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RESEARCH LETTER – Environmental Microbiology

# Short-term responses and resistance of soil microbial community structure to elevated CO<sub>2</sub> and N addition in grassland mesocosms

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**One sentence summary:** Elevated CO<sub>2</sub> alone or in combination with N addition does not affect soil microbial community structure in grassland mesocosms.

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## ABSTRACT

Nitrogen (N) addition is known to affect soil microbial communities, but the interactive effects of N addition with other drivers of global change remain unclear. The impacts of multiple global changes on the structure of microbial communities may be mediated by specific microbial groups with different life-history strategies. Here, we investigated the combined effects of elevated CO<sub>2</sub> and N addition on soil microbial communities using PLFA profiling in a short-term grassland mesocosm experiment. We also examined the linkages between the relative abundance of r- and K-strategist microorganisms and resistance of the microbial community structure to experimental treatments. N addition had a significant effect on microbial community structure, likely driven by concurrent increases in plant biomass and in soil labile C and N. In contrast, microbial community structure did not change under elevated CO<sub>2</sub> or show significant CO<sub>2</sub> × N interactions. Resistance of soil microbial community structure decreased with increasing fungal/bacterial ratio, but showed a positive relationship with the Gram-positive/Gram-negative bacterial ratio. Our findings suggest that the Gram-positive/Gram-negative bacteria ratio may be a useful indicator of microbial community resistance and that K-strategist abundance may play a role in the short-term stability of microbial communities under global change.

**Keywords:** global change; grassland; fungal/bacterial ratio; PLFA

## INTRODUCTION

Growing awareness of the key role of soil biodiversity for the maintenance of ecosystem services has led to considerable interest in the impacts of global change on belowground diversity in recent years (Bardgett and van der Putten 2014). Elevated

CO<sub>2</sub> and nitrogen (N) fertilisation or deposition are two main components of global change that may have significant consequences for microbial community structure and microbial-mediated processes. Empirical evidence suggests that N additions increase the fungal/bacterial ratio in grasslands in the

short-term (Bardgett et al. 1999), but decrease this ratio in the longer term (>1 year; Bardgett and McAlister 1999; Bradley, Drijber and Knops 2006; Clegg 2006; Deneff et al. 2009; Rousk, Brookes and Bååth 2011). In contrast, previous grassland studies have reported inconsistent responses of soil microbial community structure to elevated carbon dioxide (CO<sub>2</sub>): elevated CO<sub>2</sub> had either no effect on microbial community structure (Ebersberger et al. 2004; Gutknecht, Field and Balser 2012) or led to significant increases in fungal biomarkers and fungal/bacterial ratio (Kandeler et al. 2008; Guenet et al. 2012). In theory, increases in the fungal/bacterial ratio driven by decreased soil N availability under elevated CO<sub>2</sub> could be offset by N addition, especially in N-limited ecosystems (Lagomarsino et al. 2007). However, microbial community responses to simultaneous increases in N and CO<sub>2</sub> have attracted little attention to date (Gutknecht, Field and Balser 2012).

The response of soil microbial communities to global changes may be mediated by the plant community via changes in plant biomass or plant-induced changes in soil resource availability, such as labile C or mineral N contents (Bardgett, Freeman and Ostle 2008; Gutknecht, Field and Balser 2012; Drigo et al. 2013; Philippot et al. 2013). Moreover, resistance of the microbial community structure (i.e. the degree to which microbial composition remains unchanged in the face of a disturbance, Allison and Martiny 2008) to disturbances (i.e. events that alter directly or indirectly a community, usually through effects on the environment of the community) may depend on the relative abundance and contribution of specific functional groups and their life-history strategies (Schimel, Balser and Wallenstein 2007; Wallenstein and Hall 2011). For example, r-strategists exhibit high growth rates and consume soil labile carbon, while K-strategists present slower growth rates and are likely to out-compete r-strategists in conditions of low nutrient availability due to their higher substrate affinities (Fierer, Bradford and Jackson 2007). Numerous studies suggest that K-strategist microorganisms are more resistant to global change induced disturbances (de Vries and Shade 2013; Bischoff et al. 2016; Villa et al. 2016; Zhang et al. 2016). The fungal/bacterial ratio and Gram-positive/Gram-negative bacteria ratios have been proposed as proxies of the prevalence of K-strategists in the microbial community and are thus expected to be positively related to microbial community resistance (de Vries and Shade 2013).

