

# Interactive effects of forest die-off and drying-rewetting cycles on C and N mineralization

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## ABSTRACT

Mediterranean forests will experience more frequent and intense drought periods and extreme rainfall events in the coming decades. Concomitantly, drought-induced forest die-off is likely to increase. Changes in rainfall patterns and forest die-off directly influence soil microbial communities and activity and, consequently, carbon (C) and nitrogen (N) turnover, but their interactive effects have not yet been explored. We investigated the short-, and the long-term interactive effects of forest die-off and drying-rewetting cycles on soil C and N mineralization rates of a Mediterranean woodland. Soil samples collected under and out of the influence of holm oak (*Quercus ilex*) trees with different defoliation degrees (six healthy, six affected and six dead) were incubated under two contrasting water regimes (i.e. drying-rewetting cycles vs. constant soil moisture). Potential soil C and N mineralization responded differently to water regimes, with an overall 55% increase in C mineralization and a 22% decrease in N mineralization in the drying-rewetting cycle treatment compared to the constant moisture treatment. Holm oak decline decreased the response of C mineralization while increased the response of N mineralization to the drying-rewetting cycles at both the short- and the long-term. Moreover, N turnover showed a higher sensitivity to these environmental changes than that of C during most of the year. Our study provides solid evidence that an intensification of the drying-rewetting regimes can result in a decoupling of soil C and N cycles in Mediterranean forests and that forest die-off might enhance this decoupling at both the short- and the long-term, with important implications for the ecosystem functioning.

## 1. Introduction

Soils represent the largest carbon (C) and nitrogen (N) pools in forest ecosystems (Schlesinger and Bernhardt, 2013). When a dry soil is rewetted, a pulse of microbial activity occurs, with important consequences for soil C and N cycling at ecosystem level (Blazewicz et al., 2014; Borken and Matzner, 2009; Jarvis et al., 2007). This microbial activity pulse, named the *Birch effect* after one of its first observers (Birch, 1958), can be a major contribution to ecosystem C release into the atmosphere (Jenerette et al., 2008). The size of the pulse depends on the intensity and duration of the rainfall and the previous drought

event, as well as on the vegetation and soil organic matter content and quality (Huxman et al., 2004; Meisner et al., 2015; Morillas et al., 2017; Song et al., 2017; Wang et al., 2016). Regional- to global-scale climate change projections regarding precipitation dynamics remain relatively uncertain. However, the frequency and intensity of droughts as well as of extreme rainfall events in the Mediterranean region are expected to increase (IPCC, 2013). Also, the rainfall regimes of semiarid areas worldwide are expected to be characterized by a lower number of more concentrated rainfall events (Lafuente et al., 2018). These changes will likely increase the intensity and importance of rainfall pulses and drying-rewetting cycles in the Mediterranean basin, already

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**Table 1**

Main biogeochemical ( $n = 6$ ) and bacterial functional alpha-diversity ( $n = 5$ ) variables. Values represent the mean ( $\pm$  1SE). Statistically significant effects of defoliation ( $P_d$ ) and ecotype ( $P_e$ ) are represented by bold  $P$  values. Different letters represent significant differences among defoliation degrees in each ecotype ( $P < 0.05$ , ANOVA). Underlined values denote significant differences between ecotypes for the respective defoliation degree and season ( $P < 0.05$ ). TC = total carbon; TN = total nitrogen; SIR = substrate-induced respiration;  $H'_{\text{bact}}$  = soil bacterial functional Shannon index;  $S'_{\text{bact}}$  = soil bacterial functional richness, Sp = spring; Su = summer.

		Holm oak ecotype			Grassland ecotype			Mixed models	
		Healthy	Affected	Dead	Healthy	Affected	Dead	$P_d$	$P_e$
TC	Sp	<u>3.06 (0.72)</u>	<u>2.34 (0.28)</u>	<u>2.60 (0.31)</u>	<u>1.04 (0.08)</u>	<u>1.38 (0.21)</u>	<u>1.33 (0.18)</u>	0.925	< <b>0.001</b>
TN	Sp	<u>0.24 (0.04)</u>	<u>0.19 (0.02)</u>	<u>0.20 (0.02)</u>	<u>0.11 (0.01)</u>	<u>0.10 (0.02)</u>	<u>0.12 (0.01)</u>	0.365	< <b>0.001</b>
$\text{NH}_4^+ \text{-N}$	Sp	<u>5.28 (2.01)</u>	1.83 (0.90)	2.84 (0.85)	<u>0.10 (0.10)</u>	0.08 (0.08)	1.51 (1.36)	0.387	< <b>0.001</b>
	Su	<u>7.64 (1.41)a</u>	3.87 (0.70)ab	3.66 (0.71)b	<u>4.15 (2.22)</u>	2.68 (0.60)	4.09 (0.88)	0.293	<b>0.014</b>
$\text{NO}_3^- \text{-N}$	Sp	<u>4.96 (0.73)</u>	<u>5.59 (0.57)</u>	4.24 (0.21)	<u>2.48 (0.59)</u>	<u>2.21 (0.57)</u>	2.39 (0.38)	0.605	< <b>0.001</b>
	Su	1.07 (0.22)	1.84 (0.23)	1.75 (0.33)	1.39 (0.22)	1.35 (0.12)	1.85 (0.33)	0.095	0.894
SIR	Sp	31.5 (9.28)	38.7 (4.73)	46.0 (16.0)	17.7 (4.62)	13.8 (2.39)	39.5 (8.37)	0.073	<b>0.036</b>
	Su	20.9 (6.67)	16.1 (1.86)	15.6 (2.77)	10.1 (2.32)	8.38 (1.43)	8.42 (1.24)	0.775	< <b>0.001</b>
$H'_{\text{bact}}$	Sp	<u>4.47 (0.05)</u>	<u>4.39 (0.06)</u>	4.47 (0.07)	<u>4.09 (0.09)</u>	<u>3.90 (0.12)</u>	4.28 (0.11)	<b>0.027</b>	< <b>0.001</b>
	Su	4.17 (0.05)	<u>4.07 (0.10)</u>	3.89 (0.06)	4.02 (0.10)	<u>3.68 (0.14)</u>	3.75 (0.09)	0.052	< <b>0.001</b>
$S'_{\text{bact}}$	Sp	27.8 (0.58)	27.2 (0.80)	27.8 (0.58)	24.0 (1.52)	23.2 (1.46)	26.4 (1.17)	0.212	< <b>0.001</b>
	Su	25.0 (0.77)a	23.0 (1.55)ab	19.6 (1.63)b	21.8 (2.11)	17.8 (2.13)	16.8 (2.08)	<b>0.042</b>	<b>0.001</b>

