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Soil biota responses to long-term atmospheric CO_2 enrichment in two California annual grasslands

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Abstract Root, arbuscular-mycorrhizal (AM), soil faunal (protozoa and microarthropods), and microbial responses to field exposure to CO_2 for six growing seasons were measured in spring 1997 in two adjacent grassland communities. The grasslands showed contrasting root responses to CO_2 enrichment: whereas root length was not affected in the sandstone grassland, it was greater in the serpentine grassland, as was specific root length. AM fungal hyphal lengths were greater in the sandstone, but were unaffected in the serpentine community. This lent support to the hypothesis that there may be a tradeoff in resource allocation to more fine roots or greater mycorrhizal extraradical hyphal length. AM root infection was greater in both communities at elevated CO_2, as was the proportion of roots containing arbuscules. Our data on total hyphal lengths, culturable and active fungi, bacteria, and protozoa supported the hypothesis that the fungal food chain was more strongly stimulated than the bacterial chain. This study is one of the first to test these hypotheses in natural multi-species communities in the field.

Key words Root Hyphae · Arbuscular-mycorrhizae · Food web · Grasslands

Introduction

Terrestrial ecosystems generally respond to exposure to elevated CO_2 with an increased belowground resource allocation in the form of increased root growth and possibly higher rhizodeposition (Norby 1994; Rogers et al. 1994). Soil microbial growth is constrained by carbon availability (Paul and Clark 1989; Zak et al. 1994), and hence an increase in carbon should affect soil bacteria and fungi. Since fungi have a higher substrate use efficiency than bacteria (e.g., Zak et al. 1996), a shift in the relative contribution of these components to microbial biomass could be expected in response to increased carbon availability. If bacteria and fungi are differentially affected by elevated CO_2, an effect on higher levels of the respective bacterial and fungal soil food chains in soil might also be expected. Experimental evidence from field studies to support this hypothesis is quite sparse. Often, only total microbial biomass was measured (e.g., Rice et al. 1994; Niklaus and Körner 1996; Hungate et al. 1997). Runion et al. (1994) observed no significant changes in bacteria and fungi in elevated CO_2, and also found no effect on microarthropod and minimal effects on saprophytic nematode populations. Zak et al. (1996) were unable to detect a difference between bacterial and fungal phospholipid fatty acids in ambient and elevated CO_2. Klironomos et al. (1997) studied only the fungal food chain and found an increase in mycorrhizal hyphae and a decrease in other hyphae in elevated CO_2, but no increase in microarthropod numbers. Lussenhop et al. (1998) found no increase in microarthropod numbers in elevated CO_2, or in microbial biomass C or extraradical mycorrhizal fungal hyphal length, but an increase in the number of protozoa. This suggested stimulation of the bacterial rather than the fungal food chain. These last four studies were all performed in the field, but using a single-species monoculture. It is uncertain how bacterial and fungal food chains respond to long-term CO_2 exposure in intact multi-plant species communities.

Among the soil biota, arbuscular-mycorrhizal (AM) fungi play a special role because they provide a direct link between plant roots and the soil, functioning more as an extension of the root system than as a part of the

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heterotrophic soil microbiota (Allen 1991). Miller et al. (1995) documented a negative correlation between gross root morphology (specific root length) and extraradical hyphal length in soil for two grassland communities. Rarely have gross root morphology (for example specific root length) and extraradical mycorrhizal hyphal length been considered together for a plant community in elevated-CO_2 experiments. If more resources are allocated belowground under elevated CO_2, will these be invested (at the plant community level) equally in more fine roots and greater hyphal length, or will one be favored at the expense of the other?

The Jasper Ridge CO_2 experiment (Field et al. 1996) provided a unique opportunity for addressing these questions using a comparative approach. An identical field CO_2 exposure system was applied to two adjacent grassland communities experiencing the same climate. The sandstone and serpentine grassland ecosystems included in this study differ in soil properties (Luo et al. 1996; for example, the sandstone is generally less nutrient limited than the serpentine), plant species composition, productivity, and in their responsiveness to elevated CO_2 (Field et al. 1996). We specifically tested the following hypotheses in this study. (1) In both grasslands, elevated CO_2 will increase root length, root biomass, specific root length, and mycorrhizal root infection. (2) The grassland with the greater increase in mycorrhizal hyphae will show the smaller increase in specific root length and vice versa. (3) The fungal food chain will be stimulated more strongly than the bacterial food chain in both grasslands.