In this experiment, we investigate the combined effects of elevated CO<sub>2</sub> and N supply on soil microbial community structure in grassland mesocosms planted with *Dactylis glomerata*. Previous work published on this mesocosm experiment has shown that soil microbial processes related to C and N cycling were altered by the combined effects of elevated CO<sub>2</sub> and N addition (Niboyet et al. 2010), and that the abundance of nitrifiers was altered by experimental treatments (Simonin et al. 2015). We examine how soil PLFA profiles are affected by elevated CO<sub>2</sub> and N addition, and analyse the links between the relative abundance of r- and K-strategists and the resistance of the soil microbial community structure to these disturbances.

## MATERIALS AND METHODS

### Experimental design

The mesocosm experiment was performed in growth chambers at the Université Paris Sud (Orsay, France) and comprised two treatments in a factorial design: atmospheric CO<sub>2</sub> (ambient 381 ± 6 μmol mol<sup>-1</sup> vs elevated 645 ± 9 μmol mol<sup>-1</sup>) and N addition (no N addition vs +10 g N-NH<sub>4</sub>NO<sub>3</sub> m<sup>-2</sup>). The elevated CO<sub>2</sub> con-

centration corresponds to the intermediary IPCC projections at the horizon 2050 (Stocker 2014), whereas the N addition (equivalent to 100 kg N ha<sup>-1</sup>) is in line with local fertiliser practices (Bloor, Barthes and Leadley 2008). Each mesocosm consisted of a PVC pot (15 × 20 × 50 cm) filled with soil from a nearby grassland (pH = 8.5, 2.46 g C kg<sup>-1</sup>, organic matter content 4.26 g kg<sup>-1</sup>, 0.23 g N kg<sup>-1</sup>, cation exchange capacity 1.81 cmol kg<sup>-1</sup>). Mesocosms were sown with *Dactylis glomerata* and two mesocosms were placed in each of 12 growth chambers within a large glasshouse. CO<sub>2</sub> was manipulated at the chamber level whereas N was manipulated at the mesocosm level; each growth chamber contained one replicate mesocosm of each N treatment such that each CO<sub>2</sub> × N treatment combination was replicated six times (total of 24 mesocosms). The treatments were initiated 1 month after sowing, when the *D. glomerata* seedlings had fully emerged (Niboyet et al. 2010), and the mesocosms were harvested after 10 weeks under treatments. The aboveground (shoot) and belowground (root) plant biomass were collected, washed and oven-dried (60°C, 72 h) prior to weighing to determine dry mass. Samples from the top soil (0–10 cm) were collected at the final harvest and sieved at 2 mm, and then immediately used for measurements of soil water content, soil respiration and gross N mineralisation rates (see Niboyet et al. 2010 for additional details). In addition, soil samples were stored at -20°C for PLFA extraction.

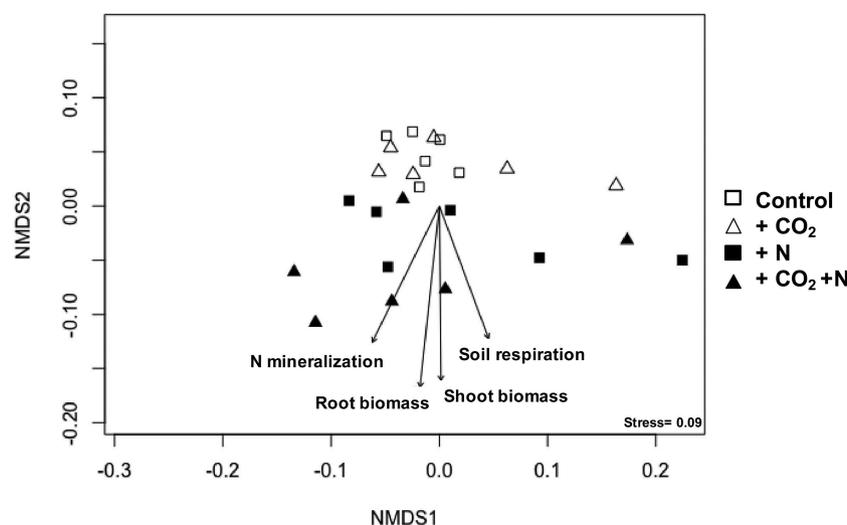
### Analysis of the soil microbial community structure by PLFA profiles

