in a $^{13}CO_2$-enriched atmosphere and studied the distribution of ^{13}C labeled assimilates between numerous pools and fluxes in soil and plants. The interactions with plant N availability were evaluated by labeling the soil with ^{15}N-enriched KNO_3.

In particular, we evaluate how warming affects:

1) the contribution of new photosynthates to metabolic activity: respiration, assimilation and storage. We hypothesize that increase in minimum daily temperatures will increase respiration expenses by night rather than day-time photosynthesis and respiration. It will be reflected in more ^{13}C being fixed in storage pools in order to replace night-time C losses, so that the differences between the treatments in this pool will be likely transient, and in higher allocation to respiration;
2) N availability: we hypothesize that during the experiment conduction (October) plants subjected to warming will be characterized by a better nutrient status. It will be ensured through increased N mineralization by the microbial community which is not constrained by water availability from Autumn to Spring. The effect could be further enhanced through a higher plant N uptake rates;
3) N translocation dynamics: we hypothesize that the better nutrient status induced by warming will also affect the N re-distribution among plant tissues, reducing the need to mobilize nitrogen from internal pools to fuel growth of new leaves which happens between October and November;
4) C and N interactions: we hypothesize that increase in N availability under warming will affect C allocation preferences, with minor C expenses for nutrient foraging and consequent minor belowground C allocation.

2. Materials and methods

2.1. Experimental site and layout

The study area is located in the Capo Caccia peninsula (northwest Sardinia, Italy; 40° 37′ N, 8° 10′ E). The geologic substrate is Mesozoic limestone and the main soil type is Terra Rossa (Lithic Xerorthent and Typic Rhodoxeralfs, USDA 1993; Chromic Cambisol, WRB 2014). The soil is rocky and shallow (20–30 cm); the texture is sandy loam with an ABC profile; pH is 7.7; soil organic matter content is 3.9% in the main rooting zone (0–10 cm); bulk density is 1.1 g cm^{-3}.

The climate is Mediterranean, and according to the nearest long-term meteorological station (Fertilia Airport N 40°38′ E 8°17′; altitude 40 m asl; distance to sea about 4 km), is characterized by a mean annual rainfall of 640 mm distributed mainly in autumn/winter (75%), with a long dry period from May to September. The mean annual temperature is 16.8 °C, and 7 °C and 28 °C are the mean minimum temperature of the coldest month (January) and the mean maximum temperature of the hottest month (August), respectively.

The vegetation cover within the experimental area consists of *Cistus monspeliensis* L. (30 ± 7%), *Helichrysum italicum* G. Don (6 ± 3%) and *Dorycnium pentaphyllum* Scop. (9 ± 5%) with sporadic presence of other shrubs (*Pistacia lentiscus* L., *C. creticus* L., *Daphne gnidium* L., etc.). Bare soil represents about 15 ± 3% of the soil surface (see de Dato et al., 2010 for further site information).

The warming treatment (*W*) was established in 2001 in three replicated parcels (n = 3). *W* consists of aluminum curtains that cover the soil and vegetation during the night so that some of the energy accumulated during the day is retained within the ecosystem (Beier et al., 2004). This simulated the effect of global warming on daily minimum temperatures. Also in 2001, three other replicate parcels were established with no treatment applied: the ecosystem was left at natural ambient conditions (Ambient, *A*). The six parcels (4 × 6 m^2) were assigned to the treatments according to a randomized block design. In autumn 2009, six metallic frames (80 × 80 cm, hereafter

Table 1
Average and minimum daily temperatures of air (Tair) and soil (Tsoil) at 10 cm depth, soil water content (SWC) and air relative humidity in Ambient (A) and Warming (W) plots measured during the experiment and averaged over the year 2012.

Parameters	21 Oct −8 Nov 2012 (experiment)				Year 2012			
	A	W	Δ W-A	p-value	A	W	Δ W-A	p-value
Tair, daily av.	15.9	16.2	0.3	< 0.001	16.2	16.5	0.3	< 0.001
Tair, min	11.7	12.2	0.5	< 0.001	9.5	10.2	0.6	< 0.001
Tsoil −10, daily av.	17.6	17.6	0.0	ns	18.0	17.8	−0.2	ns
Tsoil −10, min	16.2	16.4	0.2	< 0.01	15.7	15.8	0.1	< 0.001
Water content, %	21.6	19.7	−1.8	< 0.001	15.0	14.6	−0.4	< 0.001
Relative humidity	95.2	92.7	−2.5	< 0.001	86.0	84.2	−1.9	< 0.001
Frost days (2008–2012)	–	–			19	15	4	< 0.05

referred to as plots) each enclosing one *Cistus monspeliensis* shrub (hereafter *Cistus*), were inserted at 5 cm soil depth within each experimental parcel (three belonging to *A* and three belonging to *W*). *Cistus* plants enclosed in these plots were labeled in 2012 with ^{15}N and ^{13}C.

During the labeling-chasing period, in late October 2012, *W* treatment significantly increased daily minimum air temperatures by 0.5 °C, modifying also average air and minimum soil temperatures (Table 1). The upper soil layer was affected by the treatment, but only to a depth of 10 cm (data not shown). *W* also lowered soil water content (Table 1), probably by increasing evapotranspiration losses. The soil water content in all treatments varied substantially during the experiment due to three important rain events happened during the ^{15}N labeling day and on the 3^{d} and 8th day of the post-labeling sampling (hereafter chasing period) (Fig. S1).

2.2. Labeling and sampling

Prior to the labeling, on 21 October, *Cistus* leaves, twigs and soil were sampled in both *A* and *W* plots in order to measure the pre-labeling natural ^{13}C and ^{15}N abundance in these pools. Plot labeling with ^{15}N-enriched KNO_3 solution was performed on 22 October 2012. Each plot received 0.58 g of $K^{15}NO_3$ dissolved in 202 mL of water, equivalent to 1.3 kg N ha^{-1}. To ensure homogeneous labeling, plots were divided in 81 subsquares and 2.5 mL of labeling solution was added with the pipette to each square. Post-labeling sampling started on 23 October: the same plant samples were utilized for ^{13}C and ^{15}N analyses.

