

Effect of Elevated CO₂ and Drought on Soil Microbial Communities Associated with *Andropogon gerardii*

Issmat I. Kassem¹, Puneet Joshi¹, Von Sigler^{1*}, Scott Heckathorn¹ and Qi Wang²

¹Department of Environmental Sciences, University of Toledo, Ohio 43606, USA;

²Department of Civil Engineering, University of Toledo, Ohio 43606, USA)

Abstract

Our understanding of the effects of elevated atmospheric CO₂, singly and in combination with other environmental changes, on plant-soil interactions is incomplete. Elevated CO₂ effects on C₄ plants, though smaller than on C₃ species, are mediated mostly via decreased stomatal conductance and thus water loss. Therefore, we characterized the interactive effect of elevated CO₂ and drought on soil microbial communities associated with a dominant C₄ prairie grass, *Andropogon gerardii* Vitman. Elevated CO₂ and drought both affected resources available to the soil microbial community. For example, elevated CO₂ increased the soil C:N ratio and water content during drought, whereas drought alone decreased both. Drought significantly decreased soil microbial biomass. In contrast, elevated CO₂ increased biomass while ameliorating biomass decreases that were induced under drought. Total and active direct bacterial counts and carbon substrate use (overall use and number of used sources) increased significantly under elevated CO₂. Denaturing gradient gel electrophoresis analysis revealed that drought and elevated CO₂, singly and combined, did not affect the soil bacteria community structure. We conclude that elevated CO₂ alone increased bacterial abundance and microbial activity and carbon use, probably in response to increased root exudation. Elevated CO₂ also limited drought-related impacts on microbial activity and biomass, which likely resulted from decreased plant water use under elevated CO₂. These are among the first results showing that elevated CO₂ and drought work in opposition to modulate plant-associated soil-bacteria responses, which should then influence soil resources and plant and ecosystem function.

Key words: denaturing gradient gel electrophoresis; drought; elevated CO₂; soil microbial communities.

Kassem I, Joshi P, Sigler V, Heckathorn S, Wang Q (2008). Effect of elevated CO₂ and drought on soil microbial communities associated with *Andropogon gerardii*. *J. Integr. Plant Biol.* 50(11), 1406–1415.

Available online at www.jipb.net

During the last nine decades, the atmospheric concentration of CO₂ has increased by 36%, primarily as a result of anthropogenic activities (IPCC 2007). Increased atmospheric CO₂ concentration and related environmental changes (e.g. warming and decreased/increased precipitation) can influence the biological processes of plants and soil microorganisms inhabiting diverse ecosystems (Zak et al. 1993, 2000; Kandeler et al. 1998). Elevated CO₂ typically increases plant growth in plants with both

C₃ and C₄ photosynthetic metabolism, though photosynthetic and growth increases are generally larger in C₃ species (e.g., Sage and Monson 1999; Ehleringer et al. 2002; Wang et al. 2008 and references therein). In C₃ species, increased growth under elevated CO₂ is primarily due to decreased photorespiration (photosynthetic fixation of O₂ rather than CO₂), while in C₄ species, increased growth under elevated CO₂ is primarily due to decreased stomatal conductance and transpiration, which decreases soil water use (this also occurs in C₃ species).

As with most terrestrial ecosystems, grasslands will be impacted by future global environmental and climate changes, including elevated CO₂ (Fay et al. 2003), and this will have importance to the global ecosystem, as grasslands comprise approximately one-quarter of the Earth's land surface (Adam et al. 2000). For example, under elevated CO₂, *Andropogon gerardii*, a dominant C₄ plant in tall-grass prairies, exhibits increased above-ground biomass (19–166% under favorable conditions), photosynthesis, and root exudation (in mixed plant communities), as well as decreased stomatal conductance (in wet seasons), sap flow and evapotranspiration (Owensby et al.

Received 9 May 2008 Accepted 22 Jun. 2008

Supported by the University of Toledo Department of Environmental Sciences and grants from the National Science Foundation to S. Heckathorn and E.W. Hamilton.

*Author for correspondence.

Tel: +1 419 530 2897;

Fax: +1 419 530 4421;

E-mail: <von.sigler@utoledo.edu>.

© 2008 Institute of Botany, the Chinese Academy of Sciences

doi: 10.1111/j.1744-7909.2008.00752.x

1994, 1997, 1999; Adam et al. 2000; Williams et al. 2000, 2004). Grassland soils are naturally water-limited (Knapp 1984). Therefore, small increases in soil water content brought about by elevated CO₂ (Adam et al. 2000) can have a dramatic impact on microbial biomass production and nutrient use (Rice et al. 1994; Griffiths et al. 2003). It follows that elevated CO₂ and its subsequent impact on *A. gerardii* physiology might significantly alter critical microbial functions in grassland soils, including organic matter decomposition, carbon release, and the availability of plant nutrients (Zak et al. 2000; Alkorta et al. 2003; Griffiths et al. 2003; Arias et al. 2005). Despite this relationship, few studies have addressed the indirect effect of elevated CO₂ on both structure and activity of soil microbial communities associated with plants (Ebersberger et al. 2004).

Increased atmospheric CO₂ concentration affects the occurrence of droughts (Ward et al. 1999), which have increased in duration and intensity worldwide since the 1970s (IPCC 2007). Drought impacts ecosystem functions and plant-microbe relationships in terrestrial environments (Griffiths et al. 2003; IPCC 2007), it also affects the partitioning of carbon between the atmosphere and terrestrial systems. For example, prolonged drought in North America in 2002 reduced the amount of carbon uptake by vegetation and soil from an annual average of 650 million metric tons to 330 million metric tons (Peters et al. 2007). Since the frequency of heat and drought stresses is predicted to increase (Mearns et al. 1984), it follows that the amount of carbon sequestered by terrestrial ecosystems will likely decrease. Consequently, the combination of increased drought and CO₂ enrichment could result in a chronic impact on soil microbial communities and their relationships with plants. However, to our knowledge, the effect of this important interaction on the structure and activity of soil microbial communities has not been examined.