TC and TN are expressed in %;  $\text{NH}_4^+ \text{-N}$  and  $\text{NO}_3^- \text{-N}$  are expressed in mg N kg soil<sup>-1</sup>; SIR is expressed in mg C kg soil<sup>-1</sup> h<sup>-1</sup>.

characterized by a large intra-annual variation in soil water content that largely regulates ecosystem functioning (Gallardo et al., 2009). Whilst recent studies have suggested that small rainfall pulses and drying-rewetting processes may be the main driver of soil C and N cycling in Mediterranean environments (Rey et al., 2017; Song et al., 2017; Wang et al., 2016), the effects of drying-rewetting cycles on both C and N mineralization rates of Mediterranean forest soils have not yet been well elucidated (Wang et al., 2016).

Mediterranean forests are representative of one of the most widely distributed semi-arid areas throughout the world (Jarvis et al., 2007) and are considered biodiversity hotspots. Intensified droughts are increasing tree defoliation and mortality in these forests (Carnicer et al., 2011; Lloret et al., 2004). On the short-term, soil microbial-driven C and N mineralization could rise followed by rapid N losses due to stimulated decomposition of litter, roots and dead wood (Edburg et al., 2012; Lloret et al., 2014; Xiong et al., 2011). On the long-term, repeated drought-induced mortality events, along with an unsuccessful recruitment of the dominant tree species in these forests may lead to a vegetation succession process where trees would be replaced by understory species (Ibáñez et al., 2015; Saura-Mas et al., 2014). This succession process would have an impact on ecosystem functioning, and therefore on critical local and global ecosystem services, even more severe than the short-term direct effects of tree defoliation and mortality (Anadón et al., 2014; Ávila et al., 2016; Díaz, 2014). However, the impacts of forest die-off on soil microbial functioning both at the short- and long-term are still far from being understood (Ávila et al., 2016; Rodríguez et al., 2017). More importantly, although there is increasing evidence that multiple environmental changes can generate effects that are not predictable from single-factor studies (Doblas-Miranda et al., 2017; Matesanz et al., 2009; Morillas et al., 2015), how forest die-off may influence the response of microbial functioning to changing rainfall patterns has not been yet studied.

Herein, we aimed to investigate the short- and long-term interactive effects of forest die-off and drying-rewetting cycles on soil microbial activity, as determined by C and N mineralization rates, of a holm oak (*Quercus ilex*) woodland. To do so, we collected and biogeochemically characterized soil samples under (holm oak ecotype) and out (grassland ecotype) of the influence of selected holm oak trees with different defoliation degrees. Then, we measured soil potential C and N mineralization rates under two different water regimes (i.e. constant soil moisture vs. drying-rewetting cycles). The comparison among samples collected in the holm oak ecotype allowed us to explore the short-term effects of holm oak decline (HOD). The comparison among samples collected in both (holm oak vs. grassland) ecotypes allowed us to

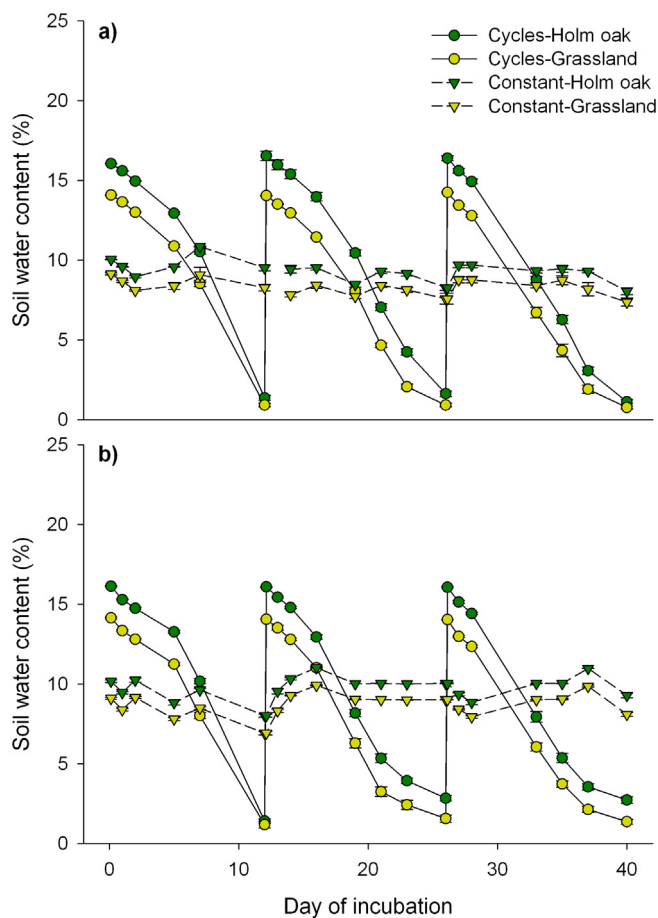
explore the HOD long-term effects under a plant succession scenery (Rodríguez et al., 2017). Based on previous studies that showed different sensitivity of N- and C-related processes to environmental changes (e.g. Durán et al., 2013; Morillas et al., 2015, 2017; Rodríguez et al., 2017; Tan and Wang, 2016), we hypothesized that soil C and N mineralization would respond differently to drying-rewetting cycles, as well as to the interaction of HOD and soil water regime. Further, given the seasonality in soil water availability of Mediterranean ecosystems, and its influence on biogeochemical cycles, we hypothesized that the response of C and N mineralization to these environmental changes would vary depending on the time of the year (spring vs. summer).