A solution of chloroform (15 mL), methanol (30 mL) and citrate buffer (12 mL) was used to extract the lipids from 10 g of freeze-dried soil (Frostegård, Tunlid and Bååth 1993). After extraction and derivatisation, the fatty acid methyl esters (FAME) were characterised based on a standard bacterial acid methyl ester (BAME) ranging from 11:0 to 20:0. We used an Agilent 6890 gas chromatograph equipped with a Flame Ionisation Detector (GC-FID) and a SGE-BPX5 column (65 m × 320 μm × 0.25 μm) to quantify the FAME extracted from the soil samples based on their retention time and mass spectral comparison. Standard fatty acid nomenclature was used following Frostegård, Tunlid and Bååth (1993). Seventeen lipids were identified and for each lipid, the relative area of the corresponding peak was calculated. The assignments of PLFA regarded as bacterial biomarkers, fungal biomarker, Gram-negative bacterial biomarkers and Gram-positive bacterial biomarkers were performed as described by O'Leary and Wilkinson (1988), Frostegård, Tunlid and Bååth (1993), Frostegård and Bååth (1996) and Zelles (1997, 1999). These biomarkers were used to compute the relative abundance of bacteria, fungi and the Gram-positive/Gram-negative and fungal/bacterial ratios.

### Statistical analysis of PLFA profiles

The experiment was represented as a fully factorial split-plot design: the whole-plot factor is the CO<sub>2</sub> treatment and the N treatment is the split-plot factor.

The PLFA patterns of the soil samples were analysed in R (R Core Team 2015) by non-metric multidimensional scaling (NMDS) with the 'Vegan: Community Ecology Package' (Oksanen et al. 2007). Bray-Curtis similarities were used to build a distance matrix for the NMDS. Permutational multivariate analysis of variance (PERMANOVA) was then used to test whether treatments altered PLFA patterns using the *adonis* function (999 permutations) of the Vegan package.



**Figure 1.** NMDS of the PLFA pattern (17 lipids) in *D. glomerata* mesocosms in the four treatments ( $n = 6$  in each treatment). Root and shoot biomass, soil respiration and gross N mineralisation rates are represented as vectors as they were found to be significant explanatory variables ( $P < 0.05$ ).

The interactive effects of elevated  $\text{CO}_2$  and N supply on the relative abundance of bacteria and fungi, and on the ratios between the relative abundance of fungal/bacterial and Gram positive/Gram negative biomarkers were assessed using PROC MIXED in SAS 9.3 (SAS Institute, Cary, NC, USA).

Correlations between NMDS scores and potential drivers of microbial community structure (plant biomass, soil respiration, gross N mineralisation and soil water content; Niboyet et al. 2010) were assessed with the *envfit* function and post hoc permutation tests ( $n = 999$ ). Soil respiration was used as an indicator of soil labile carbon, and gross N mineralisation rates as a proxy of soil N availability.

### Resistance of the soil microbial community structure

To measure the resistance of the soil microbial community structure to the disturbances, we calculated Bray-Curtis similarities in microbial communities in disturbed treatments ( $\text{CO}_2$ , N or  $\text{CO}_2 + \text{N}$ ) relative to the control treatment given by the PLFA profiles (de Vries and Shade 2013) using the *vegdist* function (Vegan in R). A similarity of 1 indicates maximum resistance, i.e. no effect of disturbance on microbial community structure, while a similarity close to 0 indicates very low resistance. Correlations between the resistance index and Gram-positive/Gram-negative ratio or fungal/bacterial ratio were examined using Proc CORR in SAS 9.3.

## RESULTS

### Elevated $\text{CO}_2$ and N addition effects on soil microbial community structure

Multivariate analysis (NMDS) indicated that N addition significantly altered the microbial community structure (PERMANOVA:  $P = 0.003$ ) (Fig. 1). In contrast, elevated  $\text{CO}_2$  had no significant effect ( $P = 0.86$ ) and no significant  $\text{CO}_2 \times \text{N}$  interactions were detected ( $P = 0.38$ ). The addition of N significantly increased the relative abundance of fungi ( $P < 0.05$ , +78%, Fig. 2A) and the fungal/bacterial ratio ( $P < 0.001$ , +95%, Fig. 2B), while  $\text{CO}_2$ , alone or in combination with the N treatment, had no effect on these variables (Fig. 2). The bacterial relative abundance (Fig. 2A), the Gram+ and Gram- relative abundance (data not shown), and

the ratio of Gram-positive/Gram-negative bacterial biomarkers (Fig. 2B) were not affected by any of the treatments.