Because of the complexity of plant-soil interactions, a multiphasic approach is necessary to understand the relationship between elevated CO₂, singly and in combination with drought, and plant/soil microbial communities. Many previous studies used traditional microbiological techniques to identify changes in soil bacteria in response to elevated CO₂ concentration. The results of these studies were conflicting, as no response, increases, and decreases in biomass were reported (Diaz et al. 1993; Zak et al. 1993, 2000; Allen et al. 2000; Williams et al. 2000; Freeman et al. 2004). These inconsistent conclusions are likely attributable to natural variation in soil microbial communities, soil type, the species of plant under study and methodological biases (Sadowsky and Schortemeyer 1997; Franklin and Mills 2003; Schmidt 2006). Although no techniques are without bias (Muyzer and Smalla 1998), the combination of molecular fingerprinting with traditional methods can yield a more comprehensive characterization of microbial communities, as compared with traditional methods alone (Torsvik and Ovreas 2002; Schmidt 2006). A popular method for characterizing impacts to complex microbial assemblages

is denaturing gradient gel electrophoresis (DGGE) analysis (reviewed in Muyzer and Smalla 1998), which has been used previously to assess the impact of elevated CO₂ on the structure of soil microbial communities. For example, studies conducted by Ebersberger et al. (2004) revealed that CO₂ enrichment resulted in significant season-specific changes in community structure, suggesting that the effect of elevated CO₂ on soil microbial communities might be modulated by the presence of other prevailing environmental parameters.

To better understand the potential effects that global climate change might have on soil microbial communities, particularly in C₄-dominated grasslands, we investigated the interactive effect of elevated CO₂ and drought on the activity and structure of the soil microbial community associated with *A. gerardii*, a dominant, drought-resistant grass of central US prairies (Knapp 1985). We hypothesized that elevated CO₂ would increase soil water availability, thereby mitigating the effect of drought on the microbial communities. To test this hypothesis, we used a multiphasic approach that incorporated molecular and traditional microbiology to assess the plant-mediated impact of elevated CO₂ and drought in the context of bacteria abundance, activity and community structure.

Results

Plant physiological responses

Leaf water potential, a measure of plant water status, decreased substantially with drought ($P < 0.001$), and there was significant drought x CO₂ interaction ($P < 0.001$), such that elevated CO₂ largely ameliorated the decrease in water potential with drought (Table 1); similar results were observed for leaf relative water content (not shown). Shoot mass was decreased ($P = 0.09$) and root mass increased by drought ($P = 0.009$), while both were unaffected by CO₂; thus, the gradual drought treatment stimulated root growth in this drought-tolerant plant species. Stomatal conductance was decreased by both drought ($P = 0.08$) and elevated CO₂ ($P = 0.001$), and there was an interactive effect ($P < 0.05$), such that stomatal conductance was lowest in the combined elevated CO₂ and drought treatment. Net photosynthesis was decreased by drought ($P < 0.016$) and increased by elevated CO₂ ($P < 0.001$), and there was a significant drought x CO₂ interaction ($P < 0.004$), such that photosynthesis did not decrease during drought under elevated CO₂.

Plant and soil C:N

The C:N ratio of plant shoots was not significantly affected by either drought or CO₂ (Figure 1); similar results were observed for both shoot %N and %C (not shown). In contrast, root C:N was decreased by drought ($P = 0.007$) and was unaffected by elevated CO₂; this decrease in C:N was caused by a decrease

Table 1. Summary of plant parameters across treatments

Treatment	Water potential (MPa)	Shoot biomass (g)	Root biomass (g)	Stomatal conductance (mol H ₂ O/m ² per s)	Photosynthesis (μmol CO ₂ /m ² per s)
Control	-1.34 ± 0.10	2.95 ± 0.38	2.75 ± 0.39	0.16 ± 0.02	22.35 ± 0.96
Drought	-3.55 ± 0.44	2.43 ± 0.13	7.26 ± 1.99	0.13 ± 0.01	16.97 ± 0.45
Elevated CO ₂	-1.51 ± 0.13	2.84 ± 0.31	2.82 ± 0.35	0.09 ± 0.01	27.2 ± 0.30
Elevated CO ₂ and drought	-1.74 ± 0.07	2.19 ± 0.22	4.66 ± 0.50	0.05 ± 0.01	29.97 ± 0.81

The results are reported as mean values ± SE across at least four replicates.

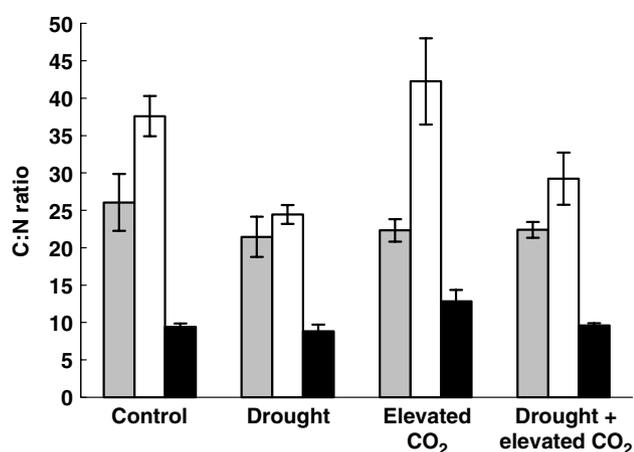


Figure 1. C:N ratio measured for shoots (■), roots (□) and bulk soil (■) under drought and elevated CO₂.

The results are reported as mean values ± standard error ($n = 3$).

in %C with drought ($P = 0.047$), as %N was unaffected by the treatments (not shown). The C:N ratio of bulk soil was increased by elevated CO₂ ($P = 0.005$) and decreased by drought ($P = 0.008$) (Figure 1). These changes were due to small, but statistically insignificant, increases in %C resulting from elevated CO₂ and decreases with drought (ambient and high CO₂), and decreases in %N with elevated CO₂ (not shown). For rhizosphere soil (adhering to roots), C:N increased with CO₂ and was unaffected by drought (not shown).

Estimation of total and active bacteria abundance

Total bacteria. The number of soil bacteria (cells/g dry soil) under control and drought treatments were statistically similar ($P = 0.48$) (Figure 2A). Likewise, the number of soil bacteria observed under the elevated CO₂ treatment was similar to that resulting from combined elevated CO₂ and drought ($P = 0.32$). However, the number of bacteria observed under conditions of elevated CO₂ (including combined elevated CO₂ and drought) were significantly higher than the number observed under other treatments including ambient CO₂ concentrations ($P < 0.0001$).

Active bacteria. The number of active bacteria present under control conditions was statistically similar to the number observed under drought conditions ($P = 0.34$) (Figure 2A). The number of active bacteria present under the elevated CO₂ treatment was significantly higher ($P < 0.05$) than the number under control and drought treatments, but not significantly different ($P = 0.32$) than the number observed following treatment with combined elevated CO₂ and drought. Additionally, the number of active bacteria observed in the combined elevated CO₂ and drought treatment was significantly higher than the number observed in control or drought treatments ($P < 0.05$).