## 2. Material and methods

### 2.1. Study site

The study was carried out in a holm oak woodland located in the central part of the Iberian Peninsula, southwest of Madrid (40°23'N, 4°11'W; 630–660 m above sea level). Climate is continental Mediterranean with mean annual temperature of 15 °C and mean annual precipitation of 558 mm (Ninyerola et al., 2005). Most rainfall concentrates from autumn to spring, while summers are warm and dry. Atmospheric N deposition in the study area is around 5–6 kg N ha<sup>-1</sup> year<sup>-1</sup> (Im et al., 2013; Ochoa-Hueso et al., 2013, p.). Soil is a Cambisol, sandy and slightly acid (pH ~ 6.3), with low total C and N content (Table 1). Aboveground vegetation is characterized by a tree density of ~180 trees ha<sup>-1</sup>, mostly composed of *Quercus ilex* ssp. *Ballota* L. (holm oak) with scarce *Juniperus oxycedrus* Sibth. & Sm (cedar). The understory is dominated by *Retama sphaerocarpa* L., *Lavandula stoechas* ssp. *pedunculata* (Mill.) Samp. ex Rozeira, and diverse pasture species (see Rodríguez et al., 2017 for more information about the study site).

This region suffered a severe drought in 2005 (European Environment Agency, 2008), with a 55% decrease in the average annual rainfall. This drought resulted in a strong event of holm oak defoliation (around 20–30% of the total population) and mortality (15%) that persists (Valladares, unpublished data). Consequently, this woodland shows a successional chronosequence that goes from a range of holm oak trees with different defoliation degree (holm oak ecotype) to a grassland ecotype, with contrasting soil biogeochemistry and microbial diversity characteristics (Table 1).



**Fig. 1.** Dynamic of soil moisture of samples collected under the holm oak and the grassland ecotype both in spring (a) and summer (b), and subjected to the drying-rewetting cycles and the constant moisture regime. Symbols and error bars represent means ( $n = 18$ ) and standard errors (1SE) of the mean, respectively.

## 2.2. Experimental design

We selected 18 holm oak trees of similar size based on its crown defoliation degree (six healthy, six affected, and six dead trees) separated at least 10 m from other trees. For each tree, we established a 5-m, north-facing transect with two sampling points, one under the tree canopy, at 0.3 m from the trunk (rhizosphere of holm oak ecotype), and one 5 m away from the trunk (rhizosphere of grassland ecotype), out of the influence of the tree (Rodríguez et al., 2011; Tang and Baldocchi, 2005). This paired-point design has been successfully used in the past (Rodríguez et al., 2017), and allowed us to explore both the short- and the long-term effects of holm oak decline (HOD). Moreover, it allowed us to measure the effect of HOD while distinguishing it from other confounding factors such as the inherent spatial variability of soils (Barba et al., 2013). At the end of spring (June) and summer (September) of 2013, one soil sample was collected from the first 10 cm of soil in each sampling point using a marked stainless steel corer (5 cm wide  $\times$  20 cm long), and kept at 4 °C until analysis. For more information about the experimental design see Rodríguez et al. (2017).

## 2.3. Soil preparation

Soil samples were sieved (2 mm mesh size), homogenized under field moist conditions and analyzed for soil water content (SWC) by oven-drying a subsample of 5 g at 60 °C to constant weight. Water holding capacity (WHC) was determined following Rey and Jarvis (2006).

## 2.4. Soil biogeochemistry and microbial diversity

Soil total C and N content was determined by dry combustion with an elemental analyzer (LECO TruSpec CN). Given the temporal stability of these two variables and the proximity of the spring and summer samplings, these analyses were carried out only for the spring samples. Soil inorganic N, microbial biomass and functional alpha-diversity of soil bacterial communities were estimated in both spring and summer soil samples as described in Rodríguez et al. (2017). Briefly, soil inorganic N was extracted by shaking fresh soil subsamples with 0.5 M  $K_2SO_4$  in an orbital shaker and filtering the suspension through a 0.45 mm Millipore filter. Then, we used these extracts to colorimetrically determine the amount of  $NH_4^+$ -N and  $NO_3^-$ -N (Durán et al., 2009). Soil microbial biomass was estimated by using the substrate-induced respiration (SIR) method (Anderson and Domsch, 1978). We estimated the functional alpha-diversity and richness of soil bacterial communities by using Biolog® EcoPlates™ (BIOLOG Inc., Hayward, CA) to assess the community-level physiological profiles (CLPP), following Flores-Rentería et al. (2016). Biolog EcoPlates is a method based on carbon substrate utilization recognized as a useful tool for comparing microbial communities. Briefly, soil microorganisms were extracted by shaking fresh soil subsamples with sterile 0.8% saline solution (NaCl) in an orbital shaker for 20 min, and left to stand at room temperature for 30 min. The supernatant was diluted into the sterile saline solution to a 1:1000 final dilution, mixed for 30 s and left to stand for 10 min. Then, a 100 ml aliquot of each diluted solution was added to each of 96 wells (arranged by triplicate for each substrate). Plates were incubated at 28 °C in a humidity-saturated environment and darkness. Optical density (590 nm), which is indicative of carbon-source utilization, was measured and recorded every 24 h during 7 days using a Victor3 microplate reader (Perkin-Elmer Life Sciences, Massachusetts, USA). Optical density (absorbance) at the day the plate reached the asymptote (120 h) was the value used in all posterior analyses. The three values for each individual substrate within a plate were averaged. Functional alpha-diversity, i.e. how diversified the species are within a site, of bacterial communities was evaluated through both the Shannon index ( $H'_{bact}$ ) and richness ( $S_{bact}$ ). Shannon index was calculated as follows (Formula 1):

$$Shannon (H') = - \sum_{i=1}^s \frac{n_i}{N} \times \ln \left( \frac{n_i}{N} \right) \quad (1)$$

where  $n_i$  is the absorbance of a specific well (C substrate) and N is the whole absorbance of the plate. Functional richness is calculated as the total number of C substrate catalyzed.

## 2.5. Soil incubation experiment: potential C and N mineralization rates

We carried out 40-day, in darkness laboratory incubations (25 °C) of the 18 soil samples collected under the holm oak (H) and under the grassland (G) ecotype both in spring and summer. Subsamples (50 g) of each soil sample were incubated at one of the two different water regimes: a constant regime with steady soil moisture at 25% WHC of soil samples (i.e. 10% and 9% SWC for H and G samples, respectively), and a cycles regime with three 13-days drying-rewetting cycles, in which soil water content oscillated between 40% WHC (16% and 14% SWC for H and G samples, respectively) and 10% WHC (4% and 3% SWC for H and G samples, respectively) (Fig. 1). Rewetting events consisted in adding  $\sim 1.8$  mm of water to reach the 40% WHC. The drying-rewetting cycles regime resembles the natural annual oscillation in soil water content at the study site, while the constant moisture regime represents a control based on the annual mean encountered at the site. The comparison between these two treatments allowed us to quantify the net effect of the drying-rewetting cycles on C and N mineralization processes (Borken and Matzner, 2009). All soil samples received the same amount of water in both regimes.

