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Community-level physiological profiles of bacteria and fungi: plate type and incubation temperature influences on contrasting soils

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Abstract

Temperature sensitivity of community-level physiological profiles (CLPPs) was examined for two semiarid soils from the southwestern United States using five different C-substrate profile microtiter plates (Biolog GN2, GP2, ECO, SFN2, and SFP2) incubated at five different temperature regimes. The CLPPs produced from all plate types were relatively unaffected by these contrasting incubation temperature regimes. Our results demonstrate the ability to detect CLPP differences between similar soils with differing physiological parameters, and these differences are relatively insensitive to incubation temperature. Our study also highlights the importance of using both bacterial and fungal plate types when investigating microbial community differences by CLPP. Nevertheless, it is unclear whether or not the differences in CLPPs generated using these plates reflect actual functional differences in the microbial communities from these soils in situ.
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Keywords: Biolog; Microbial functional diversity; Soil microbial community; Substrate utilization pattern

1. Introduction

Soil microorganisms regulate many ecosystem processes such as nutrient transformations and litter decomposition, as well as influence soil structural and hydrological properties [1–4]. Although we know a great deal about how the activity of the soil microflora affects these processes, we understand much less about the influences of the composition and structure of microbial communities [3,5]. Research linking soil microbial community structure with ecosystem function has been impeded, in part, because many of the available approaches for describing microbial communities involve large investments of time and monetary resources, and require highly specialized expertise [6].

Over the past decade, the diversity of soil microbial communities has been increasingly characterized using the utilization pattern of individual carbon (C) substrates generated with commercially available 96-well Biolog microtiter plates [6,7]. These community-level physiological profiles (CLPPs) provide a rapid and relatively inexpensive means of assessing differences in the soil microflora [8–10]. Microbial community analyses based on CLPPs have been corroborated by other microbial community measures, including plate counts [11–14], fatty acid methyl ester and phospholipid fatty acid analysis [13–19], API 20NE enzyme and C tests [20], and an array of molecular assays [19,21,22]. In addition, previous research has demonstrated that CLPPs are highly reproducible [23–25].

A variety of CLPP plate types are available commercially from Biolog, Inc. (Hayward, CA, USA), including types designed specifically for bacteria or fungi. The GN2 and GP2 plates each contain 95 unique C substrates that

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were developed for identifying pure cultures of Gram-negative and Gram-positive bacteria, respectively [26]. The GN2 and GP2 plates share 62 substrates [27], although they have been shown to be poor replicates of each other due to differences in their formulation [28]. ECO plates were developed for bacterial community analyses of environmental samples and were first described by Insam [29]. These plates contain 31 unique C substrates that are purportedly more relevant to the ecological functions these organisms perform within ecosystems [26]. However, 25 of these C substrates are also found on GN2 plates. Substrates are replicated three times within each ECO plate to help account for variability in inoculum densities derived from environmental samples [26,30]. The degree of substrate utilization in GN2, GP2, and ECO plates is measured based on color formation from a redox indicator (tetrazolium dye) [31].

Plates designed to assess fungal CLPPs, SFN2 and SFP2, have exactly the same substrates as their respective GN2 and GP2 bacterial plates, but do not include the tetrazolium dye contained in the bacterial plates, which some fungi are unable to reduce [5]. Substrate utilization in fungal plates is assessed turbidimetrically [32]. Additionally, prokaryotic antibiotics are added to the inoculating media to reduce the impact of generally faster growing bacteria on fungal substrate utilization patterns [5,32].

After inoculation with a soil/water dilution, CLPP plates are typically incubated at a constant temperature. Soil microorganisms generally exhibit optimal growth around 25°C, so most culture methods (including CLPP plates) utilize incubation temperatures near this value [33]. Biolog recommends incubating bacterial plates at temperatures ranging from 26 to 37°C, depending on the specific target organisms involved, and incubating fungal plates at 26°C (Biolog, Inc., personal communication). However, the incubation temperature used may select for organisms best able to survive and grow at that temperature [3]; thus, standard incubation temperatures that do not reflect field temperature regimes may increase the bias of CLPP patterns [23,24,30]. The effect of incubation temperature on CLPP patterns has not been addressed directly in the literature, despite this possible source of bias in the reported data [30,34]. Further, we know of no other study that has compared all five plate types or both the bacterial and fungal plate types in a single study.