Estimation of microbial biomass by quantifying soil DNA

The concentration of DNA isolated from the soils (μg DNA/g dry soil) was used to estimate soil microbial biomass (Garcia-Pichel et al. 2003). Specifically, the concentration of DNA isolated from control soils was significantly higher than that isolated from soils under drought ($P < 0.0001$) and combined elevated CO₂ and drought ($P < 0.01$) conditions. Although the concentration of DNA isolated from soils under elevated CO₂ was similar to that observed under control conditions ($P = 0.17$), it was significantly higher than in soils under drought ($P < 0.0001$) and combined elevated CO₂ and drought ($P < 0.01$) treatments. Finally, the concentration of DNA isolated from soils under drought conditions was significantly lower than from soils under combined elevated CO₂ and drought ($P < 0.05$).

Soil microbial activity

The rate of FDA hydrolysis (μg fluorescein produced per g dry soil per h) was used to estimate the overall enzymatic activity of the soil microbial community (Adam and Duncan 2001). Overall, microbial FDA hydrolysis activity in soils under the drought treatment was higher, as compared with all other treatments (all comparisons $P < 0.05$), while combined elevated CO₂ and drought resulted in the lowest activity (Figure 2C). Microbial activity under control conditions was similar to that under elevated CO₂ ($P = 0.33$), and combined elevated CO₂ and drought ($P = 0.056$) treatments. Microbial activity was significantly higher under the elevated CO₂ treatment than under combined elevated CO₂ and drought ($P < 0.05$).

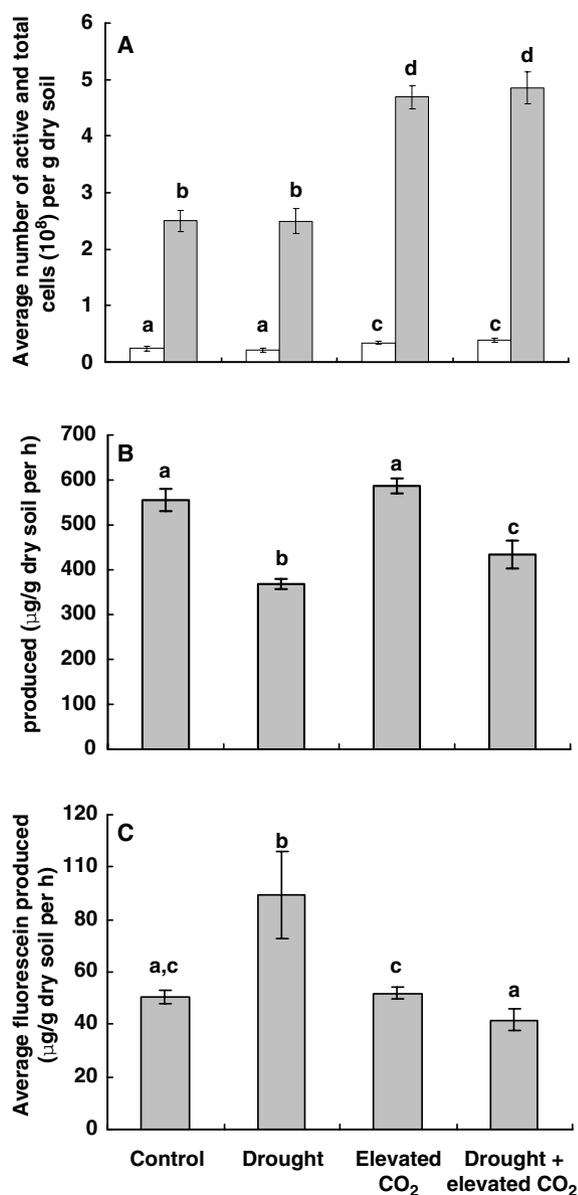


Figure 2. The effect of drought and CO₂ exposure on the enumeration and activity of the soil microbial biomass.

(A) Number of total (□) and active (□) bacteria.

(B) Microbial biomass (concentration of soil DNA).

(C) Microbial community activity (FDA analysis).

Same letter designation indicates that no statistical significance was observed ($\alpha = 0.05$). The results are reported as mean values \pm SE ($n = 3$).

Bacteria metabolic diversity and average metabolic response

The metabolic diversity of culturable bacteria (CMD) following the elevated CO₂ treatment was significantly higher than that

of all other treatments ($P < 0.05$), while the lowest CMD was measured under the drought and control treatments (Figure 3A). In addition, the CMD measured under combined elevated CO₂ and drought was higher than that measured under control conditions ($P < 0.05$), but was not significantly different than that measured under drought ($P = 0.15$) conditions. The CMD measured under the drought treatments was similar to that measured under control conditions ($P = 0.32$).

The average metabolic response (AMR) exhibited a similar trend to CMD, as the highest AMR was measured under the elevated CO₂ treatment (Figure 3B). Specifically, the AMR under elevated CO₂ was significantly higher than the AMR under the control, drought, and combined elevated CO₂ and drought treatments (all $P < 0.05$). The AMR resulting from combined elevated CO₂ and drought was similar to that measured under drought ($P = 0.22$) and control ($P = 0.299$) conditions. Finally, no significant difference in AMR was detected between control and drought treatments ($P = 0.25$).

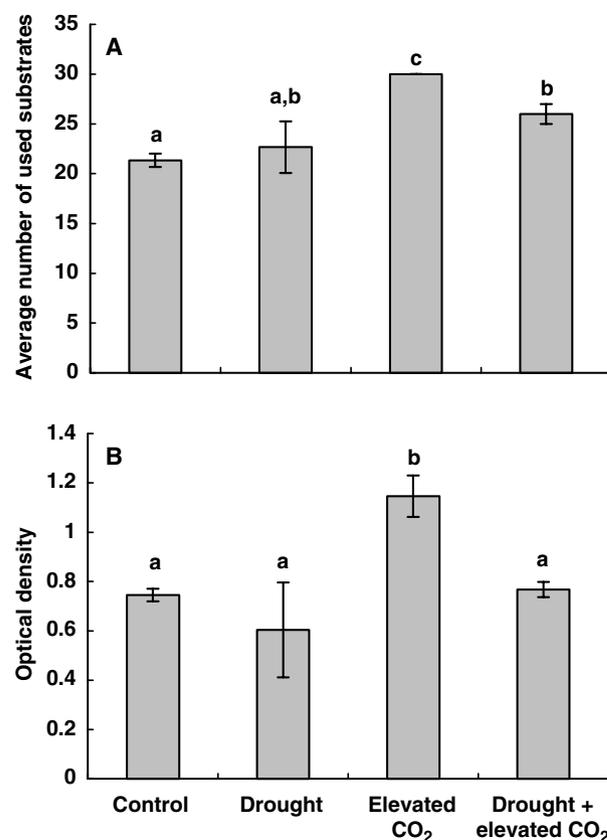


Figure 3. Response of microbial activity to drought and elevated CO₂ as assessed by (A) bacteria community metabolic diversity, and (B) average metabolic response.