A and *W* plots were labeled with $^{13}CO_2$ in pairs during midday hours (10:00-15:00) on 23 October under the same environmental conditions. Prior to labeling, the other few herbaceous species growing inside the plots were covered with opaque black plastic bags to prevent ^{13}C fixation. Transparent Lexan chambers, 80 × 80 × 60 cm (for details on chamber characteristics see Guidolotti et al. (2017)), were labeled by acidifying 2.5 g of $Na_2{}^{13}CO_3$ (99 at-% ^{13}C) dissolved in 5 mL of water with 10 mL of 5 M H_2SO_4 (Hafner et al., 2012). To ensure sufficient label uptake, the chambers were kept closed for 1.5 h. Before the labeling, plot net ecosystem exchange and ecosystem respiration were measured with Li8100 (Li Cor Bio-sciences, Lincoln, NE, USA) coupled to the labeling chamber. During the labeling, temperature, relative humidity and radiation inside the chamber were continuously monitored by a HydroClip S3 probe (Rotronic, Basserdorf, Switzerland) and Apogee quantum sensor (Apogee Instruments Inc., Logan, UT, USA), respectively. Chamber temperature increased up to few degrees while the chamber was closed, but the values remained below the maximum daily temperatures the plants experience during the growing season. Light conditions inside the chamber were reduced by only 10% due to the chamber wall and were kept constant during the labeling time (data not shown).

Immediately after chamber opening, one shoot of *Cistus* per plot was sampled and divided into: twigs, summer standing leaves (hereafter old leaves) and new winter leaves (hereafter new leaves). The material was placed into liquid N for transportation and stored at −80 °C before being processed and analyzed. Soil was sampled with a corer (diameter 2 cm) in three sub-replicates per plot to a depth of 10 cm. These sub-replicates within each plot were pulled together in one sample. All soil cores were maintained at 5 °C during transportation and stored at −20 °C before being further processed. All samples were taken at day 0 (immediately after labeling) and 1, 2, 3, 4 and 16 days after labeling.

The isotopic composition of the CO_2 efflux from the soil was measured in two replicates per plot with a Keeling plot approach (Keeling, 1958, 1961). Static opaque soil chambers (~1 L) were pushed 2 cm deep into the soil 4 days prior to labeling. At the sampling time, chambers were closed with a lid and 4 air samples at 5 min intervals were taken with a syringe and transferred to pre-evacuated 10 mL vials. Gas samples were analyzed for CO_2 concentration and isotope composition on an IRMS (Isoprime, Cheadle, UK) coupled to a Multiflow (Isoprime, Cheadle, UK) within 10 days after sampling. To determine the absolute CO_2 concentration in the sampled air, a calibration curves were constructed between known CO_2 concentrations and the peak area on the mass 44 and 45 estimated by IRMS. Soil respiration measurements were performed with the same frequency as the plant sampling. Additionally, pre-labeling "background" values were determined on the air collected prior to labeling.

The isotopic composition of shoot CO_2 efflux was measured on the attached shoots including new and old leaves. Leaf gas exchange was measured with a Li6400 portable photosynthesis system equipped with an opaque chamber (6400-179 22L Lighted Conifer Chamber, Li-Cor Bio-sciences, Lincoln, NE, USA). Before the air collection, shoots were maintained for 30 min in the dark as suggested by Gratani et al. (2008). The air in the inlet was supplied at 400 ppm from the tank with a known C isotope composition. Instantaneous measurements of shoot respiration were performed with Li6400 at a reference temperature of 25 °C. The air in the leaf chamber outlet flushed a vial of 10 mL for 3 min. Two vials were sampled successively from each shoot. δ^{13}C of shoot respiration was determined with a two-end mixing model:

$$\delta^{13}C = \frac{(\delta Cout*[Cout]) - (\delta Cin*[Cin])}{([Cout] - [Cin])}$$

where $\delta Cout$ and δCin are δ^{13}C measured in the outlet and inlet of the chamber, and $[Cout]$ and $[Cin]$ are the CO_2 concentrations in the same samples. CO_2 efflux was measured once a day as previously described each time on the same shoots.

2.3. Analyses

Water-soluble organics were extracted from twigs, old and new leaves for isotope analyses as described in Brugnoli et al. (1998). Briefly, approximately 100 mg of plant material was reduced to a fine powder, suspended in demineralized water and shaken for 1 h at 50 °C. After 5 min of centrifugation at 12000*g*, the supernatant was collected and the extraction repeated twice. After freeze-drying, the extracts were processed on an IRMS (Isoprime, Cheadle, UK) coupled to an elemental

analyzer (NA1500, Carlo Erba, Milan, Italy) for $\delta^{13}C$ determination. Extracts were weighed to determine the total amount of soluble assimilates in the sample. The remaining non-soluble fraction material was freeze-dried and analyzed for $\delta^{13}C$. $\delta^{15}N$ was analyzed on the grounded and freeze-dried leaves and twigs.

Soil microbial biomass carbon and nitrogen (MBC and MBN) were determined by the fumigation-extraction method (Vance et al., 1987). Briefly, N and organic C were extracted with 0.05 M K_2SO_4 from fumigated and non-fumigated soil samples and successively measured using a CNS analyzer (Vario EL, Elementar Analysensysteme, Hanau, Germany). MBC and MBN were calculated with a conversion factor of 0.45 for C (Wu et al., 1990) and 0.54 for N (Brookes et al., 1985). K_2SO_4 extractable C (EC) and N (EN) and the associated $\delta^{13}C$ and $\delta^{15}N$ values were used as a proxy for dissolved organic matter and, in the case of C–for root exudates (Blagodatskaya et al., 2014). Fine roots (< 2 mm) collected from soils were oven-dried at 60 °C and analyzed for $\delta^{13}C$ and $\delta^{15}N$.