### Relationships between PLFA profiles and plant and soil variables

The correlations with the NMDS axis 2 indicate that the effect of the N treatment on the soil microbial community structure seemed to be mainly related to a gradient in root and shoot biomass and in soil respiration (a proxy of soil labile C availability) and gross N mineralisation rates (a proxy of soil mineral N availability) (Fig. 1, Table S2, Supporting Information). In this experiment, N addition had a positive effect on root and shoot biomass ( $P < 0.001$  in both cases), especially under elevated  $\text{CO}_2$  ( $P = 0.02$  and  $P = 0.003$ , respectively, Table S1, Supporting Information). Elevated  $\text{CO}_2$  and N addition had positive effects on soil respiration ( $P = 0.03$  and  $P < 0.001$ , respectively, Table S1, Supporting Information). N addition also increased gross N mineralisation rates when combined with elevated  $\text{CO}_2$  ( $P = 0.03$ , Table S1, Supporting Information).

### Resistance of the soil microbial community structure to the environmental change

The resistance of the PLFA profiles to the disturbances (i.e. to the imposed increases in  $\text{CO}_2$ , N or in both  $\text{CO}_2$  and N) decreased with the fungal/bacterial ratio (Fig. 3A) but increased with the ratio of Gram-positive/Gram-negative bacteria (Fig. 3B). These correlations also tended to be observed when considering each disturbance separately (Table S3, Supporting Information).

## DISCUSSION

Increases in fungal biomass after the addition of N have been frequently observed in short-term studies, i.e. in studies conducted over several months (Bardgett et al. 1999; Gallo et al. 2004), where the progressive inhibition of growth or the decrease of mycorrhizal fungal biomass by N has not already occurred (Bardgett and McAlister 1999; Gallo et al. 2004; Treseder 2008). Our results confirm an N-induced increase in both the relative abundance of fungi and the fungal/bacterial ratio. Stimulation

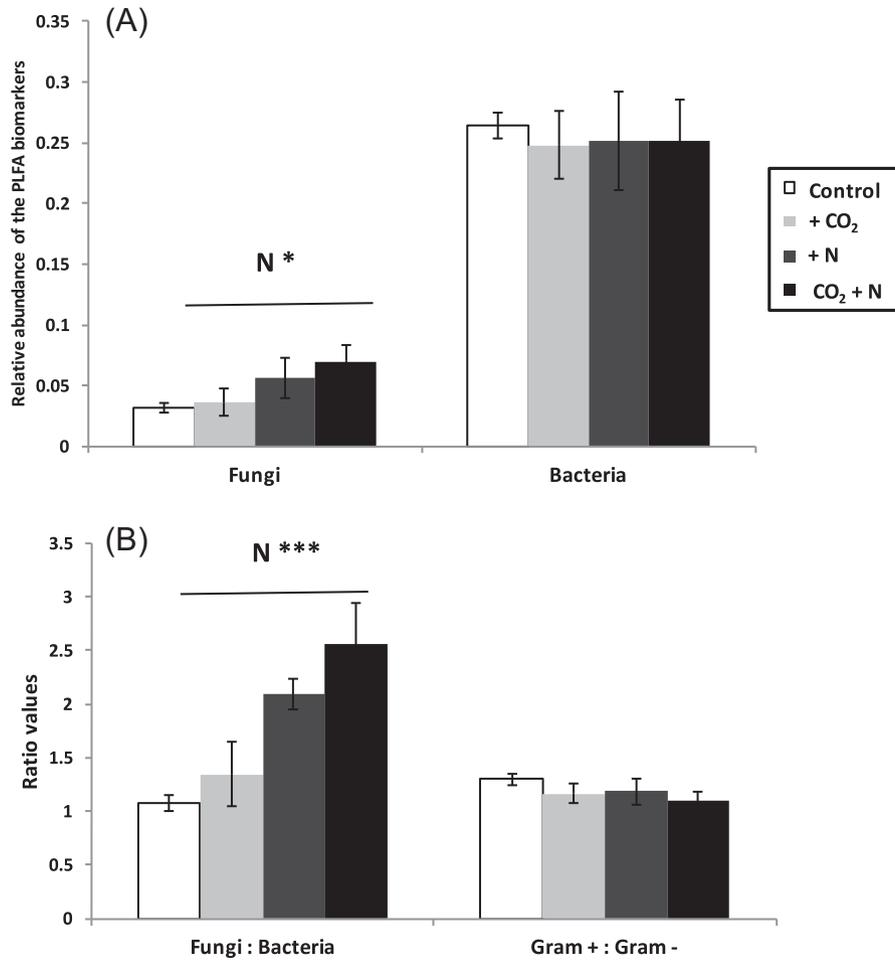


Figure 2. Relative abundance of the bacterial and fungal biomarkers (A) and ratios of the PLFA relative abundance of fungi/bacteria and Gram-positive/Gram-negative bacteria (B) in the four treatments. The means and standard errors ( $n = 6$ ) are presented. Significant effects are indicated (\* $P < 0.05$ , \*\*\* $P < 0.001$ ).