To estimate potential C mineralization rates, we periodically measured the heterotrophic respiration rate ( $R_H$ ) during the incubation by placing each sample inside a 1 l gastight glass jar with a lid connected to a portable, closed-chamber soil respiration system (EGM-4, PP systems, MA, USA) during 60 s. Then, we used the ideal gas law equation to convert and extrapolate the net  $CO_2$  increase (ppm) to mass of C ( $m$ ) in the headspace of the jar (Formula 2):

$$m = \frac{ppm \times P \times V \times M}{R \times T} \quad (2)$$

where  $P$  and  $V$  are, respectively, the air pressure (ATM) and the known headspace volume in the jar (l),  $M$  is the atomic mass of carbon ( $g \text{ mol}^{-1}$ ),  $R$  is the universal constant of gases ( $0.08206 \text{ ATM l mol}^{-1} \text{ K}^{-1}$ ) and  $T$  is the temperature (K) at the measurement time.

In both water regimes, we carried out  $R_H$  measurements before, and 3 h after the first soil rewetting (Day 0), as well as on days 1, 2, 5, 7, 12, 19, 26, 33 and 40. In the case of the drying-rewetting cycles regime, we rewetted soils on days 12 and 26 (after they reached 10% WHC) and measured  $R_H$  3 h after rewetting and on days 13, 14, 27 and 28. We estimated the potential C mineralization rate for each water regime ( $C_m$ ) by interpolation of  $R_H$  between measurement dates. Potential C mineralization was normalized both by dry soil mass ( $mg \text{ C-CO}_2 \text{ kg}^{-1} \text{ soil day}^{-1}$ ) and by soil initial C content ( $g \text{ C-CO}_2 \text{ kg}^{-1} \text{ C day}^{-1}$ ).

We estimated the pulse effect of each rewetting event on microbial respiration ( $R_{Hpulse}$ ) as follows (Formula 3):

$$R_{Hpulse} = R_{Hmax} - R_{H0} \quad (3)$$

where  $R_{Hmax}$  is the maximum heterotrophic respiration rate we observed after the rewetting event and  $R_{H0}$  is the heterotrophic respiration rate measured right before the rewetting event.

Potential net N mineralization rates ( $N_m$ ) for each water regime were estimated by assessing the increase in total inorganic N after the 40 days of incubation. To do so, we measured soil inorganic N ( $NH_4^+ - N + NO_3^- - N$ ) before and after the incubation of soil samples. Inorganic N was extracted by shaking fresh soil subsamples (5 g) with 25 ml of 0.5 M  $K_2SO_4$  for 1 h at 200 rpm in an orbital shaker and filtering the suspension through a 0.45 mm Millipore filter. Then, we used these extracts to colorimetrically determine the amount of  $NH_4^+ - N$  and  $NO_3^- - N$  as described by (Durán et al., 2009). Potential N mineralization was normalized both by dry soil mass ( $mg \text{ N kg}^{-1} \text{ soil day}^{-1}$ ) and by initial C content ( $mg \text{ N kg}^{-1} \text{ C day}^{-1}$ ). Temporal dynamics of net N mineralization were not measured.

Finally, to explore the interactive effect of holm oak decline (HOD) and the change in soil water regime from constant moisture to drying-rewetting cycles, we estimated the percent change in  $C_m$  and  $N_m$  for each sample as follows (Formula 4):

$$\text{Percent change} = \left( \frac{X_{cy} - X_{cte}}{X_{cte}} \right) * 100 \quad (4)$$

where  $X_{cy}$  is the mineralization rate in the cycles regime and  $X_{cte}$  is the mineralization rate in the constant regime.

### 2.6. Statistical analyses

The effects of defoliation degree and ecotype, as well as the interaction among them, on soil biogeochemistry and bacterial functional alpha-diversity variables were assessed for each season using linear mixed-effects models. Tree identity was considered as a random factor to account for the likely spatial dependency of the two ecotypes (holm oak and grassland) selected for the same tree. Similarly, the effects of water regime, defoliation degree and ecotype, as well as the interactions among them, on potential C and N mineralization rates ( $C_m$  and  $N_m$ , respectively) were assessed for each season using linear mixed-effect models with tree as a random factor. We also used linear mixed-effects models to test the general effect of defoliation degree on  $C_m$  through the

incubation for each season and ecotype separately, and the general effect of defoliation degree, ecotype and season on the percent change of  $C_m$  and  $N_m$  and  $R_{Hpulse}$ . Temporal dependencies (repeated measures) were considered by using tree as a random factor.

Simultaneous tests for general linear hypotheses (multiple comparisons of means: Tukey contrasts) were performed to test pairwise statistical differences between ecotypes for each defoliation degree, season and water regime. Subsequently, and due to the strong influence of the ecotype factor, we used one-way ANOVA to evaluate the effect of defoliation degree within each ecotype, water regime and season level separately, as well as on the potential C mineralization rate for each day individually. Pairwise statistical differences among defoliation degrees were tested using the Tukey's test.

All statistical analyses were performed in R 3.1.1 (R Core Team, 2014). Linear mixed-effects models and simultaneous tests for general linear hypotheses were performed using the statistical package lme4 (Bates et al., 2015) and multcomp (Bretz et al., 2011), respectively.

## 3. Results

### 3.1. Effect of defoliation and ecotype on soil biogeochemistry and microbial functional diversity

Defoliation significantly decreased  $NH_4^+ - N$  and bacterial functional richness ( $S_{bact}$ ) in soil samples collected under the holm oak ecotype in summer (Table 1). Neither total C and N nor microbial biomass (SIR) were significantly affected by the defoliation degree (Table 1). All variables were significantly higher in the holm oak than in the grassland ecotype (Table 1), except for the  $NO_3^- - N$  concentration of soil samples collected in summer, which did not show significant differences.