The results are reported as mean responses \pm standard error ($n = 3$).

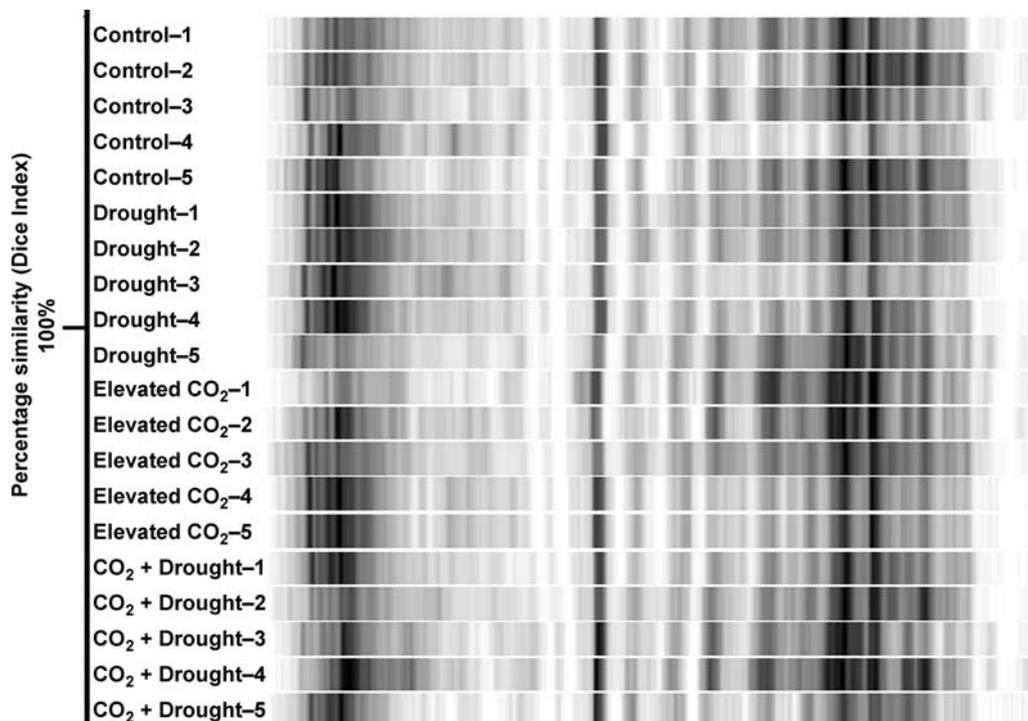


Figure 4. Unweighted pair-group with arithmetic means algorithm (UPGMA) dendrogram showing the percent similarity of soil bacteria communities subjected to CO₂ and drought treatments.

Bacteria community structure

Denaturing gradient gel electrophoresis analysis of soil bacteria resulted in fingerprints that exhibited an average of 48 dominant bands, which did not change significantly ($P < 0.05$) across treatments. All profiles were 100% similar (Dice coincidence index), exhibiting no treatment-based differences (Figure 4).

Discussion

The overall objective of this study was to determine the impact of elevated atmospheric CO₂, with and without drought stress, on soil microbial communities, especially bacteria, associated with *A. gerardii*. Our data provide evidence that soil water availability, soil carbon and nitrogen levels, or C:N ratio might be primary factors influencing the activity and abundance of soil bacteria associated with *A. gerardii*. Specifically, elevated CO₂ appeared to decrease/mitigate the negative impacts of drought on microbial communities by decreasing the stomatal conductance of *A. gerardii* (Table 1), which subsequently increased water availability to soil microorganisms. This corroborates previous findings that showed decreases in stomatal conductance in *A. gerardii* (Owensby et al. 1997; Adam et al. 2000; Williams et al. 2000), increases in soil water content (Wullschlegler et al. 2002; Lecain et al. 2003) and soil C:N ratio under elevated CO₂

(Williams et al. 2000; Phillips et al. 2006), which collectively were predicted to result in increased microbial growth (Zak et al. 1993, 2000). Our findings show that the increased soil C:N ratio (Figure 1) was driven by a small increase in %C under elevated CO₂ and a small decrease in %N under drought. Since elevated CO₂ increased photosynthesis, but did not increase plant C:N or mass, the increase in soil C:N ratio and %C probably resulted from increased root carbon exudation.

Griffiths et al. (2003) and Whiteley et al. (2003) showed that water stress decreased the number of culturable bacteria in grassland soils. However, our data showed that moderate drought did not appear to impact the number of total and active bacteria (Figure 2A). Since direct counts of "active" cells have been shown to actually provide an indication of cell viability rather than activity (Creach et al. 2003), it is likely that drought decreased the culturability of the soil bacteria, which would support the results reported by Griffiths et al. (2003) and Whiteley et al. (2003). As shown by DNA quantification, drought appeared to decrease soil microbial biomass, whereas the addition of elevated CO₂ exhibited a mitigating effect, significantly increasing microbial biomass and the abundance of active and total bacteria (Figure 2A,B). This response can be at least partially explained by noting that water stress can result in bacteria lysis (Fierer et al. 2003), which subsequently releases nutrients that feed surviving microorganisms and increase their activity (Figure 2C). An alternative explanation is that drought induced

an increase in the concentration and diversity of intercellular enzymes capable of FDA hydrolysis in the soil matrix (Halverson et al. 2000). Although the FDA assay provides an appropriate measure of overall microbial activity, it should be noted that this assay is not bacteria-specific (Breeuwer et al. 1995). Therefore, other soil organisms such as lichens and fungi could have contributed to the observed increase in overall activity (Palmer and Friedmann 1990; Gaspar et al. 2001). Regardless of the cause, increased activity following drought appears to be aided by the tolerance of the grassland microbial communities to conditions of water limitation (Knapp 1984; Griffiths et al. 2003). It was notable that communities exposed to combined elevated CO₂ and drought exhibited significantly lower FDA activity than those under drought stress alone. This result also suggested that elevated CO₂ played a role in decreasing/mitigating the effect of drought, likely by limiting evapotranspiration and cell lysis or by increasing root exudation by *A. gerardii*.