Materials and methods

Field experiment

This research was conducted at the Jasper Ridge Biological Preserve near Stanford, Calif. (37°24′N, 122°13′W, 100 m elevation). The site has a Mediterranean-type climate with cool, wet winters and warm, dry summers. At the site, two different grassland communities exist adjacent to each other. The serpentine grassland occurs on serpentine-derived soils, and sandstone grassland on sandstone-derived soils. The CO_2 experiment in the field consisted of 30 plots of 0.33 m^2 area in each of the two grassland communities. Three treatments were started in January 1992, replicated ten times in both grasslands: no-chamber controls, open-top chambers (cylindrical, 1 m tall) with ambient CO_2 and open-top chambers with elevated CO_2 (ambient + 350 $\mu l\ l^{-1}$). Comprehensive details on the design of the Jasper Ridge experiment are given in Field et al. (1996). At the end of the growing season, in early April 1997, after six growing seasons of continuous fumigation with elevated CO_2, a soil sample was obtained from each chambered plot to a depth of 15 cm with a corer (7.9 cm diameter), cooled on ice, and stored in polyethylene bags.

Root and mycorrhizal measurements

Roots (≤ 1 mm) were hand picked from a 10-g soil subsample per core (standardized picking time per sample: 45 min), dried to constant weight at 65°C, and root length was measured (using dried roots) according to Tennant (1975). Specific root length was calculated by dividing root length by root weight for a sample. A random subsample of these roots (> 25 2-cm-long pieces) was also used to measure fungal root infection. Dried roots were washed in tap water, cleared in 10% KOH (90°C) for 45 min, acidified in 1% HCl for 15 min, stained in 0.05% Trypan Blue in lactoglycerol (90°C) for 30 min, and then stored in lactoglycerol (Brundrett 1994). The root fragments were mounted on slides, and at each gridline intersect at ×200 magnification, presence and absence of fungal infection (AM and non-mycorrhizal fungal infection) was noted (Rillig et al. 1998). For each sample, 200 gridline intersects were examined. AM fungal hyphae were distinguished from non-mycorrhizal fungal hyphae as described in detail in Rillig et al. (1998). The presence of arbuscules and vesicles was noted.

Hyphae were extracted from a 4-g soil subsample by an aqueous extraction and membrane filter technique modified after Jakobsen et al. (1992). Soil samples were mixed and suspended in 100 ml of deionized water, to which 12 ml of a sodium hexametaphosphate solution (35 $g\ l^{-1}$) was added. The soil suspensions were shaken for 30 s (end-over-end), left on the bench for 30 min, and then decanted quantitatively through a 38-μm sieve to retain hyphae, roots, and organic matter. The material on the sieve was sprayed gently with deionized water to remove clay particles, and then transferred into a 250-ml Erlenmeyer flask with 200 ml of deionized water. The flask was shaken vigorously by hand for 5 s, left on the bench for 1 min, and then a 2-ml aliquot was taken and pipetted onto 25-mm Millipore filters. The material on the filter was stained with 0.02% Trypan Blue in lactoglycerol for 5 min, rinsed with deionized water and transferred to microscope slides. Hyphae were distinguished at ×200 magnification into mycorrhizal and non-mycorrhizal hyphae according to Miller et al. (1995) using similar criteria as for internal hyphae. Hyphal extraction efficiency was determined by re-extracting hyphae in every discarded fraction according to Miller et al. (1995) and hyphal length values were corrected accordingly (extraction efficiency averaged 85%).