### 3.2. Effect of water regime on potential C and N mineralization rates

Water regime had significant and contrasting effects on  $C_m$  and  $N_m$  (Table 2). Drying-rewetting cycles increased  $C_m$  (55% increase in average) while decreased  $N_m$  (22% decrease in average) with respect to the constant soil moisture treatment (Fig. 2). Percent changes of  $C_m$  and  $N_m$  were not significantly different between seasons considering both

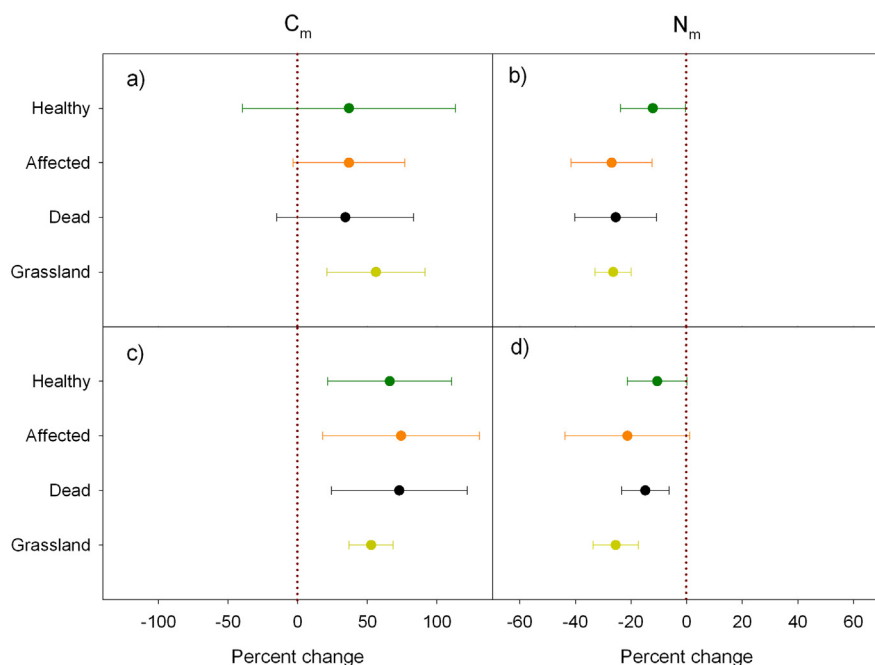
**Table 2**

Statistical results of mixed models to test the effect of defoliation degree, ecotype and water regime on the potential C and N mineralization rates ( $C_m$  and  $N_m$ , respectively) of soil samples collected in spring and summer. Statistically significant effects of defoliation (D), ecotype (E) and water regime (WR), as well as significant interactions among factors, are represented by bold  $P$  values.

Mixed models	Spring			Summer		
	$\eta^2$	df	$P$	$\eta^2$	df	$P$
$C_m$						
D	1.169	2	0.558	3.532	2	0.171
E	21.466	1	< <b>0.001</b>	14.490	1	< <b>0.001</b>
WR	17.437	1	< <b>0.001</b>	100.766	1	< <b>0.001</b>
D x E	1.565	2	0.457	1.465	2	0.481
D x WR	3.289	2	0.193	2.638	2	0.267
E x WR	0.566	1	0.452	6.279	1	<b>0.012</b>
D x E x WR	3.290	2	0.193	1.414	2	0.493
$N_m$						
D	1.504	2	0.472	0.448	2	0.799
E	114.411	1	< <b>0.001</b>	23.097	1	< <b>0.001</b>
WR	61.164	1	< <b>0.001</b>	59.935	1	< <b>0.001</b>
D? x ?E	3.808	2	0.149	0.479	2	0.787
D? x ?WR	5.126	2	0.077	1.576	2	0.455
E? x ?WR	3.268	1	0.071	4.334	1	<b>0.037</b>
D? x ?E? x ?WR	2.681	2	0.262	6.018	2	<b>0.049</b>

Variables expressed as  $mg \text{ C-CO}_2$  or  $N \text{ kg}^{-1} \text{ soil day}^{-1}$ .





**Fig. 2.** Percent change in potential C and N mineralization rates ( $C_m$  and  $N_m$ , respectively) between the drying-rewetting cycles and the constant soil moisture regime treatments. Dots are the mean of soil samples collected under healthy, affected and dead trees and under the grassland ecotype both in spring (a–b) and summer (c–d) and subjected to the 40-day laboratory incubation ( $n = 6$ ). Error bars indicate the 95% confidence interval. Positive values indicate that drying-rewetting cycles had a positive effect compared to the constant soil moisture treatment, negative values indicate the opposite.

ecotypes together ( $\chi^2 = 1.92$ ,  $P = 0.166$  and  $\chi^2 = 0.66$ ,  $P = 0.417$ , respectively). The temporal dynamics of C mineralization in each water regime were similar for all soil samples regardless of the ecotype, defoliation degree and season (Fig. 3a). Carbon mineralization boosted in each rewetting event respect to the constant soil moisture regime (Fig. 3a), with  $R_{Hpulse}$  mean values of 27.0, 9.9 and 5.7 mg C-CO<sub>2</sub> kg soil<sup>-1</sup> h<sup>-1</sup> (Fig. 4; Table S1).

### 3.3. Effect of defoliation on the response of potential C and N mineralization rates to different water regimes (HOD short-term effects)

Soil samples collected in the holm oak ecotype showed significant changes in  $C_m$  with water regime only in summer ( $P < 0.05$ ), with no differences among defoliation degrees in any season (Fig. 2). Considering each water regime separately, we found a significant and negative effect of HOD on  $C_m$  of soil samples collected in summer and subjected to the water cycles regime (Table 3). Soil samples collected under affected and dead trees showed a trend to lower values of  $C_m$  compared to soils collected under healthy trees (30% decrease), with significant differences ( $P < 0.01$ ) one day after the first rewetting (Fig. 3b). Soil samples collected under affected and dead trees also showed values of  $R_{Hpulse}$  for the second rewetting event marginally lower than those of soils collected under healthy trees ( $P = 0.06$ ; Table S1). This negative effect of HOD was only marginally significant when we expressed  $C_m$  on a C basis (Table S2). In the case of  $N_m$ , we found significant negative effects of water regime ( $P < 0.05$ ) for soils collected under affected and dead trees in both seasons, but never for samples collected under healthy trees (Fig. 2). Considering each water regime separately, HOD did not significantly affect  $N_m$  (Table 3).