It is established that bacteria in grassland soils are tolerant to water limitation (Fierer et al. 2003; Griffiths et al. 2003). Therefore, it was not surprising that following the drought treatment, the degree and diversity of substrate use by culturable soil bacteria was unchanged relative to the control (Figure 3A,B). In contrast, these parameters significantly increased under elevated CO₂, which could result from CO₂-driven increases in root carbon exudation and soil water content (Diaz et al. 1993; Dhillon et al. 1996). The combination of elevated CO₂ and drought appeared to moderate the degree and diversity of substrate use, likely due to several interacting factors including a balance between changes in root biomass, soil water availability and soil C:N. Collectively, these results show that multiple environmental factors can work in opposition as well as in concert to modulate the activity of bacteria. Our metabolic analysis was intended not as an absolute measure of *in situ* microbial activity, but as an index by which to compare treatment impacts. It should be noted that this analysis does not select for the soil microbial community as a whole, but for a subset of the community that will exhibit growth on a defined substrate (Konopka et al. 1998). Therefore, while our results do not reflect the contribution of all bacteria, they can provide a valuable index of relative functional potential and an effective means to compare the culturable subsets of differing communities of bacteria (Haack et al. 1995).

Previous studies have indicated that grassland microbial communities were resistant to structural changes following exposure to either elevated CO₂ (Bruce et al. 2000; Ebersberger et al. 2004) or water stress (Griffiths et al. 2003). While corroborating those findings, our results also expand on them by providing evidence that the interactive effect of elevated CO₂ and drought on the bacteria community structure was also limited. Despite the likely stimulatory effect of CO₂ enrichment on root exudation and its subsequent impact on the activity of some soil bacteria, exudation might not provide enough carbon to drive gross structural changes in the bacteria community (Bruce

et al. 2000). Additionally, Griffiths et al. (2003) predicted that although sudden changes in soil moisture potential might result in bacteria death, gradual soil drying could result in adaptation to water stress. This phenomenon would explain the limited impact to the bacteria community structure of the drought treatment, which was designed to mimic the slow onset of natural drought. The addition of elevated CO₂ might further offset the impact of drought by effectively increasing water availability and therefore allowing soil bacteria a further opportunity to adapt to water stress. While DGGE analysis is an effective method for detecting substantial changes in diverse microbial assemblages (Muyzer et al. 1993), it should be noted that subtle changes to complex communities might go undetected (Bruce et al. 2000; McCaig et al. 2001; Ebersberger et al. 2004). Therefore, we maintain that drought and elevated CO₂, singly and in combination, had a limited effect on the gross community structure of bacteria associated with *A. gerardii*.

While previous studies have more or less addressed the individual impact of drought and elevated CO₂ on microbial communities (Bruce et al. 2000; Griffiths et al. 2003; Ebersberger et al. 2004), their interactive effects have not been investigated. Our results provide evidence that elevated CO₂ can mitigate the negative impacts of drought on the biomass and activity of soil microbes as well as certain plant properties. Furthermore, this study revealed that elevated CO₂ and drought, singly and in combination, had a limited impact on the microbial community structure. As atmospheric CO₂ concentrations increase along with the frequency of droughts (Mearns et al. 1984), a delicate interplay between detrimental and mitigating effects will impact the response of microbial communities. Therefore, the impact of increased CO₂ and drought on ecosystem functions should not be examined as independent phenomena, but in concert, as well as with other drivers of environmental changes.

Materials and Methods

Experimental design

Andropogon gerardii Vitman (big bluestem) was germinated from seeds collected at the Konza Prairie Research Natural Area (Manhattan, KS, USA). Following germination, the seedlings were transferred to pots (53 cm deep × 10 cm diameter) containing soil collected from mesic prairies in the Oak Opening Metropark (Toledo, OH, USA) that were dominated by *A. gerardii*. Prior to use, the soil was homogenized by thorough hand mixing while wearing sterile gloves and sieved (2 mm opening size). The plants were arranged into four groups each containing five replicate pots. The plants were subjected to one of four treatments for 70 d: (i) control (incubation at 370 μmol/mol CO₂, ambient concentration); (ii) elevated CO₂ (incubation at 700 μmol/mol CO₂, approximately double the ambient concentration); (iii) moderate drought (Heckathorn et al. 1997)

(following one month of daily watering, no water was provided for 40 d); and (iv) both elevated CO₂ treatment and drought combined. The elevated CO₂ treatments were conducted in environment chambers (E-36HO, Percival Scientific, Perry, IA, USA) equipped to maintain desired light and temperature levels and CO₂ concentration. No fertilizer was added throughout the experiment and all groups were treated with the same temperature regime (30 °C day and 22 °C night). Light levels were maintained at 700–1 000 μmol/m² per s PPFD (photosynthetic photon flux density) during the experiment.

Measurement of plant parameters

The stomatal conductance to water vapor and net photosynthesis (i.e. CO₂ uptake) was measured using a portable infrared gas analyzer (Li-Cor 6400, Lincoln, NE, USA) according to the method of Heckathorn et al. (1996). Leaf water potential was measured using a pressure chamber (Heckathorn et al. 1997) before harvesting the plants for further analyses. Relative water content was measured after harvesting the plants, and was calculated from the fresh, dry, and turgid weight of plant leaves. Turgid weight was determined after soaking the leaves in distilled water overnight at low-light conditions, while dry weight was measured after incubating the leaves at 70 °C for 72 h. Dried roots and shoots were ground separately using a mortar and pestle, and sieved (0.5 mm mesh-pore) for C:N elemental analysis using a Perkin-Elmer 2400 CHNS/O Analyzer (Series II), following the manufacturer's specifications. All analyses were carried out in triplicate at the end of the incubation period.

Soil processing and storage

After plant collection, the bulk and rhizosphere soils from each pot were sieved (2 mm) and thoroughly homogenized by hand mixing (using sterile gloves). Soil samples were assessed in duplicate for C:N elemental analysis as described above. Soils used for bacteria counts and enzymatic activity assays were stored at 4 °C and processed within 12 h of collection, while soil samples for DNA-related experiments were immediately frozen at –70 °C for subsequent analyses.