2.4. Stocks

To estimate the leaf area and the leaf biomass of the studied plants, all shoots belonging to each plant were counted and three representative shoots from each plant were sampled. Shoot leaf area was determined after scanning by a software for image analysis (Skyeleaf, Powys, UK) and leaf mass was determined after drying the leaves at 70 °C until constant weight. Total leaf area and total leaf mass of the plants were obtained by multiplying the shoot leaf area and shoot leaf mass (average value of the sampled shoots) by the number of shoots counted. To estimate *Cistus* woody biomass, we used allometric relationships specifically developed for *Cistus* in the experimental site (de Dato et al., 2008). The belowground biomass was estimated by daily sampling of three 2 cm-diameter soil cores to a depth of 10 cm depth. Roots collected from the cores were oven-dried at 60 °C and weighed.

2.5. Calculations

The relative distribution of ^{13}C between pools and fluxes was estimated as the percentage of ^{13}C recovered from all measured compartments immediately after labeling (day 0). For this, all isotopic δ were first converted to atom% notations. Further, the excess ^{13}C in the pool of interest at a certain time ($excess^{13}Ct$) was calculated considering the mass of the pool (C_{pool}, g m^{-2}), atom% measured in the sample (atom $\%_s$) and background atom% (atom$\%_{bg}$) values:

$$excess^{13}C_t = \frac{atom\%_s - atom\%_{bg}}{100} * C_{pool}$$

For soil CO_2 efflux and shoot CO_2 efflux, the C_{pool} is expressed in g C plot^{-2} h^{-1}. To estimate the daily recovery from these fluxes, we used linear interpolation between the adjacent daily estimates of excess ^{13}C in a flux.

To calculate a weighted recovery of a pool at each sampling time ($^{13}_{t}C_{rec}$, % of recovered ^{13}C), excess13Ct of a pool was related to the reference recovery of a plot measured immediately after labeling ($excess^{13}C_0$), which was taken as 100% (Hafner et al., 2012).

$$^{13}_{t}C_{rec} = \frac{excess^{13}C_t}{excess^{13}C_0} * 100$$

$Excess^{13}C_0$ was determined as a sum of the label excess from all pools and fluxes at time *0*. The results of a previous experiment performed in the same site demonstrated that the $\delta^{13}C$ peak of soil CO_2, measured in the first hours after labeling, is affected by the $^{13}CO_2$ diffused into the soil pores during the labeling procedure and, therefore, is not of a biological origin (Gavrichkova et al., submitted, Subke et al., 2009). Accordingly, soil respiration measured in the first 24 h and affected by a back-diffusion of the CO_2 from the soil pores was excluded from the calculations.

We applied the following equation to estimate the final allocation of the label between pools and fluxes by the end of the chasing period (Cfinal):

$$C_{final} = \sum_{14}^{0} excess^{13}C_{tresp} + \sum_{14} excess^{13}C_{tpool}$$

where $\sum_{14}^{0} excess^{13}C_{tresp}$ is the sum of the ^{13}C excess in shoot and soil respiration at each sampling time and $\sum_{14} excess^{13}C_{tpool}$ is the sum of the ^{13}C excess in aboveground and belowground pools at the last sampling, 16 days after labeling. The values of $\sum_{14}^{0} excess^{13}C_{tresp}$ and $\sum_{14} excess^{13}C_{tpool}$ were further expressed as percentages of Cfinal.

The weighted recovery of ^{15}N and its final allocation between pools was calculated by the same algorithms as for ^{13}C.

2.6. Statistical analyses

Statistical analyses were performed using the software STATISTICA 7 for Windows (StatSoft Inc., Tulsa, OK, USA). The effects of Warming treatment on climatic parameters were tested by a Wilcoxon signed rank test, verifying whether the differences between the mean daily or minimum values (Warming-Control) could be assumed to be higher than zero. Analysis of variance (ANOVA) was applied to estimate the significance of differences between the *W* and *A* treatment in C belowground stocks and CO_2 fluxes where the normality criterion was satisfied. In the case of significant differences between the *W* and *A* treatment, a post-hoc Tukey's HSD test was performed. The non-parametric Mann-Whitney *U*-test was used to estimate significant differences between the treatments for ^{13}C and ^{15}N enrichment of pools and fluxes, C allocation and tissue N contents in every time step of the chasing period as well as for aboveground C and N stock values

Table 2
Above- and belowground C and N stocks and fluxes (Mean ± SE) in Ambient and Warming plots. MBC – microbial biomass C, MBN – microbial biomass N, EC – extractable C, excess $^{13}C_0$ excess of C measured in all pools and fluxes at time 0–immediately after the labeling, excess $^{15}N_{16}$–excess of N in all pools 16 days after the labeling.

Stock	Ambient	Warming	Unit	p-value
Leaves	26 ± 7	29 ± 5	g C m^{-2}	ns
Branches	57 ± 8	89 ± 9	g C m^{-2}	ns
Roots, 0–10 cm	51 ± 13	54 ± 7	g C m^{-2}	ns
Shoot CO_2 efflux	0.24 ± 0.01	0.28 ± 0.02	g C m^{-2} h^{-1} tissue	< 0.05
MBC, 0–10 cm	134 ± 8	161 ± 9	g C m^{-2}	< 0.05
MBN, 0–10 cm	16.4 ± 1.3	18.9 ± 1.0	g N m^{-2}	< 0.05
EC, 0–10 cm	34 ± 3	36 ± 2	g C m^{-2}	ns
Soil CO_2 efflux	0.51 ± 0.06	0.66 ± 0.07	g C m^{-2} h^{-1}	ns
Photosynthesis	3.1 ± 0.67	2.0 ± 0.35	g C m^{-2} h^{-1} tissue	ns
excess $^{13}C_0$	0.22 ± 0.06	0.39 ± 0.02	g C m^{-2}	< 0.05
excess $^{15}N_{16}$	0.0028 ± 0.0013	0.0019 ± 0.002	g N m^{-2}	ns