In this study, we examined the effect of incubation temperature on the CLPP produced by five plate types: GN2, GP2, ECO, SFN2, and SFP2. Specifically, we addressed the following hypotheses: (1) CLPPs change as a result of incubation temperature; (2) fluctuating incubation temperatures that mimic diel temperature regimes experienced by soil microbial communities in the field produce different CLPPs than those generated from incubation of plates at a constant temperature with the same average temperature; and (3) CLPP plate types vary in their ability to distinguish among different soils.

2. Materials and methods

2.1. Site descriptions and soil sampling

We used four contrasting soils representing two major vegetation types present in the Colorado Plateau region of the southwestern United States. The Sunset Crater (SC) site (35°23′34″N, 111°25′43″W) was adjacent to Sunset Crater National Monument at an elevation of approximately 1850 m and within the pinyon-juniper woodland vegetation zone. Soils at the SC site are derived from recently deposited (< 1100-year-old) basaltic ash, cinders, and flows. The soils belong in the U.S. Department of Agriculture (USDA) Soil Taxonomic subgroup of cindery, mesic, Typic Ustorthents; these soils are relatively nutrient poor and have low water storage capacities [35]. Mean annual precipitation is 550 mm and mean annual air temperature is 11.8°C (http://www.lwf.ncdc.noaa.gov). This site has approximately 40% total vegetative cover, with piñon pines (*Pinus edulis*) comprising 80% of all trees [36].

The Gus Pearson Natural Area (GPNA) is contained within the U.S. Forest Service Fort Valley Experimental Forest (35°16′11″N, 111°44′30″W). The GPNA site is at an elevation of about 2200 m within the ponderosa pine vegetation zone. The soil at GPNA is derived from flow and cinder basalt and is classified in the fine, smectitic Typic Argiboroll USDA Soil Taxonomic family. The mean annual precipitation is about 570 mm, and the mean annual air temperature is 7.5°C [37]. The vegetation consists almost entirely of uneven-aged ponderosa pine (*Pinus ponderosa*) in the overstory and a variety of bunchgrasses in the understory [38].

Mineral soils (0–5-cm depth) were sampled at both sites in January 2001. Samples were taken from under five mature pinyon (SC site) or ponderosa (GPNA site) canopies and from five intercanopy areas at each site. At the SC site, intercanopy areas were essentially devoid of vegetation, while bunchgrasses dominated intercanopy areas at the GPNA site. The five samples were composited within canopy-types at each site, giving four distinct soils. We chose these sites because previous data suggest that the structure and function of the soil microbial communities under tree canopies and in intercanopy spaces differ dramatically at each site [39,40]. Soil samples were stored intact at 4°C for a week, then sieved moist through a 4-mm mesh sieve. Twenty grams of soil from each soil type were adjusted to field capacity (−33 kPa water potential [38]; A. Classen and S. Hart, unpublished data). Soils were then pre-incubated in glass jars fitted with thin polyethylene film (to maintain wetness while allowing for gas exchange) for 25 days in growth chambers using the fluctuating temperature regime for the site (see below).

2.2. Plate preparation

Three different microtiter plates (GN2, GP2 and ECO)

were used to describe bacterial CLPPs, and two distinct plate types (SFN2 and SFP2) were used to assess fungal CLPPs. Bacteria were extracted from 4 g of soil with 36 ml of 50 mM K_2HPO_4 buffer that had been adjusted to pH 6. Soil suspensions were then shaken for 30 min on a reciprocal shaker. After settling for 30 min., an 8-ml aliquot of the supernatant was diluted in 792 ml of inoculating solution for a final 1:1000 dilution (A.C. Kennedy, USDA-ARS, personal communication). The inoculating solution consisted of 0.40% NaCl, 0.03% Pluronic F-68, and 0.01% (w/w) Gellan Gum dissolved in deionized water (Biolog, Inc., personal communication). All solutions, transfer equipment, and glassware were sterilized with an autoclave prior to use.

Fungal extractions were performed using the protocols outlined above except the inoculating solution also contained streptomycin sulfate and chlortetracycline to limit bacterial growth. These two antibiotics were added after the inoculating solution was sterilized. The amount added provided 1 μg of streptomycin sulfate and 0.5 μg chlortetracycline per microtiter plate well [5]. Although we did not test the efficacy of this treatment, these same antibiotic concentrations are commonly used in isolation media to prevent bacterial contaminants [41]. Furthermore, Dobranic and Zak [5] found no bacterial growth when well material was streaked onto nutrient agar plates using these same antibiotic concentrations.