Estimation of the abundance of total and active bacteria

Three grams of each soil sample were added to 150 mL of 10 mmol/L sodium phosphate buffer (pH 7.0) and shaken for 1 h. CTC (5-cyano-2, 3-di-4-tolyl-tetrazolium chloride) (Polysciences, Inc., PA, USA) (200 μL) was added to 800 μL of soil slurry (5 mmol/L final concentration) and incubated for 16 h in the dark (Gruden et al. 2003). Cells were fixed by adding formaldehyde (2% final concentration) and incubating for 5 min (Kepener et al. 1994) at room temperature, followed by the addition of Tween 80 (0.1% final concentration). Samples were incubated for 5 min at room temperature and then sonicated for

4 min (45 s pulses at 160 W) (Griebler et al. 2001), to separate bacteria from soil particles. The samples were centrifuged at 50g for 12 min and 800 μL of supernatant was transferred into a new tube, and then stained with 0.5% Picogreen (Quant-iT dsDNA reagent, Molecular Probes) for 5 min (Kepener et al. 1994; Griebler et al. 2001). The supernatant was filtered onto GE polycarbonate (PCTE) filter membranes (pore size 0.2 μm) (Washington DC, USA) under low vacuum pressure. The filters were transferred onto microscope slides for examination. Cells were visualized with fluorescence microscopy (1 000× magnification) to enumerate total cells (those stained with Picogreen) and active cells (cells that reacted with CTC). Ten random fields were selected or an equivalent of a minimum of 200 bacterial cells was counted for each slide. The results were reported as total- and active bacteria per gram dry soil.

Fluorescein diacetate hydrolysis assay

Overall microbial activity was measured by determining the rate of fluorescein diacetate (FDA) hydrolysis. Three grams (wet weight) of soil were suspended in 15 mL of 60 mmol/L sodium phosphate buffer (pH 7.6). FDA (150 μL of a 1 000 μg/mL solution, dissolved in acetone) was added to each sample. Two controls were also used, including one that contained soil and buffer only (to account for background fluorescence in the absence of FDA), and another prepared without soil, containing only buffer and FDA (to determine the rate of background fluorescein production in the absence of soil). All samples were incubated in a shaking water bath (30 min at 200 r/min) at 30 °C, following which enzymatic activity was stopped by briefly shaking with 15 mL of 2:1 chloroform/methanol. Each sample was then subjected to centrifugation for 5 min at 3 000g followed by filtration of the supernatant through filter paper (Whatman, No. 3). The resulting solution was measured for absorbance at OD₄₉₀.

The absorbance of each sample was compared with those of fluorescein standard solutions prepared as described by Adam and Duncan (2001). All samples and controls were assayed in triplicate and the results were expressed as μg of fluorescein produced per gram dry soil per hour.

Bacteria metabolic diversity and average metabolic response

One hundred microliters of each soil (diluted to contain approximately 10⁴ bacteria) sample were added to each well of a BIOLOG Ecoplate (Garland and Mills 1991). Plates were incubated in a humidified chamber for 48 h at 25 °C, after which the optical density (OD₅₉₀) was determined using a Model 680 microplate reader (Bio-Rad, CA, USA). Raw and background absorbance data were collected and analyzed using Microsoft Excel to measure two parameters. First, the AMR, which provides an index of the overall carbon substrate

use by culturable bacteria, was determined by calculating the average OD₅₉₀ per plate. Second, CMD was used to estimate the metabolic diversity of culturable bacteria. CMD refers to the number of differing carbon sources used by the bacteria and was calculated by summing the number of wells in which the absorbance exceeded an OD₅₉₀ of 0.2 (Lohner et al. 2007).

DNA isolation from soil

Approximately 0.8 g (wet weight) of each soil, 0.5 mL of 0.1 mm glass beads (Biospec Products Inc., OK, USA) and 1 mL of sterile DNA extraction buffer (pH 8) (50 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.6), 5% sodium dodecyl sulfate (SDS)) were added to a 2 mL microcentrifuge tube and shaken in a bead beater (Fastprep, Bio101, Thermosavant, MN, USA) for 30 s at 5.5 m/s (Sigler and Zeyer 2004). DNA isolation was carried out using phenol (pH 8)/chloroform extraction (as described in Sambrook and David 2001). Further DNA purification was carried out by passing the DNA solution through a column containing PVPP (polyvinylpyrrolidone, Acros Organics, NJ, USA) (Kuske et al. 1998). Isolated DNA was quantified by measuring optical density at A₂₆₀ and diluted with DNA-grade water to a concentration of 50 µg/mL before further use.

Polymerase chain reaction and denaturing gradient gel electrophoresis analysis

Polymerase chain reaction (PCR) analysis was carried out to target the V3 region of the 16S rRNA gene by combining 0.2 µM of forward primer (341f 5'-CCTACGGGAGGCAGCAG-3') and reverse primer (534r 5'-ATTACCGCGGCTGCTGG-3') (Muyzer et al. 1993), 1 µL of DNA template, 1× PCR buffer (Fisher Scientific, PA, USA), 1.5 mmol/L MgCl₂, 1.5 mg/mL bovine serum albumin (BSA), 0.2 mmol/L dNTP mix, 0.02 U/µL of *Taq* polymerase, and DNA-grade water to a final volume of 50 µL. To facilitate the separation of PCR products during DGGE analysis, a GC-clamp (Muyzer et al. 1993) was attached to the 5' end of the forward primer. All sets of reactions included a positive control (*Escherichia coli* DNA) and a negative control containing no template. PCR products (5 µL) were screened by electrophoresis in 1% agarose gel stained with 0.5 µg/mL ethidium bromide. DGGE analysis was carried out according to the method of Sigler et al. (2004) using a DCode Universal Mutation Detection System (Bio-Rad) in gels containing a denaturant gradient of 40–55% (100% denaturant represents 7 M urea and 40% vol/vol formamide). PCR and DGGE analysis of the bacterial communities were replicated four times in separate analyses. Analysis of the fingerprint patterns was carried out using GelCompar II software (Version 3.5, Applied Maths), with a band position tolerance of 1% and optimization of 3% (Sigler and Pasutti 2006). Fingerprint comparison was facilitated by using a customized DGGE marker, which was developed from DNA isolated from several bacteria strains

commonly used in our laboratory. The DGGE markers were loaded such that a maximum of two samples separated each marker lane. Similarities among fingerprints were identified by constructing a matrix of the Dice coincidence index (Dice 1945). A dendrogram describing the similarity matrix was created using cluster analysis, which was carried out using the unweighted pair-group with arithmetic means algorithm (UPGMA).

Statistical analysis

Differences in means were assessed with two-way ANOVA followed by Tukey's test to identify significant differences among treatments. Significance was determined at $\alpha = 0.05$.

Acknowledgements

We thank Malak A. Esseili for her critical review of the manuscript. We acknowledge the support of the Lucas County Metroparks, who provided access to soil sampling sites.