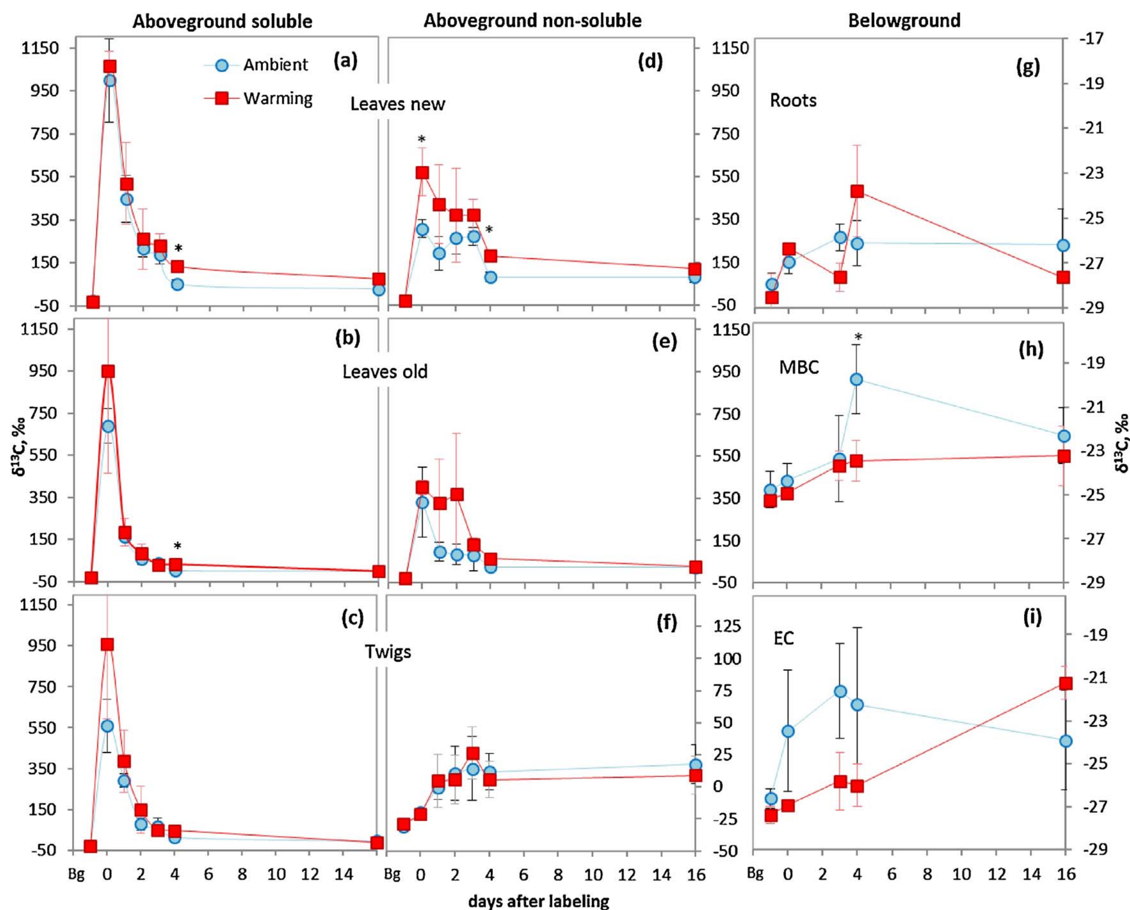

Fig. 1. $\delta^{13}C$ (Mean ± SE) of aboveground pools in Ambient and Warming plants: soluble organics in (a) – new leaves, (b) – old leaves, (c) – twigs. Non-soluble organics in (d) – new leaves, (e) – old leaves and (f) – twigs. $\delta^{13}C$ in belowground pools: (g) – roots, (h) – microbial biomass (MBC), (i) – extractable C (EC). Bg – background values measured prior to labeling. Significant differences between the treatments at $p < 0.05$ are highlighted with "*".

reported in Table 2 (n = 3).

3. Results

3.1. Carbon allocation to aboveground

Aboveground C stocks tended to be higher in *W* than in *A*, although the differences were not statistically significant (Table 2). Gas exchange measurements performed prior to the labeling on a plant level showed no differences in assimilation activity between the treatments (p = 0.82), but more ^{13}C was recovered immediately after the labeling from *W* plants (Table 2). Complexly, enrichment of aboveground pools and fluxes tended to be higher in *W* plants. (Figs. 1 and 2).

The labeled leaf-soluble organics in old and new leaves showed similar values between the two treatments (Fig. 1a and b). The maximal initial enrichment of the soluble fraction was two to three times higher compared to the non-soluble one (Fig. 1a, b, d, e). On the 4th day of the chasing period a higher $\delta^{13}C$ was measured in many pools in *W* plants ($p < 0.05$). The non-soluble fraction of new leaves was more enriched immediately after labeling in *W* than in *A* plots. $\delta^{13}C$ of both fractions extracted from twigs at any time of the chasing period was similar between treatments (Fig. 1c, f). $\delta^{13}C$ of the non-soluble fraction slowly depleted over time in leaves, whereas in twigs the label gradually accumulated (Fig. 1d–f). $\delta^{13}C$ in all aboveground fractions was similar in the last two samplings, suggesting that a steady state in label distribution was reached already on day 4 after labeling.

The highest ^{13}C enrichment among all pools and fluxes was measured for shoot respiration (Fig. 2a). $\delta^{13}C$ of the shoot respiration flux was significantly higher in *W* plots, especially in the first days after labeling. This difference was also reflected in absolute shoot respiration rates (Table 2).

3.2. Assimilates allocation to belowground

Root biomass of *Cistus* was similar between *W* and *A* plots (Table 2). ^{13}C enrichment of roots was minimal compared to the background, with similar values between treatments (Fig. 1g). Instead, considerable ^{13}C amounts were recorded in the CO_2 efflux from the soil, with higher $\delta^{13}C$ values for *A* plots (Fig. 2b). This difference between treatments was maintained during almost the whole chasing period, even when the data points affected by back-diffusion of the CO_2 from the soil pores were excluded. Absolute soil CO_2 efflux, in contrast, was similar between treatments, with a trend toward higher respiration for *W* plots (Table 2).