Soil faunal and microbial measurements

Microarthropods were extracted from soil using a modification of the Berlese funnel extractor method (Moldenke 1994). Within 24 h of sampling, 0.5 kg of soil was placed into large plastic funnels lined with wet cheesecloth and moistened. The funnels were arranged in series under 40-W light bulbs. Animals were collected for 7 days into beakers placed under the funnels, killed, rinsed with tap water, and stored in 50% ethanol. The isolated microarthropods were counted under a dissecting scope (× 50), and the numbers were expressed as individuals per kilogram of dry soil. Protozoa (ciliates, amoebae, and flagellates) were determined with a most-probable-number protocol (Ingham 1994). Active fungal length and bacteria were measured using the europium (III) thenoyltrifluoroacetonate differential fluorescent staining procedure and the fluorescein diacetate (FDA) method following Morris et al. (1997). Similar results were obtained with both methods, and only the FDA results are presented. Additionally, fungal colony-forming units were measured (for the sandstone soil only; for seven chambers per treatment) with a soil dilution method (Warcup 1955) and a soil-washing protocol (Gams and Domsch 1967). For the soil dilution method, serial dilutions were made using sterile water. One milliliter of soil suspension was plated onto Cooke Rose Bengal agar (Difco, Michigan) using seven non-vented petri dishes per soil sample. Twenty sequential washings with sterile water were carried out for the soil-washing procedure, samples were shaken vigorously on a wrist-action shaker, and per petri plate, four small soil particles of approximately equal size were transferred under sterile conditions onto Cooke Rose Bengal agar (ten plates per sample). Only small particles were plated since slow-growing fungi may be overgrown when larger particle sizes are used (Bååth 1988). Colonies were counted in each case after incubation in the dark at room temperature for 10 days. The soil-washing technique favors the isolation of fungi originating from hyphae, since the washing process supposedly removes the spores present in soil (Bisset and Widden 1972). The soil dilution method captures both spores and hyphae. By using both methods, we could test if putative treatment differences arose purely through a change in sporulation.

Statistical analysis

Data were analyzed using 2×2 factorial ANOVAs with the fixed factors CO_2 (ambient and elevated) and plant community (sandstone and serpentine). Parametric assumptions of normality (Shapiro-Wilks *W*-test) and homogeneity of variances (Levene's test) were checked, and data were $\log_{10}$ or arcsine square root transformed when necessary. All statistical tests were performed using the SYSTAT 7.0 software (SPSS, Chicago,Ill.).

Results

Root responses to elevated CO_2 followed different patterns in the two grasslands (Table 1; *P*-values in Table 2). Whereas in the sandstone grassland, root length was not affected, in the serpentine, root length was 78% greater under elevated CO_2 relative to ambient. Specific root length was also higher in the serpentine grassland, since root weight only increased by 49%. Conversely, in the sandstone, specific root length decreased in elevated CO_2. AM hyphal lengths followed an inverse pattern to the root morphology responses, with a strong increase in the sandstone and no significant change in the serpentine.

AM fungal root infection differed between the two grasslands at ambient levels, and in both communities there was a significant and large increase under elevated CO_2. The proportion of root with arbuscules, the short-lived symbiotic organ of carbon and nutrient exchange, was tenfold greater under CO_2 enrichment in the sandstone grassland, and almost threefold greater in the serpentine. Vesicles were only found in a few of the roots examined and their abundance showed no trend across the treatment (data not shown). The proportion of roots infected with non-mycorrhizal fungi was significantly less under elevated CO_2 in both grasslands. Reflecting the enhancement of percent AM infection, and the increased root length, the infected root length per gram of soil was significantly greater under elevated CO_2 in both grasslands.

In the sandstone grassland, the AM hyphal length to root length ratio increased, primarily because hyphal length was greater at elevated CO_2 while root length was unchanged. However, AM hyphal length per *infected* root length did not change significantly in the sandstone grassland. The latter ratio directly relates root-internal AM fungal presence with extraradical proliferation, i.e., how many hyphae are produced per infection unit. In contrast, both ratios in the serpentine were lower with CO_2 enrichment, because of increased root length in this grassland.

Data pertaining to the fungal and bacterial food chains are presented in Table 3 (*P*-values in Table 4), with additional information on culturable fungal propagules from the sandstone in Table 5. Total hyphal

Table 1 Means (with SEs in parentheses) of root and arbuscular-mycorrhizal (AM) parameters under ambient and elevated atmospheric CO_2 in the sandstone and serpentine grassland