References

- Adam G, Duncan H (2001). Development of a sensitive and rapid method for the measurement of total microbial activity using fluorescein diacetate (FDA) in a range of soils. *Soil Biol. Biochem.* **33**, 943–951.
- Adam NR, Owensby CE, Ham JM (2000). The effect of CO₂ enrichment on leaf photosynthetic rates and instantaneous water use efficiency of *Andropogon gerardii* in the tallgrass prairie. *Photosynth. Res.* **65**, 121–129.
- Allen AS, Andrews JA, Finzi AC, Matamala R, Richter DD, Schlesinger WH (2000). Effects of free-air CO₂ enrichment (FACE) on below-ground processes in a loblolly pine (*Pinus taeda*) forest. *Ecol. Appl.* **10**, 437–448.
- Alkorta I, Amezaga I, Albizu I, Aizpurua A, Onaindia M, Buchner V, Garbisu C (2003). Molecular microbial biodiversity assessment: a biological indicator of soil health. *Rev. Environ. Health.* **18**, 131–151.
- Arias ME, Gonzalez-Perez JA, Gonzalez-Vila FJ, Ball AS (2005). Soil health: a new challenge for microbiologists and chemists. *Int. Microbiol.* **8**, 13–21.
- Breeuwer P, Drocourt JL, Bunschoten N, Zwietering MH, Rombouts FM, Abee T (1995). Characterization of uptake and hydrolysis of fluorescein diacetate and carboxyfluorescein diacetate by intracellular esterases in *Saccharomyces cerevisiae*, which result in accumulation of fluorescent product. *Appl. Environ. Microbiol.* **61**, 1614–1619.
- Bruce KD, Jones TH, Bezemer TM, Thompson LJ, Ritchie DA (2000). The effect of elevated atmospheric carbon dioxide levels on soil bacterial communities. *Glob. Change Biol.* **6**, 427–434.
- Creach V, Baudoux AC, Bertru G, Rouzic BL (2003). Direct estimate of active bacteria: CTC use and limitations. *J. Microbiol. Method.* **52**, 19–28.

- Dhillion SS, Roy J, Abrams M** (1996). Assessing the impact of elevated CO₂ on soil microbial activity in a Mediterranean model ecosystem. *Plant Soil* **187**, 333–342.
- Diaz S, Grime JP, Harris J, McPherson E** (1993). Evidence of a feedback mechanism limiting plant response to elevated carbon dioxide. *Nature* **364**, 616–617.
- Dice LR** (1945). Measures of the amount of ecological association between species. *Ecology* **26**, 297–302.
- Ebersberger D, Wermbter N, Niklaus P, Kandeler E** (2004). Effects of long term CO₂ enrichment on microbial community structure in calcareous grassland. *Plant Soil* **264**, 313–323.
- Ehleringer JR, Cerling TE, Dearing MD** (2002). Atmospheric CO₂ as a global change driver influencing plant-animal interactions. *Integr Comp. Biol.* **42**, 424–430.
- Fay PA, Carlisle JD, Knapp A, Blair JM, Collins SL** (2003). Productivity responses to altered rainfall patterns in a C₄-dominated grassland. *Oecologia* **137**, 245–251.
- Fierer N, Schimel JP, Holden PA** (2003). Influence of drying-rewetting frequency on soil bacterial community structure. *Microb. Ecol.* **45**, 63–71.
- Franklin RB, Mills AL** (2003). Multi-scale variation in spatial heterogeneity for microbial community structure in an eastern Virginia agricultural field. *FEMS Microbiol. Ecol.* **44**, 335–346.
- Freeman C, Kim SY, Lee SH, Kang H** (2004). Effects of elevated atmospheric CO₂ concentrations on soil microorganisms. *J. Microbiol.* **42**, 267–277.
- Garcia-Pichel F, Johnson SL, Youngkin D, Belnap J** (2003). Small-scale vertical distribution of bacterial biomass and diversity in biological soil crusts from arid lands in the Colorado plateau. *Microb. Ecol.* **46**, 312–321.
- Garland JL, Mills AL** (1991). Classification and characterization of heterotrophic microbial communities on the basis of patterns of community level sole-carbon-source utilization. *Appl. Environ. Microbiol.* **57**, 2351–2359.
- Gaspar ML, Cabello MN, Pollero R, Aon MA** (2001). Fluorescein diacetate hydrolysis as a measure of fungal biomass in soil. *Curr. Microbiol.* **42**, 339–344.
- Griebler C, Mindl B, Slezak D** (2001). Combining DAPI and SYBR green II for the enumeration of total bacterial numbers in aquatic sediments. *Int. Rev. Hydrobiol.* **86**, 453–465.
- Griffiths RI, Whiteley AS, O'Donnell AG, Bailey MJ** (2003). Physiological and community responses of established grassland bacterial populations to water stress. *Appl. Environ. Microbiol.* **69**, 6961–6968.
- Gruden CL, Khijniak A, Adriaens P** (2003). Activity assessment of microorganisms eluted from sediments using 5-cyano-2,3-ditoly tetrazolium chloride: a quantitative comparison of flow cytometry to epifluorescent microscopy. *J. Microbiol. Methods* **55**, 865–874.
- Haack SK, Garchow H, Klug MJ, Forney LJ** (1995). Analysis of factors affecting the accuracy, reproducibility, and interpretation of microbial community carbon source utilization patterns. *Appl. Environ. Microbiol.* **61**, 1458–1468.
- Halverson LJ, Jones TM, Firestone MK** (2000). Release of intracellular solutes by four soil bacteria exposed to dilution stress. *Soil Sci. Soc. Am. J.* **64**, 1630–1637.
- Heckathorn SA, DeLucia EH, Zielinski RE** (1997). The contribution of drought-related decreases in foliar nitrogen concentration to decreases in photosynthetic capacity during and after drought in prairie grasses. *Physiol. Plant.* **101**, 173–182.
- Heckathorn SA, Poeller GJ, Coleman JS, Hallberg RL** (1996). Nitrogen availability alters patterns of accumulation of heat stress-induced proteins in plants. *Oecologia* **105**, 413–418.
- Intergovernmental Panel on Climate Change (IPCC)** (2007). Contribution of working group I to the fourth assessment report of the Intergovernmental Panel on Climate Change. In: Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB et al., eds. *Climate Change 2007: The Physical Science Basis*. Cambridge University Press, Cambridge.
- Kandeler E, Tschermo D, Bardgett RD, Hobbs PJ, Kampichler C, Jones TH** (1998). The response of soil microorganisms and roots to elevated CO₂ and temperature in a terrestrial model ecosystem. *Plant Soil* **202**, 251–262.
- Kepener JR, Raymond L, Pratt JR** (1994). Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. *Microbiol. Rev.* **58**, 603–615.
- Knapp AK** (1984). Post-burn differences in solar radiation, leaf temperature and water stress influencing production in a lowland tallgrass prairie. *Am. J. Bot.* **71**, 220–227.
- Knapp AK** (1985). Effect of fire and drought on the ecophysiology of *Andropogon gerardi* and *Panicum virgatum* in a tallgrass prairie. *Ecology* **66**, 1309–1320.
- Konopka A, Oliver L, Turco RF** (1998). The use of carbon substrate utilization patterns in environmental and ecological microbiology. *Microb. Ecol.* **35**, 103–115.
- Kuske CR, Banton KL, Adorada DL, Stark PC, Hill KK, Jackson PJ** (1998). Small-scale DNA sample preparation method for field PCR detection of microbial cells and spores in soil. *Appl. Environ. Microbiol.* **64**, 2463–2472.
- Lecain DR, Morgan JA, Mosier AR, Nelson JA** (2003). Soil and plant water relations determine photosynthetic responses of C₃ and C₄ grasses in a semi-arid ecosystem under elevated CO₂. *Ann. Bot. (Lond)* **92**, 41–52.
- Lohner RN, Sigler V, Mayer CM, Balogh CA** (2007). Comparison of the benthic bacterial communities within and surrounding *Dreissena* clusters in lakes. *Microb. Ecol.* **54**, 469–477.
- McCaig AE, Glover LA, Prosser JI** (2001). Numerical analysis of grassland bacterial community structure under different land management regimens by using 16S ribosomal DNA sequence data and denaturing gradient gel electrophoresis banding patterns. *Appl. Environ. Microbiol.* **67**, 4554–4559.
- Mearns LO, Katz RW, Schneider SH** (1984). Extreme high temperature events: changes in their probability with changes in mean temperature. *J. Clim. Appl. Meteorol.* **23**, 1601–1613.
- Muyzer G, de Waal EC, Uitterlinden AG** (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis

- analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**, 695–700.
- Muyzer G, Smalla K** (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek* **73**, 127–141.
- Owensby CE, Auen LM, Coyne PI** (1994). Biomass production in a nitrogen-fertilized, tallgrass prairie ecosystem exposed to ambient and elevated levels of CO₂. *Plant Soil* **165**, 105–113.
- Owensby CE, Ham JM, Knapp AK, Auen LM** (1999). Biomass production and species composition change in a tallgrass prairie ecosystem after long-term exposure to elevated atmospheric CO₂. *Glob. Change Biol.* **5**, 497–506.
- Owensby CE, Ham JM, Knapp AK, Bremer D, Auen LM** (1997). Water vapour fluxes and their impact under elevated CO₂ in a C₄-tallgrass prairie. *Glob. Change Biol.* **3**, 189–195.
- Palmer RJ Jr, Friedmann EI** (1990). Water relations and photosynthesis in the cryptoendolithic microbial habitat of hot and cold deserts. *Microb. Ecol.* **19**, 111–118.
- Peters W, Jacobson AR, Sweeney C, Andrews AE, Conway TJ, Matarie K et al.** (2007). An atmospheric perspective on North American carbon dioxide exchange: carbon tracker. *Proc. Natl. Acad. Sci. USA* **104**, 18925–18930.
- Phillips DA, Fox TC, Six J** (2006). Root exudation (net efflux of amino acids) may increase rhizodeposition under elevated CO₂. *Glob. Change Biol.* **12**, 561–567.
- Rice CW, Garcia FO, Hampton CO, Owensby CE** (1994). Soil microbial response in tallgrass prairie to elevated CO₂. *Plant Soil* **165**, 67–74.
- Sadowsky MJ, Schortemeyer M** (1997). Soil microbial response to increased concentrations of atmospheric CO₂. *Glob. Change Biol.* **3**, 217–224.
- Sage RF, Monson RK** (1999). *C₄ Plant Biology*. Academic Press, San Diego.
- Sambrook J, David DW** (2001). *Molecular Cloning: A Laboratory Manual (3-Volume Set)*. Cold Spring Harbor Laboratory Press, New York.
- Schmidt TM** (2006). The maturing of microbial ecology. *Int. Microbiol.* **9**, 217–223.
- Sigler V, Pasutti L** (2006). Evaluation of denaturing gradient gel electrophoresis to differentiate *Escherichia coli* populations in secondary environments. *Environ. Microbiol.* **8**, 1703–1711.
- Sigler WV, Miniaci C, Zeyer J** (2004). Electrophoresis time impacts the denaturing gradient gel electrophoresis-based assessment of bacterial community structure. *J. Microbiol. Methods* **57**, 17–22.
- Sigler WV, Zeyer J** (2004). Colony-forming analysis of bacterial community succession in deglaciated soils indicates pioneer stress-tolerant opportunists. *Microb. Ecol.* **48**, 316–323.
- Torsvik V, Ovreas L** (2002). Microbial diversity and function in soil: from genes to ecosystems. *Curr. Opin. Microbiol.* **5**, 240–245.
- Wang D, Heckathorn SA, Barua D, Joshi P, Hamilton EW, LaCroix JJ** (2008). Effects of elevated CO₂ on the tolerance of photosynthesis to acute heat stress in C₃, C₄, and CAM species. *Am. J. Bot.* **95**, 165–176.
- Ward JK, Tissue DT, Thomas RB, Strain BR** (1999). Comparative responses of model C₃ and C₄ plants to drought in low and elevated CO₂. *Glob. Change Biol.* **5**, 857–867.
- Whiteley AS, Griffiths RI, Bailey MJ** (2003). Analysis of the microbial functional diversity within water-stressed soil communities by flow cytometric analysis and CTC+ cell sorting. *J. Microbiol. Methods* **54**, 257–267.
- Williams MA, Rice CW, Omay A, Owensby CE** (2004). Carbon and Nitrogen pools in a tallgrass prairie soil under elevated Carbon dioxide. *Soil Sci. Soc. Am. J.* **68**, 148–153.
- Williams MA, Rice CW, Owensby CE** (2000). Carbon dynamics and microbial activity in tallgrass prairie exposed to elevated CO₂ for 8 years. *Plant Soil* **227**, 127–137.
- Wullschleger SD, Tschaplinski TJ, Norby RJ** (2002). Plant water relations at elevated CO₂- implications for water-limited environments. *Plant Cell Environ.* **25**, 319–331.
- Zak DR, Pregitzer KS, Curtis PS, Teeri JA, Fogel R, Randlett DL** (1993). Elevated atmospheric CO₂ and feedback between carbon and nitrogen cycles. *Plant Soil* **151**, 105–117.
- Zak DR, Pregitzer KS, King JS, Holmes WE** (2000). Elevated atmospheric CO₂, fine roots and the response of soil microorganisms: a review and hypothesis. *New Phytol.* **147**, 201–222.

(Handling editor: Jiquan Chen)