Absolute amounts of C and N in microbial biomass were higher in *W* plots (Table 2). Negligible ^{13}C amounts were recovered in microbial biomass, and values were higher in *A* plots on day 4 after labeling (Fig. 1h). EC, a proxy of root exudates, also tended to be more enriched in *A* soils (Fig. 1i) except at the last measurement.

3.3. Budget and recovery of assimilated carbon

Shoot CO_2 efflux was complexly a predominant sink for assimilates in *W* plots (51% of C_{final}); whereas in *A* plots the major part of the label remained incorporated in aboveground biomass. The proportion of label allocated to shoot respiration was especially high within the first day after labeling in both treatments (Fig. 3b). On a daily basis, the ^{13}C recovery in leaves was similar between the treatments, and significant

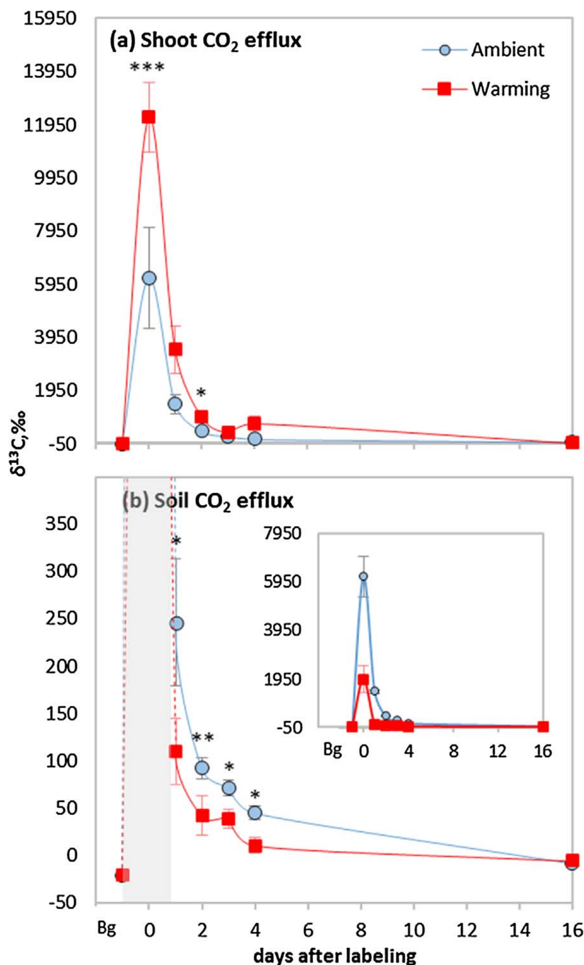

Fig. 2. $\delta^{13}C$ (Mean ± SE) in Ambient and Warming plots of (a) shoot respiration and (b) soil respiration without including values measured immediately after the labeling, which were affected by back-diffusion of the label from the soil pores. Grey area indicates a time when the contribution of back diffusion of the $^{13}CO_2$ from the soil pores is considerable. Insert: $\delta^{13}C$ in soil respiration: all available data. Bg – background values measured prior to labeling. Significant differences between the treatments at $p < 0.05$ are highlighted with "*", at $p < 0.01$–with "**" and at $p < 0.001$–with "***".

differences were recorded between day 4 and 16 of the chasing period (Fig. 3a). By the end of chasing a higher proportion of ^{13}C remained sequestered in leaves of *A* plants (10% in *A* and 5% in *W*, $p < 0.05$) with all ^{13}C being stored in the non-soluble leaf fraction (Fig. 3a). Branches were the only pool where label was accumulating in time thanks to transfer and conversion of ^{13}C to non-soluble form encompassing storage and structural C (Fig. 3c). It was a predominant sink in *A* plots (38% of C_{final}) and a second big sink in *W* plots (31% of C_{final}) with no difference between the treatments. Final allocation to belowground was higher in *A* plots (23% in *A* and 12% in *W*, $p < 0,05$, Fig. 4a) with major part of ^{13}C being respired in 16 days of chasing (Fig. 3d). Just 1–2% of C_{final} was re-distributed between main belowground pools: roots, soil and microbial biomass (data not shown).

3.4. ^{15}N uptake

The total pre-labeling N content was significantly higher in old leaves and twigs of *W* versus *A* plants, but not in new leaves (Fig. 5a–c, $p < 0.05$). Differences between treatments decreased at the beginning of the chasing period due to an increase in *A* plants, which was coupled with changes in SWC (Fig. 6a).

^{15}N label appeared gradually in the aboveground pools (Fig. 5d–f): a small increase in tissue $\delta^{15}N$ was already measured in leaves and twigs on day 1 (two days after N labeling), but the values increased rapidly starting from day 2 (three days after N labeling). Between day 4 and 16, ^{15}N reached a steady state only in twigs. Belowground, roots and microbial biomass were substantially enriched already on first day after adding ^{15}N (Fig. 5g–f). Peaks of ^{15}N enrichment of MBN and roots were reached at different times in *A* and *W* plots. By the end of the chasing period, more than half of the ^{15}N recovered (N_{final}) in plant tissues was in the roots. No between-treatment differences in the distribution of ^{15}N between pools were measured (Fig. 4b).

4. Discussion

4.1. Changes of C allocation under warming

Our data partially confirm the first hypothesis: the gain of newly assimilated C in *W* over *A* treatment during the labeling was achieved mainly through conversion of recently assimilated ^{13}C to storage pools, encompassed in non-soluble leaf fraction (Fig. 1). Additional C was merely temporary stored in this fraction and was further utilized to fuel metabolic processes, primary shoot CO_2 efflux (Fig. 4, Table 2). Higher call for reserves and their higher night-time respiratory-driven reduction has been reported in studies with similar climate manipulation designs (Rustad et al., 2001; Turnbull et al., 2002; Welker et al., 2004; An et al., 2005). We suggest that the observed differences between treatments in allocation of ^{13}C to respiration are also underestimated because we don't possess nigh-time respiration rates when the effect of treatment on temperature is at its maximal rate. The fact that night-time warming affected shoot fluxes measured during the day at a constant temperature of 25 °C is instead in contrast to the first hypothesis and points on indirect effect of night-time warming on plant activity. Indirect effects are likely linked to changes in nutrient availability with warming (Hypothesis 2 and 4). Hence, higher shoot respiration during the day hours in *W* plots should be connected to higher N availability and content observed in leaves and twigs (Fig. 5a–c). Respiration-tissues N content interaction was reported previously in many studies (Reich et al., 1998, 2008; Atkin et al., 2005). We also highlight that our experiment covers a particular phase of the Cistus phenology – recovery and growth after the end of the summer drought. Growth is an energy-demanding process characterized by additional respiration expenses (Rambal et al., 2004). We however don't possess enough experimental data to confirm or reject a more intensive growth during the experiment conduction for plants subjected to warming which could potentially explain the observed differences in label allocation to respiration.