Soil dilutions were placed into sterile wells then transferred to the plates using an 8-channel pipettor. Bacterial inoculations were accomplished by transferring 150 μl of the soil dilution to each of the 96 wells on the microtiter plates (Biolog, Inc., personal communication). Fungal inoculations used only 100 μl per well ([5,32] and Biolog, Inc., personal communication). All work during plate preparation was done under a laminar-flow hood to minimize the risk of contamination. All plates were placed in polyethylene bags to reduce desiccation while incubating in the dark in growth chambers (see below).

2.3. Temperature regimes

Incubation temperature regimes were selected based on the greatest diel soil temperature range observed at each site (5–7.5-cm mineral soil depth [38]; A. Classen, unpublished data), which occurred in the intercanopy areas during the summer. Using the field temperature pattern for that day at each site, we devised five temperature treatments for incubating the CLPP plates: (1) the average daily soil temperature of the site (32°C for SC, 25°C for GPNA); (2) the maximum soil temperature of the site (48°C for SC, 39°C for GPNA); (3) the minimum soil temperature of the site (16°C for SC, 5°C for GPNA); and (4) a fluctuating temperature regime which mimicked the sinusoidal diel soil temperature regime of the site, and had the same average value as treatment 1. We also included a 'standard' temperature treatment of 25°C as a control, which is similar to the incubation temperatures employed in most studies using CLPP plates for microbial community analyses. Because the average and standard temperature treatments were the same for GPNA, there were only four temperature treatments for soils from the GNPA site.

2.4. Plate reading and data analysis

For bacterial plates (GN2, GP2, and ECO), optical density at both 590 and 750 nm were read on an Emax plate reader (Molecular Devices, Inc., Sunnyvale, CA, USA) at 0, 24, 48, 72, and 120 h. Fungal plates (SFN2 and SFP2) were read at 750 nm during the same time intervals as for bacterial plates, with the additional time interval of 168 h. The final values used to denote activity in each well for the bacterial plates were the 590 nm values (color development plus turbidity) minus the 750 nm values (turbidity only), after correcting for readings in the A1 (control) well at these wavelengths (Biolog, Inc., personal communication). Final values for the fungal plates were the 750 nm optical density readings minus the A1 well optical density. Well optical density values that were negative or under 0.06, the detection limit of the spectrophotometer (Biolog, Inc., personal communication), were set to zero. We analyzed data from the ECO plates in two different ways: by averaging the three values for individual substrate use within a plate (i.e., $n = 3$; denoted as ECO P), and by treating each of these within-plate replicates as if they were plate replicates (i.e., $n = 9$; denoted as ECO R).

We plotted corrected color (bacterial plates) or turbidity (fungal plates) development of the entire plate versus read time to select the optimal periods for analysis for each plate type (data not shown). Bacterial plates visually appeared to show fungal growth in a majority of the plates after 72 h, so we chose the 72-h incubation period for evaluating bacterial plates. Turbidity development in the fungal plates was generally much slower than color development in the bacterial plates; hence, we used the longest incubation period (168 h) for the analyses of CLPPs for fungi. These incubation times are similar to those used in other CLPP studies at comparable incubation temperatures [5,32,42].

We normalized the data by dividing the color or turbidity development of each well by the total color or turbidity development of the entire plate. Hence, after normalization, the sum of all of the individual well values from a plate equaled one. This normalization procedure served two purposes. First, it provided a simple method for reducing the influence of differences in initial inoculum densities on the generated CLPPs, thus improving comparisons among contrasting soil types [6,31,43]. Second, it allowed for unbiased comparisons of CLPPs for a given soil across different temperature treatments. Without normalization, we would have been unable to separate the effect of temperature on enzyme kinetics from the effect

of temperature on preferential organism selection in the CLPP analyses.

We used non-metric multidimensional scaling (NMDS) ordination to test for potential differences in CLPPs. These methods have been shown to be the most robust among current statistical methods for the analysis of community data [44]. NMDS is a non-parametric analytical technique that is applied to the dissimilarity matrix calculated among the different substrates using the Bray–Curtis dissimilarity coefficient [45]. CLPP data were not transformed prior to analysis. Comparisons between treatment groups and sites were made using an analysis of similarity (ANOSIM) statistical test. This test ranks the elements of the Bray–Curtis dissimilarity matrix computed between all samples, and calculates the statistic:

$$R = (r_B - r_W)/[N(N-1)/4]$$

where N is the total number of replicates across all groups, r_B is the average ranked dissimilarity between every pair of replicates from different groups and r_W is the average ranked dissimilarity for every pair of replicates within the same group. Statistical analyses were conducted using DECODA software [46].