Variable	Sandstone		Serpentine	
	Ambient	Elevated	Ambient	Elevated
Root length (m g^{-1} soil)	0.211 (0.014)	0.226 (0.013)	0.149 (0.007)	0.266 (0.016)
Root weight ($\times 10^{-3}$ g g^{-1} soil)	0.73 (0.09)	0.90 (0.06)	0.59 (0.05)	0.88 (0.09)
Specific root length (m g^{-1})	309.8 (24.1)	254.9 (11.9)	257.8 (10.9)	316.2 (26.6)
Fractional AM root infection	0.44 (0.04)	0.76 (0.05)	0.54 (0.03)	0.84 (0.02)
Fractional arbuscular infection	0.02 (0.01)	0.21 (0.03)	0.11 (0.02)	0.31 (0.02)
Fractional non-mycorrhizal fungal infection	0.21 (0.04)	0.11 (0.03)	0.25 (0.04)	0.09 (0.02)
AM root length (m g^{-1} soil)	0.09 (0.01)	0.17 (0.01)	0.08 (0.01)	0.22 (0.01)
AM hyphal length (m g^{-1} soil)	24.45 (1.12)	45.57 (0.82)	39.24 (2.25)	37.86 (1.39)
AM hyphal length/AM root length (m m^{-1})	282.7 (23.5)	287.3 (31.2)	513.6 (66.2)	175.1 (41.1)
AM hyphal length/root length (m m^{-1})	121.3 (10.9)	207.8 (13.8)	278.3 (26.1)	146.2 (8.2)

Table 2 *F*- and *P*-values from 2×2 (CO_2 concentration; grassland community) factorial analyses of variance for root and AM parameters. *P*-values < 0.05 are *italicized*

Variable	Community		CO_2		Community × CO_2	
	F	*P*	*F*	*P*	*F*	*P*
Root length	0.70	0.40	23.96	*< 0.0001*	14.15	*< 0.0001*
Root weight	1.12	0.29	9.92	*0.003*	0.75	0.39
Specific root length	0.05	0.81	0.01	0.92	8.26	*0.007*
AM root infection	5.28	*0.02*	69.52	*< 0.0001*	0.12	0.73
Arbuscular infection	21.73	*< 0.0001*	86.02	*< 0.0001*	0.06	0.81
Non-mycorrhizal fungal infection	0.08	0.77	15.40	*0.0004*	1.05	0.31
AM root length	3.55	0.06	108.86	*< 0.0001*	8.21	*0.007*
AM hyphal length	5.61	*0.02*	43.61	*< 0.0001*	56.66	*< 0.0001*
AM hyphal length/AM root length	2.35	0.13	18.58	*< 0.0001*	19.61	*< 0.0001*
AM hyphal length/root length	9.30	*0.004*	2.13	0.15	48.97	*< 0.0001*

Table 3 Means (SEs in parentheses) of soil microbial and microfaunal parameters under ambient and elevated atmospheric CO_2 in the sandstone and serpentine grassland

Variable	Sandstone		Serpentine	
	Ambient	Elevated	Ambient	Elevated
Total hyphae (m g^{-1} soil)	30.1 (1.0)	50.5 (1.3)	44.4 (2.3)	41.1 (1.3)
Active hyphal length (m g^{-1} soil)	0.98 (0.11)	0.62 (0.13)	0.43 (0.06)	0.42 (0.06)
Active fungal biomass ($\times 10^{-6}$ g g^{-1} soil)	12.6 (1.4)	7.9 (1.7)	8.6 (1.2)	8.5 (1.3)
Microarthropods (kg^{-1} soil)	63.8 (7.3)	133.1 (12.5)	61.7 (12.0)	86.0 (7.9)
Active bacteria ($\times 10^{8}$ g^{-1} soil)	1.21 (0.24)	1.10 (0.2)	0.44 (0.03)	0.52 (0.06)
Bacterial biomass ($\times 10^{-6}$ g g^{-1} soil)	24.1 (4.8)	22.1 (5.0)	8.9 (0.6)	10.4 (1.3)
Protozoa (g^{-1} soil)				
Flagellates	2202 (537)	2753 (624)	3496 (715)	3242 (1481)
Amoebae	1100 (265)	828 (188)	1109 (478)	684 (198)
Ciliates	71.8 (46.6)	57.1 (28.4)	8.0 (3.0)	4.96 (3.5)

Table 4 *F*- and *P*-values from 2×2 (CO_2 concentration; grassland community) factorial analyses of variance for soil microbial and microfaunal parameters. *P*-values < 0.05 are *italicized*