### 3.4. Effect of ecotype on the response of potential C and N mineralization rates to different water regimes (HOD long-term effects)

Potential C and N mineralization rates were always significantly higher ( $P < 0.001$ ) in the holm oak ecotype than in the grassland ecotype regardless of the water regime, season and defoliation degree (Tables 2 and 3). These ecotype effects disappeared when we expressed these variables on a C basis (Table S2).

Potential C and N mineralization rates of the grassland ecotype responded in the same direction to the water regime change than those of

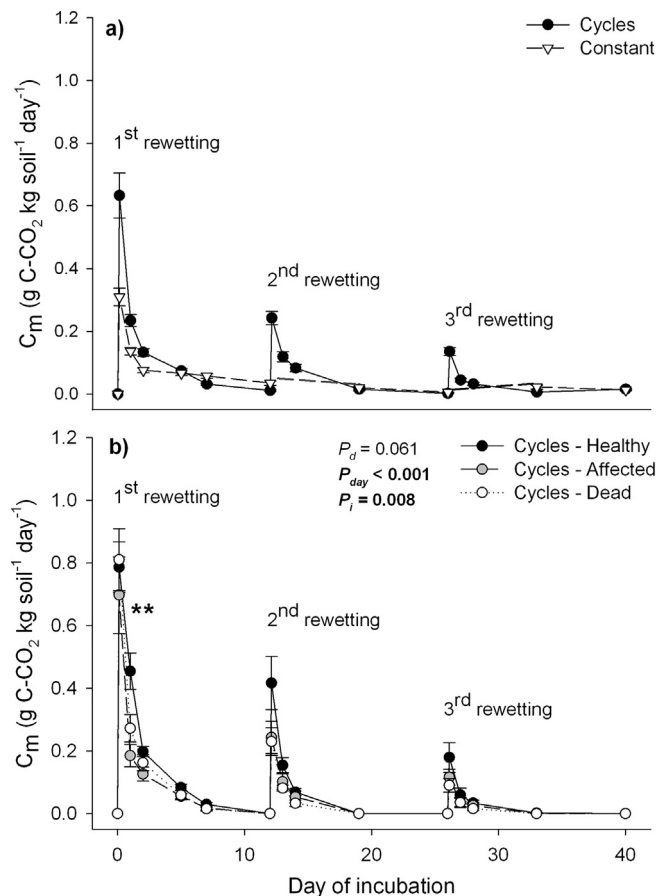
the holm oak ecotype (with increased C mineralization and decreased N mineralization) with significant ( $P < 0.05$  in all cases) percent changes regardless of the season and the defoliation degree (Fig. 2). We found a significant ecotype x water regime interaction ( $P < 0.05$ ) in the  $C_m$  and  $N_m$  of soil samples collected in summer (Table 2), with an average increase in  $C_m$  higher in the holm oak (71%) than in the grassland (53%) ecotype, and an average decrease in  $N_m$  lower in the holm oak (16%) than in the grassland ecotype (26%).

## 4. Discussion

### 4.1. Response of potential C and N mineralization to drying-rewetting cycles

The three rewetting events triggered rapid increases in microbial respiration within the first 24 h. Not surprisingly, the CO<sub>2</sub> pulses were progressively lower and slightly shorter throughout the consecutive rewetting events, which can be largely explained by substrate depletion as incubation progresses (Song et al., 2017). Rainfall pulses wet the soil surface, making labile C and dead microbial biomass accumulated over the dry period available for microbial decomposition (Blazewicz et al., 2014; Rey et al., 2017; Rey et al., 2005) and producing changes in soil microbial communities (Barnard et al., 2013; Evans and Wallenstein, 2014). Additionally, water physical CO<sub>2</sub> displacement from soil pore spaces may also contribute to soil CO<sub>2</sub> pulses in the first hours upon rewetting (Rey et al., 2017). Independently of the mechanisms behind the observed CO<sub>2</sub> pulses, our results corroborated the rapid and strong response of semiarid soils to even small rainfall pulses after a drought event (e.g. Rey et al., 2017; Song et al., 2017; Wang et al., 2016). This rapid CO<sub>2</sub> pulse after a dry soil is rewetted (Birch effect) is of great interest due to its important implications for soil C stocks (Blazewicz et al., 2014; Borken and Matzner, 2009), ecosystem C balances (Ma et al., 2012) and global warming (Jarvis et al., 2007). This is particularly true in Mediterranean and semiarid ecosystems where water availability is the main limiting factor of ecosystem functioning (e.g. Jarvis et al., 2007; Rey et al., 2017; Song et al., 2017).

In our study, the observed decrease in N mineralization with the drying-rewetting cycles respect to the constant moisture treatment disagreed with results from previous laboratory studies (see Borken and Matzner, 2009). While those studies used extended wetting and extreme and short drying periods, we simulated the natural



**Fig. 3.** Temporal dynamics of potential C mineralization rate of: a) all soil samples together ( $n = 36$ ) for each one of the water regimes (drying-rewetting cycles vs. constant soil moisture); and b) samples collected in summer under healthy, affected and dead trees (holm oak ecotype) and subjected to the drying-rewetting cycles regime ( $n = 6$ ). Symbols and error bars represent means and standard errors (1SE), respectively. Significant differences among defoliation degrees for each one of the incubation days individually are denoted by: \*\* =  $P < 0.01$ .  $P_d$ ,  $P_{day}$  and  $P_i$  show the significant level of defoliation degree, incubation day and the interaction of both, respectively (mixed models).

Mediterranean climatic conditions by using short and small rewetting events between relatively long drying periods. The different length and intensity of the drying and rewetting events could largely explain these contrasting results, as other studies using drying-rewetting cycles that simulated natural conditions also found decreases in net N mineralization (Morillas et al., 2015). Decreased N mineralization rates might be explained by substrate and/or water availability limitation, changes in soil microbial community composition and/or activity (Stark and Firestone, 1995), higher N immobilization (Gallardo et al., 2009) and increased N denitrification (Morillas et al., 2015) during the drying-rewetting cycles regime. Values of ammonium (higher in summer than in spring) and nitrate (higher in spring than in summer) at the studied site suggest that there would be enough ammonium for autotrophic nitrifiers in soil to act, which points out to a more likely water limitation of the nitrification. We did not register a significant effect of the water regimes on microbial biomass (substrate-induced respiration, Table S3), but the drying-rewetting cycles could have favored a soil microbial community with higher requirements of N and therefore lower C:N ratios (Mouginot et al., 2014). Alternatively, whilst it is unlikely that our soils experienced the anaerobic conditions traditionally considered necessary for denitrification, recent studies have suggested that denitrification can be relevant even at high soil  $O_2$

concentrations (Morse et al., 2015a, 2015b). Our study does not allow us to confirm which of these mechanisms drives the observed decrease in N mineralization. However, it clearly shows the potential negative effect of drying-rewetting cycles on the cycling and availability of these nutrients, particularly as drought periods between erratic rainfall events are expected to be more intense in Mediterranean forests due to climate change.