We performed our analyses based on the utilization of all of the individual substrates of a plate and on the utilization of substrate groups (carbohydrates, carboxylic acids, amino acids, amines and amides, polymers, and miscellaneous) within a plate [47]. Zak et al. [47] found that analysis of substrate groups provided additional insight into microbial community differences among sites beyond those obtained by analyzing the individual substrates alone.

Shannon's Diversity Indices (SDIs) were also calculated for each plate type as a measure of the diversity of the microbial communities active during plate incubation [3]. SDI accounts for both the richness and evenness of CLPPs [3]. Diversity values were compared within each site (SC and GPNA) by ANOVA (analysis of variance) using JMP (version 3.2.6, SAS Institute, Inc., Cary, NC, USA). An alpha level of < 0.05 was used to denote statistical significance.

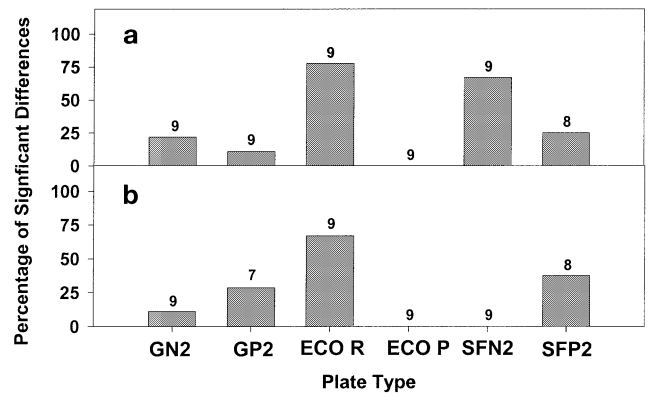

Fig. 1. Comparison of CLPPs based on individual substrates (a) or substrate groups (b) across the different temperature treatments. CLPPs were analyzed using NMDS ordination and ANOSIM. Data shown denote the percent of cases when statistically significant differences were found between the standard temperature and the other treatments. ECO R refers to the use of all replicates from the ECO plate type, while ECO P indicates that within-plate replicates were averaged before analysis. The number above each bar indicates the total number of comparisons made; these numbers differed among the plate types because some plates showed no color or turbidity development above detection limits and, therefore, were removed from the analyses.

3. Results

3.1. Effect of incubation temperature

We evaluated the effect of incubation temperature on CLPPs based on individual substrates and substrate groups using several different approaches. First, we compared the number of times statistically significant differences occurred in CLPPs between the standard temperature and other temperature regimes (i.e., maximum, minimum, fluctuating, and average) for the four different soil types (Fig. 1). For each bacterial plate type, the number of significant differences produced by each incubation temperature was minimal for individual substrates and substrate groups. The ECO plate type showed the greatest sensitivity to temperature regime when all replicates were used (ECO R). However, when the within-plate replicates

Table 1
Mean total color (GN2, GP2, ECO) or turbidity (SFN2, SFP2) development in the five plate types incubated at the constant (CON) and fluctuating (FLUC) temperature regimes with the same average temperature

Plate type	Soil type							
	SC canopy		SC intercanopy		GPNA canopy		GPNA intercanopy	
	Temperature regime		Temperature regime		Temperature regime		Temperature regime	
	CON	FLUC	CON	FLUC	CON	FLUC	CON	FLUC
GN2	21.2	10.1	25.1	7.93	44.1	50.4	ND[a]	45.1
GP2	83.5	6.78	10.3	6.83	33.2	30.7	ND	10.6
ECO	20.1	11.7	23.2	7.40	39.4	42.9	ND	34.4
SFN2	12.1	10.9	32.0	25.7	32.2	16.8	16.5	9.29
SFP2	8.29	7.20	14.1	9.91	11.2	8.64	7.84	6.59

All data are in optical density units.
[a]ND denotes no data.