Variable	Community		CO_2		Community × CO_2	
	F	*P*	*F*	*P*	*F*	*P*
Total fungal hyphal length	2.45	0.12	29.41	*< 0.0001*	56.16	*< 0.0001*
Active fungal hyphal length	14.86	*0.0005*	3.63	0.06	3.33	0.07
Active fungal biomass	1.48	0.23	2.83	0.10	2.49	0.12
Microarthropods	5.84	*0.02*	21.22	*< 0.0001*	4.91	*0.03*
Active bacteria/biomass	14.41	*0.0005*	0.007	0.93	0.24	0.62
Protozoa (total)	0.61	0.43	0.01	0.89	0.25	0.61
Flagellates	0.94	0.33	0.02	0.87	0.19	0.66
Amoebae	0.05	0.82	1.29	0.26	0.06	0.80
Ciliates	4.4	*0.04*	0.10	0.74	0.04	0.83

Table 5 Mean (SE in parentheses) fungal colony-forming units from soil dilution plating (expressed as per gram soil) and soil washing (expressed as per plated soil particle) under ambient and elevated CO_2 in the sandstone grassland. *F*- and *P*-values are from analyses of variance (*italics* highlights significance at $P < 0.05$)

Variable	Ambient	Elevated	*F* (*P*)
Colony-forming units ($\times 10^{4}$ g^{-1} soil)	1.56 (0.07)	2.66 (0.13)	11.05 (*0.006*)
Colony-forming units (particle^{-1})	1.64 (0.03)	2.06 (0.02)	103.6 (*< 0.0001*)

length was higher at elevated CO_2 in the sandstone grassland because of the greater length of AM hyphae. There was no significant change in the serpentine. Active fungal hyphal length and biomass were unaffected by treatment in both grasslands. Table 5 shows the results for culturable fungal propagules in the sandstone grassland only. Using both the soil dilution plating and soil-washing techniques, we found a significantly higher number of colony-forming units, and hence in the proportion of culturable fungi. Microarthropod (mites and collembola) numbers were 108% greater in the sandstone and 39% greater in the serpentine grassland. The number of active bacteria, bacterial biomass and the number of protozoa (divided into flagellates, amoebae, and ciliates) were all unaffected by CO_2 treatment.

Discussion

After long-term CO_2 exposure, we measured root characteristics and AM parameters, as well as several aspects of bacterial and fungal food chains in two multi-species natural grasslands. Our data lent support to all three of our hypotheses.

Root length and root biomass increases under elevated CO_2 are common (e.g., Rogers et al. 1994), and also occurred in our study. In the serpentine community, root weight increased less than root length, resulting in a higher specific root length, i.e., more fine roots. Conversely, root weight also increased in the sandstone grassland, but root length did not, i.e., roots were on average coarser. We hypothesized that AM hyphal length should be greater with CO_2 enrichment, as has been found in the field in other studies (Klironomos et al. 1998). This was the case in the sandstone grassland, but not in the serpentine, where hyphal length was unaffected.

Our hypothesis regarding an inverse relationship between fine-root and hyphal production was supported by these data, but much more drastically than expected. The two grasslands appear to exhibit different resource allocation strategies (hyphae vs fine roots). This is an important result to be taken into consideration in CO_2 field studies, since if only one of the two parameters is measured, significant responses of ecosystems may be

overlooked. It is interesting to speculate whether there is a general tradeoff between a stimulation (of production or activity) of fine roots and AM fungal hyphal length in elevated CO_2. Such differential resource allocation, if it occurs as a function of plant species, may help explain the variability among the responses of different plant species to CO_2.