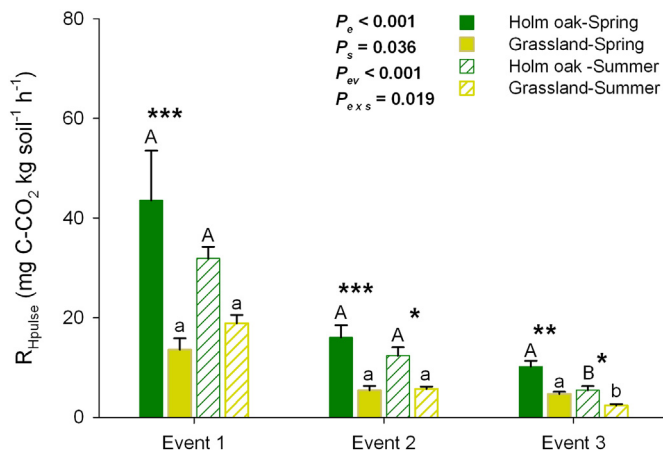
Overall, the drying-rewetting cycles treatment led to a 55% increase in the potential C mineralization rate but a 22% decrease in the potential N mineralization rate compared with the constant moisture treatment. These results confirmed our hypothesis about potential C and N mineralization rates responding differently to drying-rewetting cycles. More importantly, these results support recent studies suggesting a different sensitivity and response of C- and N-cycling to environmental changes (Durán et al., 2013; Morillas et al., 2015, 2017; Rodríguez et al., 2017; Tan and Wang, 2016). A different response in C and N mineralization could eventually lead to a decoupling of the C and N cycles, with the subsequent major effects on ecosystem function, such as asynchronies in N supply and demand or a decrease in the capacity of forest to sequester C (Fernández-Martínez et al., 2014; Finzi et al., 2011; Rodríguez et al., 2014).

#### 4.2. Short- and long-term effects of HOD on the response of potential C and N mineralization rates to drying-rewetting cycles

In our study, whereas differences among defoliation degrees in soils collected under holm oak trees indicate short-term (years) responses to holm oak decline, differences between ecotypes (holm oak vs. grassland) might be indicative of longer-term (decades/centuries) changes in ecosystem functioning. On the short term, HOD did not significantly affect the response of soil C mineralization to the water regime change, but we did find a negative effect of defoliation degree on the  $C_m$  of summer soil samples subjected to the cycles treatment. Further, we observed a negative, long-term HOD effect on the response of soil C mineralization to the water regime change, as soils collected in summer under the grassland ecotype showed lower increases in the  $C_m$  compared to those collected under the holm oak ecotype. Such lower response of soils from the grassland than from the holm oak ecotype could be largely explained by the lower soil total C content in the grassland samples (Table 1). Soils collected below different defoliation degrees did not show significant differences in soil C content. However, the fact that the differences observed in  $C_m$  among defoliation degrees were only marginal when we expressed this variable on a C basis points out both soil C content and quality as possible drivers of those differences.

Microbial communities less diverse and with different historical water regimes may have also contributed to curtail the response of C mineralization to the drying-rewetting events (Wang et al., 2016). For instance, soil bacterial communities showed decreasing functional richness with HOD both at the short- and at the long-term (Table 1). In a previous study carried out in the same site, Rodríguez et al. (2017) found a HOD-driven ecotype convergence in the soil microbial community tightly linked to a cascade effect where *Q. ilex* is being replaced by understory species (savannization process). Thus, soil microbial communities under affected and dead holm oaks, as well as under the grassland, are likely to be similar, and therefore have a similar response to environmental changes. Further, the decreased canopy cover could have selected soil microbial communities better adapted to both drying and wetting stress (Edburg et al., 2012), thus minimizing their response to them (Borken and Matzner, 2009).

Regarding potential N mineralization rates, only soil samples collected under affected and dead holm oaks showed a significant decrease in the cycles regime compared to the constant moisture treatment (Fig. 2). Further, summer soil samples collected under the grassland ecotype showed a decrease in N mineralization 38% higher than those collected under the holm oak ecotype. Thus, HOD apparently



**Fig. 4.** Pulse effect of the three rewetting events on microbial respiration ( $R_{Hpulse}$ ) of soils samples collected in spring (plain bars) and summer (stripped bars) under the holm oak (green bars) and the grassland (yellow bars) ecotypes. Bars and error bars represent means ( $n = 18$ ) and standard errors (1SE), respectively. Different letters within each ecotype represent significant differences between seasons ( $P < 0.05$ ). Asterisks indicate significant differences among ecotypes ( $* = P < 0.05$ ;  $** = P < 0.01$ ;  $*** = P < 0.001$ ).  $P_e$ ,  $P_s$ ,  $P_{ev}$  and  $P_{e \times s}$  show the significant level of ecotype, season and event, and the interaction between ecotype and season, respectively (mixed models). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

modulated the response to the water regime change at both the short- and the long-term. The higher sensitivity of N mineralization rates to the water regime change under a HOD scenery might be related to the lower functional richness and differences in soil microbial composition above discussed. More importantly, these results suggest that the interactive effect of the projected changes in water regime and forest die-off is likely to exacerbate the drying-rewetting-driven decrease in N availability discussed in the previous section.

The differences in the direction and intensity of the response of potential C and N mineralization to the interactive effects of water regime and holm oak decline supports our first hypothesis, and evidence a higher probability of C and N decoupling with the interaction of both environmental changes. All these results stress the importance of studying the interactive effects of multiple environmental changes in the ecosystem functioning (Matesanz et al., 2009).