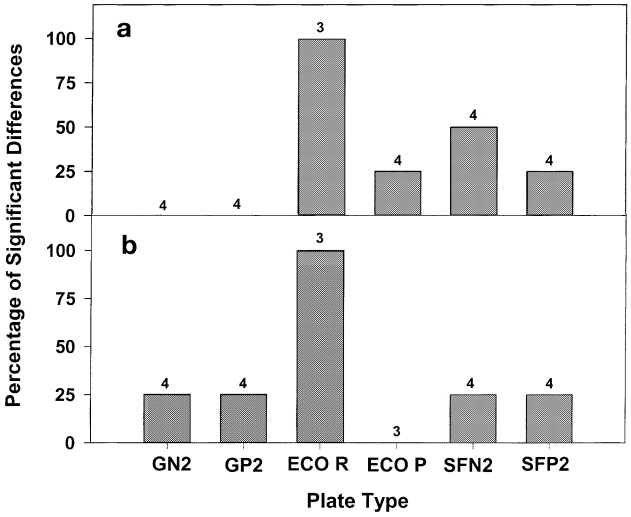

Fig. 2. Comparison of CLPPs based on individual substrates (a) or substrate groups (b) between the constant temperature treatment and the fluctuating temperature treatment with the same average temperature. CLPPs were analyzed using NMDS ordination and ANOSIM. Data shown denote the percent of cases when statistically significant differences were found. ECO R refers to the use of all replicates from the ECO plate type, while ECO P indicates that within-plate replicates were averaged before analysis. The number above each bar indicates the total number of comparisons made; these numbers differed among the plate types because some plates showed no color or turbidity development above detection limits and, therefore, were removed from the analyses.

were averaged (ECO P), this sensitivity to incubation temperature was lost.

The incidence of significant differences in CLPPs between standard, maximum, minimum, fluctuating, and average temperature regimes also was generally low for the fungal plates (Fig. 1). The SFN2 plate showed significant differences almost two-thirds of the time when CLPPs based on individual substrates were compared, but no differences were found between standard and the other temperature treatments when comparisons were made at the substrate group level. The SFP2 plates showed significant differences in about one-quarter of the cases regardless of whether CLPPs were compared based on individual substrates or on substrate groups.

We also assessed temperature effects by comparing CLPP patterns generated when plates were incubated at a constant, 'average' temperature with those produced in a fluctuating regime that had the same average temperature (Fig. 2). We found few significant differences in individual substrate or substrate group CLPPs between these two temperature regimes. However, when the ECO plates were analyzed using all available replicates (ECO R), we found differences 100% of the time between the two temperature regimes. Again, these differences essentially disappeared when the within-plate replicates were averaged (ECO P). When the constant and fluctuating temperature regimes were compared based on total color development of bacterial plates, plates incubated at the constant tem-

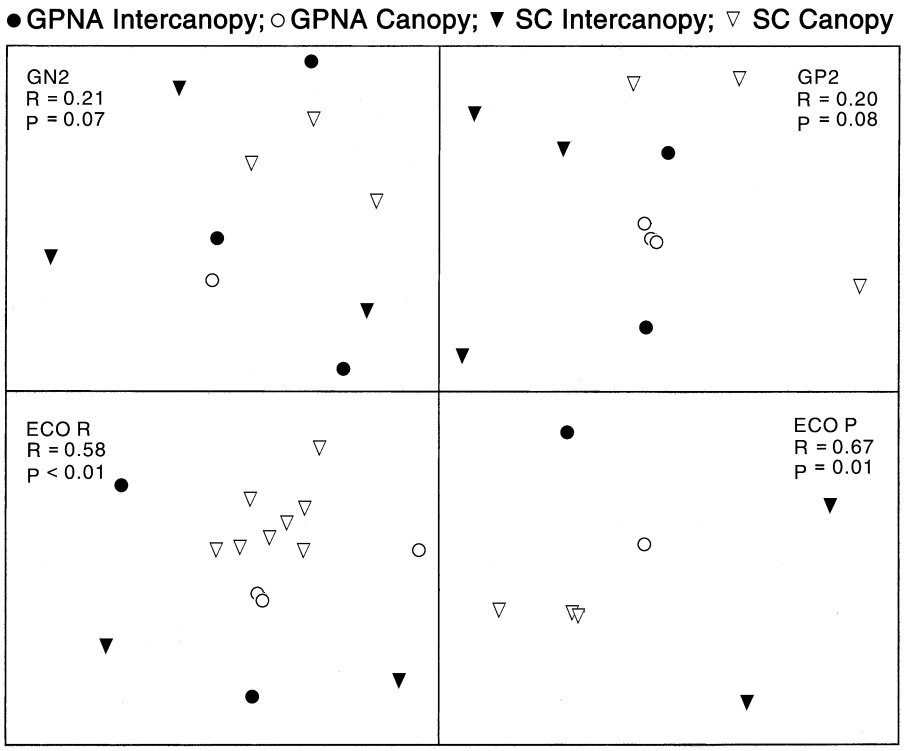

Fig. 3. Non-metric multidimensional ordination of the CLPPs generated from different soils and Biolog plate types for bacteria. ECO R indicates that all replicates were used, while ECO P indicates that within-plate replicates were averaged before analysis.