The greater ^{13}C losses in the form of shoot CO_2 efflux, coupled to less ^{13}C remaining stored in *Cistus* biomass, might limit the C sequestration capacity of the ecosystem under warming (Fig. 4). To compensate and ensure ecosystem success in future climate, *Cistus* should assimilate more C over the vegetation period in confront to ambient conditions. Our photosynthesis data are contradictory: photosynthesis rates were very variable between single replicates and tended to be higher in *A*, whereas more label was recovered in *W* plants (excess13C0, Table 2). Llorens et al. (2004) reported that night-time warming did not affect the photosynthetic performance in four shrub ecosystems across the north-south European gradient. The authors hypothesized that the temperature increase during the day was too weak to produce any direct effects on plant performance. Nonetheless, two Mediterranean shrubs under warming did demonstrate a higher potential photochemical efficiency of photosystem II, and one shrub showed an increased carboxylation efficiency in autumn and winter. This highlights alleviation of phototosynthetic constraints during the cold season with rising temperatures (Llorens et al., 2003). In our study, shoot respiration under *W* was not limited at low photosynthetic active radiation (PAR) rates as compared to *A*. This indicates, among others, a higher carboxylation efficiency and/or more efficient light harvesting (Fig. 6b). The higher N availability is generally associated with higher

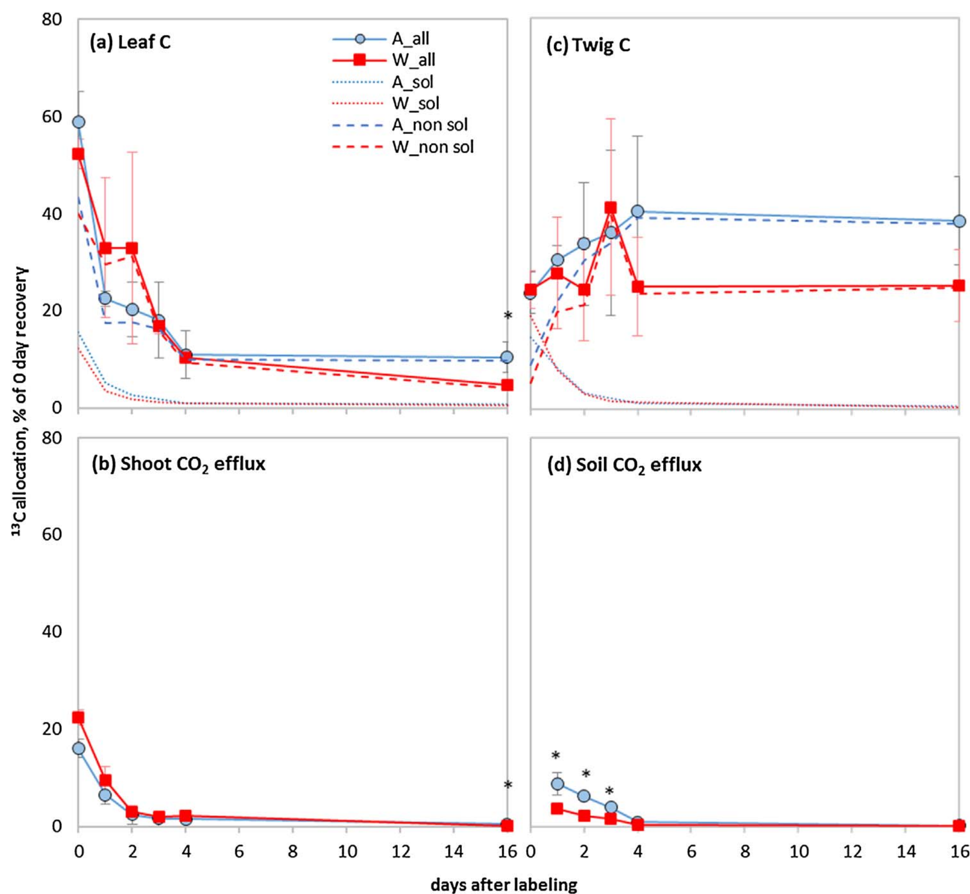

Fig. 3. ^{13}C recovery (Mean ± SE) above- and belowground pools and fluxes in Ambient (A) and Warming (W) plots: (a) ^{13}C in soluble (sol) and non-soluble (non sol) leaf fractions and in a sum of both (all); (b) ^{13}C in shoot CO_2 efflux; (c) ^{13}C in soluble (sol) and non-soluble (non sol) twig fractions and in a sum of both (all); (d) ^{13}C in soil CO_2 efflux. Significant differences between the treatments at $p < 0.05$ are highlighted with "*".

stomatal and mesophyll conductance, higher chlorophyll concentrations and better Rubisco carboxylation efficiency (e.g. Zhao et al., 2005). Combined to the reduction in the number frost days, which would likely extend the growing season in *W* plots, it should exert a positive effect on Cistus productivity on a seasonal basis. An accurate analyses of parameters related to productivity (e.g. leaf length, number of brachiblasts, total leaf area and the net ecosystem exchange on the yearly basis) is required to verify whether major C losses are effectively balanced by major C incomes in the conditions of future climate.

4.2. N availability

The higher MBC and MBN measured in *W* plots, coupled to a likely increased enzymatic performance under increased temperature (Blagodatskaya et al., 2016; Razavi et al., 2017), points to a higher mineralization potential and higher nutrient availability in *W* soils.