The underlying mechanisms for a tradeoff of resource investment into hyphae or fibrous roots (which can be thought of as ecologically similar strategies for increasing surface area for resource acquisition) are not understood. Possible mechanisms for the observed results of this comparative study are difficult to give since the two ecosystems differ in a variety of traits. Soil (e.g., nutrient status, bulk density), plant (e.g., species, physiology), and fungal (isolate) factors can influence the production of AM hyphae (Smith and Read 1997). One hypothesis is that, under certain circumstances, hyphae could be 'cheaper' to produce than roots (Fitter 1987). For example, Miller et al. (1995) reported that in a prairie and pasture community during a period of drought, specific root length was reduced without a reduction in AM hyphal length. Recovery occurred in both plant communities by increasing AM hyphal length, and not by increasing specific root length. This study, as well as ours, suggests that AM hyphal production and gross root morphology may be coordinated at the plant community level. It is possible that elevated CO_2 altered the production of fine roots versus hyphae by means of altered plant carbon supply to roots. Other root factors that may influence AM hyphal production, such as root architecture (e.g., Fitter 1987) and root hair length (Schweiger et al. 1995), may have also been affected by CO_2 enrichment, and potentially to a different degree in the two grasslands. Furthermore, the two grasslands may differ in AM fungal species composition. There is evidence from pot studies that stimulation of AM hyphal production under elevated CO_2 is a function of fungal isolate (Klironomos et al. 1998; but see Sanders et al. 1998).

Differential investment into hyphae versus fibrous roots may have important ramifications for nutrient and carbon cycling, for example if decomposition and turnover rates are different for AM hyphae and fine roots. However, to our knowledge, no data exist on comparative turnover rates and carbon costs of AM hyphae and roots in the same natural ecosystem.

Root infection responses to elevated CO_2 have rarely been reported at the plant community level for complex natural communities, but mostly for single-species experiments (e.g., Dhillion et al. 1996), or per plant species for co-occurring plants (Monz et al. 1994; Rillig et al. 1998). Whitbeck (1994) observed no or no consistent percent colonization responses to CO_2 in these same serpentine and sandstone grasslands. The increases under elevated CO_2 we observed here were likely a function of the long duration of the study, potential plant and fungal community changes, and physiological adaptations at the individual plant level. It is well known that plant species differ in fractional root infection under ambient conditions (for example in the sandstone community; Rillig et al. 1998). Therefore, the large and clear increases in fractional root infection in our study were probably also a consequence of the large soil samples taken, averaging over spatial variability in plant and fungal species composition. Interestingly, for both plant communities, non-mycorrhizal fungal root infection decreased significantly, a response previously found for individual plants grown in pots (Rillig et al. 1998). The root infection results suggest that there may be increased demand for the functions of mycorrhizal fungi in both grasslands under elevated CO_2, and that these functions may be nutritional (arbuscules) in nature and/or related to protection of roots against colonization by non-mycorrhizal fungi (Newsham et al. 1995).

To predict potential feedbacks in the plant-soil system at elevated CO_2, it is also important to understand changes in the soil food web (e.g., Couteaux et al. 1991). We presented data to support the hypothesis that the fungal food chain was more strongly stimulated than the bacterial food chain under elevated CO_2. In fact, we found no evidence for a change within the bacterial food chain. There were no detectable changes to the number of bacteria or protozoa in either of the two communities, but fungal hyphal length and microarthropod abundance were increased under elevated CO_2 in both grasslands. Additionally, the number of culturable fungal propagules derived from hyphae and from a hyphae/spore combination was increased in the sandstone grassland under elevated CO_2.

Klironomos et al. (1996) reported a similar stimulation of the fungal versus the bacterial food chain for a pot study with the shrub *Artemisia tridentata*. Under low nutrient conditions, only fungal biomass increased with elevated CO_2, but in fertilized soil, both bacterial and fungal biomass rose. Microarthropod numbers increased with elevated CO_2 only in the high-nutrient treatment, and remained unchanged in the low-nutrient experiment. Nematode abundance increased under both nutrient conditions. Newton et al. (1995) used a managed pasture community transferred into pots and a controlled environment to measure microbial biomass (not separated into bacteria and fungi), nematodes and enchytraeids. The only significant change was an increase in enchytraeid numbers in elevated CO_2. Using simplified model ecosystems in a controlled environment facility, Jones et al. (1998) found a change in fungal species composition (but not biomass) and an increase in collembola numbers in elevated CO_2. Conversely, bacterial biomass was unaffected, but protozoa and nematodes were not measured in their study.

To conclude, direct comparison of the response of two natural grasslands to elevated CO_2 has uncovered a potentially important tradeoff in resource allocation to fine roots versus AM hyphae. We also provided some of the first evidence for AM root infection increases under enriched CO_2 at the community level, and for a preferential stimulation of the soil fungal versus the bacterial food chain in multi-species communities in the field.

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