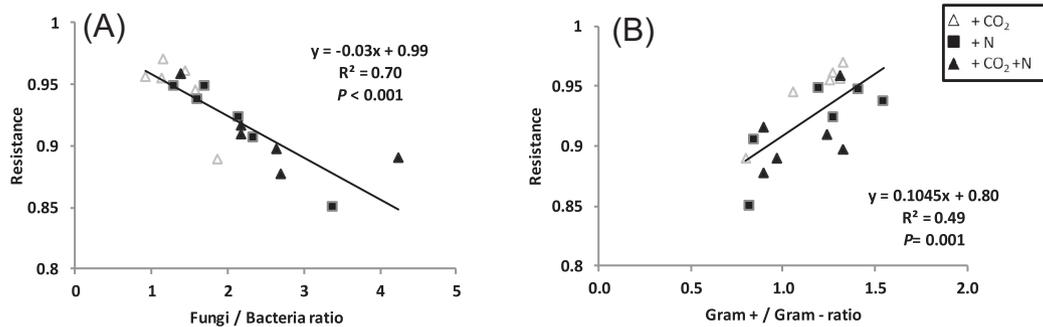


Figure 3. Pearson's correlations between ratios of the PLFA relative abundance of fungi/bacteria (A) and Gram-positive/Gram-negative bacteria (B) and the resistance of the soil microbial community structure to the treatments (elevated CO<sub>2</sub>, N addition and elevated CO<sub>2</sub> combined with N addition). The linear regressions are drawn and the associated equations, R-square and P values are given.

of root and shoot biomass by the supply of N likely resulted in an increase in C inputs via rhizodeposition (Niboyet et al. 2010), as suggested by the large increase in soil respiration with N addition. The resulting higher C availability seems to have benefited fast-growing fungi, specialised in the metabolism of large quantities of labile C substrates, and not bacteria (Chigineva, Aleksandrova and Tiunov 2009; de Graaff et al. 2010; de Vries and Caruso 2016). In our experiment, elevated CO<sub>2</sub> did not alter the microbial community structure, in agreement with other short- and long-term studies (Zak et al. 1996; Ebersberger et al.

2004; Gutknecht, Field and Balsler 2012). The absence of CO<sub>2</sub> effects and of interactive effects with N addition might reflect the lack of negative-CO<sub>2</sub> effects on soil mineral N availability in this study.

Microbial functional traits, and especially the relative abundance in a community of r- and K-strategists, may be useful indicators of the response of the soil microbial community structure to stressors, such as global environmental changes. In our study, the Gram-positive/Gram-negative ratio was positively correlated with the resistance of the microbial community

structure; assuming that the Gram positive/Gram negative ratio is a proxy of the presence of K-strategists, these results are consistent with the idea that K-strategists promote microbial community resistance (de Vries and Shade 2013). In our study, the Gram-positive/Gram-negative ratio only explained 49% of the variance of the resistance to the treatments, which is probably related to the limited effects of the treatments on the Gram-positive/Gram-negative ratio.

Surprisingly, the fungal/bacterial ratio, which is potentially also an indicator of the proportion of K-strategists, was negatively correlated with the resistance of the microbial community structure. One possible explanation is that the fungal community comprises both r- and K-strategists, and that in this short-term experiment, the fungal community was dominated by fast-growing fungi (i.e. r-strategists) that experienced drastic changes in composition and abundance. Our findings thus suggest that the Gram-positive/Gram-negative bacteria ratio may be a useful indicator of microbial community resistance to elevated CO<sub>2</sub> and N addition and that a higher relative abundance of K-strategist may promote the stability of the microbial community under global change. Other functional and taxonomic traits of soil microbial communities such as genome size, 16S rDNA gene copy number or mean generation time (Fierer, Bradford and Jackson 2007; Leff et al. 2015) should also be considered to confirm the role of K-strategists for the resistance of the soil microbial community structure to disturbances under future environmental changes.

## SUPPLEMENTARY DATA

Supplementary data are available at [FEMSLE](#) online.

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