Hypothesized higher availability of N in plots subjected to warming was confirmed by soil N and leaves N content. Our results are in accordance with literature data reporting warming to stimulate N mineralization and increase the availability of mobilized N for plants (Rustad et al., 2001; Melillo et al., 2002; Sardans et al., 2008a,b; Blagodatskaya et al., 2016). Furthermore, our data suggest that better nutrient status of leaves in *W* plants is not linked to more efficient root N uptake as suggested in literature (Rustad et al., 2001; Welker et al., 2004; An et al., 2005): both, ^{15}N enrichment (Fig. 5d–f) and ^{15}N recovery (Table 2) were similar between *A* and *W* treatments; on the contrary, the former tended to show higher N uptake rates. Hence, higher N availability remains the primary cause of tissues N differences.

4.3. N translocation dynamics

To unravel changes in internal N dynamic under warming, ^{15}N data and tissues N content should be analyzed in synergy. Differences in tissues N content have levelled out after a considerable rain event happened during the ^{15}N labeling which interrupted one week of the rainless period and increased N content in *A* plants (Fig. 5a–c, Fig. S1). The increase in leaf N content happened fast, just in one day, in confront to 3 days necessary to transport ^{15}N from belowground to leaf tissues. The increase in leaf N after the rain event in *A* hence is not directly fueled by soil or root N, but rather by reallocation of N from woody tissues. The sampled apical twigs also demonstrated an initial increase in N after the rain event. This temporal increase would then represent a flush of N from standing below woody tissues in the direction of leaves. In fact, as opposed to the leaves, the N content in twigs further declined. Indeed, N is normally redistributed between senescent and growing tissues (Milla et al., 2005), vegetative and reproducing organs (Schiltz et al., 2005) or between leaves at different heights in order to optimize photosynthetic efficiency (Field, 1983). We suggest that the interruption of the one-week period without rain stimulated plant growth in *A* plots and consequently N demand was met by re-distribution of the internal reserves between tissues. The fact that N content in *W* plants was independent from soil water variation (Fig. 6a) suggests that the internal leaf N pool was likely sufficient to support growth as suggested by the 3[d] hypothesis.

Considering that for plants of *Cistus* height only few hours would be enough for the xylem flow to deliver water and nutrients from the roots to the leaves (Windt et al., 2006), a three-day delay in ^{15}N appearance aboveground was unexpected. A series of N transformations in soil-root phase could be responsible for that. Plants generally obtain most of N from mycorrhiza symbionts (Van Der Heijden et al., 2008) but it is not possible to separate roots from mycorrhiza during the analyses. We therefore hypothesize that mycorrhiza could have retained ^{15}N before sending it to the plant tissues. It is in agreement with the observation that mineral N taken up by mycorrhiza exhibit a series of conversions to

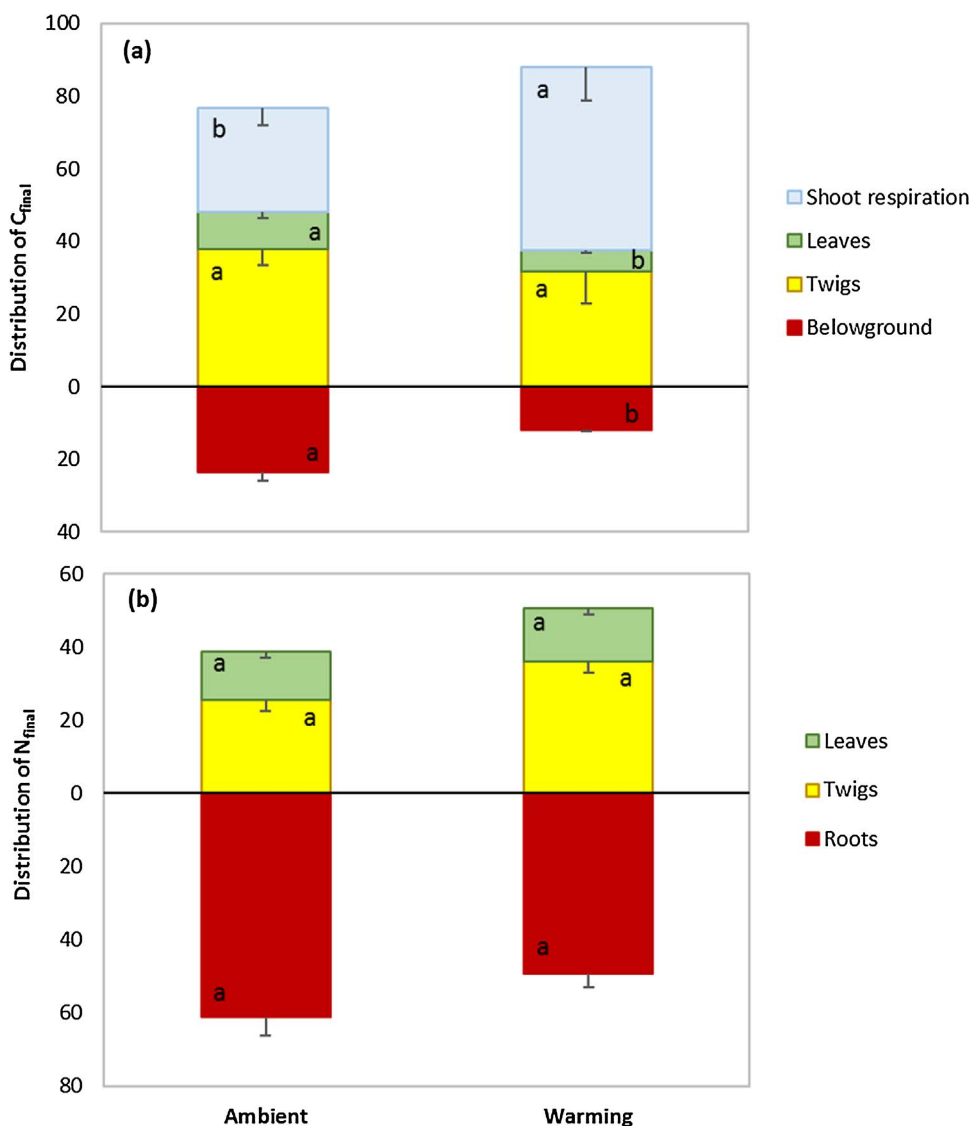

Fig. 4. Partitioning of ^{13}C (a) and (b) ^{15}N (Mean ± SE) between pools and fluxes 16 days after labeling in Ambient and Warming plots. Letters represent differences between treatments in respective pools and fluxes at $p < 0.05$.

organic N and ammonium prior being transferred to the roots (Müller et al., 2007). Second, the reduction and assimilation of mineral N forms taken up by plants proceeds in many species in roots and could be also responsible for the observed delay (Gavrichkova and Kuzyakov, 2008).