**Table 3**

Means ( $\pm$  1SE) of potential C and N mineralization rates ( $C_m$  and  $N_m$ , respectively) of soil samples collected in spring and summer under the holm oak (H) and the grassland (G) ecotype of healthy, affected and dead trees and subject to the two water regimes (constant moisture vs. drying-rewetting cycles). Statistically significant effects of defoliation ( $P_d$ ) and ecotype ( $P_e$ ) are represented by bold  $P$  values. Underlined values denote significant differences between ecotypes for the respective defoliation degree and water regime ( $P < 0.05$ ).

	Constant moisture			Mixed models		Drying-rewetting cycles			Mixed models		
	Healthy	Affected	Dead	$P_d$	$P_e$	Healthy	Affected	Dead	$P_d$	$P_e$	
<b>Spring</b>											
$C_m$	H	<u>93.2 (26.6)</u>	52.8 (5.33)	<u>82.6 (22.5)</u>	0.491	< <b>0.001</b>	<u>96.4 (22.1)</u>	72.8 (13.2)	<u>92.2 (18.2)</u>	0.799	< <b>0.001</b>
	G	<u>34.3 (5.43)</u>	37.1 (8.46)	<u>21.2 (4.78)</u>			<u>39.3 (4.66)</u>	40.1 (5.83)	<u>39.2 (5.86)</u>		
$N_m$	H	1.52 (0.09)	1.87 (0.15)	1.84 (0.20)	0.403	< <b>0.001</b>	<u>1.33 (0.10)</u>	<u>1.34 (0.07)</u>	<u>1.35 (0.14)</u>	0.535	< <b>0.001</b>
	G	0.87 (0.10)	0.70 (0.06)	1.05 (0.21)			<u>0.66 (0.07)</u>	<u>0.48 (0.05)</u>	<u>0.74 (0.11)</u>		
<b>Summer</b>											
$C_m$	H	<u>35.7 (7.10)</u>	<u>23.6 (7.32)</u>	<u>23.6 (5.79)</u>	0.189	<b>0.009</b>	<u>53.3 (3.58)</u>	34.2 (6.79)	36.0 (5.11)	<b>0.034</b>	< <b>0.001</b>
	G	<u>21.1 (5.45)</u>	<u>15.1 (1.29)</u>	<u>15.2 (2.14)</u>			<u>29.6 (6.45)</u>	22.0 (2.55)	24.2 (3.31)		
$N_m$	H	1.73 (0.24)	<u>1.82 (0.29)</u>	1.74 (0.23)	0.637	< <b>0.001</b>	<u>1.52 (0.16)</u>	1.33 (0.11)	<u>1.46 (0.18)</u>	0.541	< <b>0.001</b>
	G	1.31 (0.32)	<u>0.91 (0.06)</u>	1.30 (0.18)			0.97 (0.24)	0.77 (0.08)	<u>0.84 (0.15)</u>		

$n = 6$ ; variables expressed as  $\text{mg C-CO}_2$  or  $\text{N kg}^{-1}$  soil  $\text{day}^{-1}$ ; Values of constant moisture regime obtained from Rodríguez et al. (2017).

#### 4.3. Season as modulator of the response of potential C and N mineralization rates to drying-rewetting cycles and HOD

Our results confirmed the role of season as modulator of the microbial functioning response to drying-rewetting cycles and holm oak decline, particularly for the holm oak ecotype and the C mineralization process. Summer was the season when drying-rewetting-driven increased soil  $\text{CO}_2$  emissions under holm oaks were significant. Similarly, HOD short-term effects on the response of potential C mineralization and long-term effects on the response of potential C and N mineralization to drying-rewetting cycles were more noticeable in summer. Increased C mineralization due to rewetting after summer drought has been widely observed (Borken and Matzner, 2009; Song et al., 2017). However, to our knowledge, this is the first study exploring how forest die-off affects the response of soil microbial functioning to drying-rewetting cycles, as well as the temporal variability of that response. The higher impact of HOD on the response of potential C and N mineralization rates to drying-rewetting cycles in summer agrees with the results from a study carried out in Mediterranean *Quercus suber* forests (Ávila et al., 2016). That study explains the lower likelihood to detect differences in C and nutrient cycling between healthy and declining or dead trees in these ecosystems during spring due to the homogenizing effect of grass (Tang and Baldocchi, 2005). However, we here show that, at the short-term, potential N mineralization responded consistently and independently of the season to the water regime and the HOD and water regime interaction. This suggests a higher sensitivity of the N mineralization process to environmental changes and strengthens the need for further research on the largely unknown response of N cycle related-processes to global change scenarios.

The lack of seasonal differences in the response of potential C and N mineralization to the water regime change in the grassland ecotype also suggests a more extensive sensitivity of its soil microbial community to climate change. The projected more frequent, hotter and longer drought events in these forests structured by a single, strongly dominant tree species could drive to a scenario where trees would be replaced by the smaller understory species (Saura-Mas et al., 2014; Ibáñez et al., 2015). Herein, we show that in such plant succession scenario, this type of Mediterranean ecosystems would be less capable to sequester (Table 1), and therefore, mineralize C and N in soil (Table 2). More importantly, this plant succession would likely make Mediterranean woodlands more vulnerable to the projected intensification of drying-rewetting cycles, compromising important ecosystem functions, such as soil organic matter mineralization and nutrients turnover.



## 5. Conclusions

Our study add new evidence on the direction in which Mediterranean forest soils will respond to forecasted changes in rainfall regimes, revealing asymmetric responses in C and N mineralization. As rainfall pulses and soil drying-rewetting cycles are predicted to be more intense in the Mediterranean region in the next decades, such asymmetry is likely to result not only in important soil C losses but also in significant decreases in N availability for plants and microorganisms, with important implications for ecosystem functioning and services. Further, our study provides new and valuable insights on the hitherto unexplored interactive effects of drying-rewetting cycles and forest die-off on soil C and N mineralization. We show that tree decline and mortality might enhance the probability of C and N decoupling at both short- and long-terms, by minimizing the response of C mineralization but accentuating the response of N mineralization to drying-rewetting cycles, with a higher sensitivity of the N than of the C mineralization during most of the year. Further research is needed however to gain a better understanding of the mechanism behind these asymmetric responses of C and N mineralization, as well as of the implications of these two global changes on the microbial functioning of Mediterranean forests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.geoderma.2018.07.003>.

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