4.4. C and N interactions

As hypothesized, belowground C allocation was lowered by the treatment. We connect it with the increase of the N availability and alleviation of the nutrient constrains discussed before. Foraging for N is generally achieved through roots growth and through stimulation of microbial mineralization with rhizodeposition and hence requires investments of C into root tissues (Hamilton et al., 2008; Gavrichkova et al., 2010; Schmitt et al., 2013). Furthermore, tissues N content affects cytokinines production which are involved in source-sink regulations and influence the phloem loading/unloading rates (Van der Werf and Nagel, 1996; Roitsch and Ehneß, 2000; Gessler et al., 2004). An increase in the strength of the root sink relative to the leaf sink under N and cytokinines deprivation was proposed (Van der Werf and Nagel, 1996). Both processes, forage for nutrients and N-cytokinines interactions, affect belowground functioning by the same mechanisms – weakening the belowground sink in *W* compared with *A*. For *Cistus* under warming carbohydrate limitation should be viewed as an optimization of C redistribution between the pools.

A negative effect of warming on ^{13}C enrichment of soil CO_2 efflux and of microbial biomass, coupled to a null effect on the absolute rates of soil respiration, could be viewed as a decoupling of microbial activity from the supply of recently assimilated C from *Cistus* root exudates and as a more favorable microbial and plant nutrient status under warming. Contrary, the synchronization of ^{15}N and ^{13}C peaks in microbial biomass in *A* soil ($R^2 = 0.55$, $p < 0.05$, not shown) confirms instead a tight link between microbial metabolism and root activity (e.g. rhizodeposition) in this treatment: microbial N uptake is stimulated by root rhizodeposition, as demonstrated also by Kuzyakov and Xu (2013).

4.5. Conclusions

A prolong duration of the experimental warming – 11 years – allows structural adaptations for plants and a steady state status in the response to a long-term environmental perturbation. This is the striking difference of our experiment with other climate manipulation studies. The experimental warming shifted the ^{13}C allocation of *Cistus* to major losses through shoot CO_2 efflux and formation of short-living reserves. A major increase in N availability induced by warming contributed to elevated respiration expenses of shoots. Such a scenario may constrain plant productivity if photosynthesis remains unchanged. On contrast, the major increase in N availability decreased belowground C allocation and belowground respiration costs. The last in combination with

Fig. 5. N content (%, Mean ± SE) in Ambient and Warming plots of (a) new leaves; (b) old leaves and (c) twigs. $\delta^{15}N$ (Mean ± SE) in (d) new leaves; (e) old leaves, (f) twigs; (g) roots; (h) N in microbial biomass (MBN) (i) in extractable N (EN). Bg – background values measured prior to labeling. Significant differences between the treatments at $p < 0.05$ are highlighted with "*".

extended growing season (fewer frost days) and likely positive effect of N availability on plant photosynthetic performance should instead improve plant C gain. Both C assimilation and C losses, are intimately linked to plant N availability, which is very variable in the Mediterranean climate characterized by rainfalls from autumn to spring and long rain-free periods in summer. The success of this species in reacting to the predicted temperature increase will largely depend on the balance between N inputs through soil organic matter mineralization and nitrification on the one hand and N losses with leaching and run-off on the other.

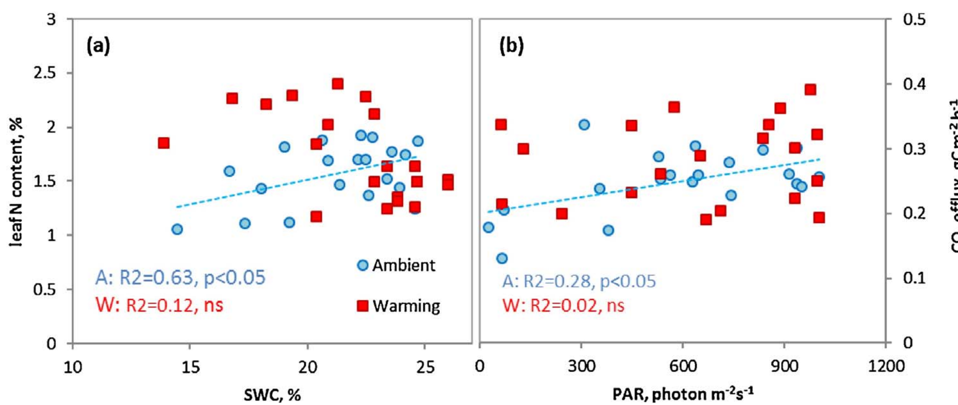

Fig. 6. (a) Leaf N content plotted against soil water content (SWC, all available data); (b) shoot respiration plotted against photosynthetic active radiation (PAR) averaged over 2 h before respiration measurements (all available data) in Ambient (A) and Warming (W) plots.

Acknowledgements

The authors thank Luciano Spaccino for helping with field sampling and isotope composition measurements. The research leading to our results was supported by European Community's Seventh Framework Programme under the project “An Integrated Network on Climate Research Activities on Shrubland Ecosystems”, in short INCREASE, grant agreement no. 227628. The present work is a part of the “CARBOSOIL” project funded by the Autonomous Province of Trento (Italy) under the “Marie Curie Action – COFUND PostDoc 2010 Incoming” program.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2017.07.013.

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