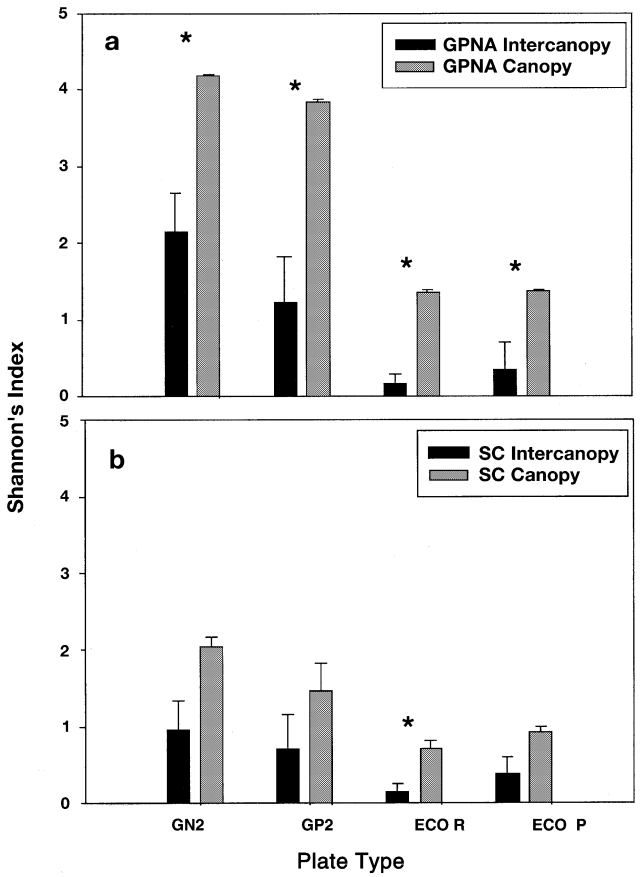

Fig. 4. SDI of carbon substrate use by the bacterial community from GPNA (a) and SC (b) sites generated using three different Biolog plate types. ECO R refers to analysis using all replicates; ECO P indicates that within-plate replicates were averaged before analysis. Vertical bars denote one standard error of the mean. Asterisks between bars indicate significant differences between the soils taken from canopy and intercanopy areas within a given plate type.

perature generally had higher color development than plates incubated at the fluctuating temperature (Table 1).

The constant and fluctuating regimes provided similar CLPPs for both fungal plate types (Fig. 2). As with bacterial plates, the constant temperature regime showed greater total substrate utilization than the fluctuating temperature regime in the fungal plates (Table 1).

3.2. Effect of plate type

We compared the ability of the three bacterial and the two fungal plate types to distinguish between the microbial communities of the four distinct soil types used in this study. CLPPs were evaluated using NMDS ordination followed by ANOSIM analyses and also by SDI values. To simplify our analyses, we only used data from plates incubated under the standard temperature regime for these comparisons.

Soil type differences were only weakly expressed in the CLPPs generated by both GN2 and GP2 plates ($R = 0.21$, $P = 0.07$, and $R = 0.20$, $P = 0.08$, respectively; Fig. 3). However, the ECO plate type was better able to distinguish the CLPPs among the soil types both when within-plate replicates were used as individual replicates (ECO R, $R = 0.58$, $P = 0.01$; Fig. 3), and when within-plate replicates were averaged (ECO P, $R = 0.67$, $P = 0.01$; Fig. 3). Paired comparisons of the CLPPs among soil types resulted in unique patterns of significant differences for each plate type (data not shown).

All three bacterial plates produced similar patterns in SDI and were equally able to distinguish between the soil types (canopy and intercanopy) of the two sites (SC and GPNA; Fig. 4). Bacterial SDI values for the intercanopy areas at both sites were lower than the associated canopy areas. Bacterial SDI values from the ECO R and ECO P analyses were also similar. Bacterial SDI values from the ECO plates were lower than those from the GN2 and GP2 plates as a result of the reduced number of substrates tested with ECO plates.

Both fungal plates produced similar CLPPs and were equally able to distinguish between the different soils (Fig. 5). The relative separation power of both plates, SFP2 and SFN2, was rather low ($R = 0.30$, $P = 0.03$ and

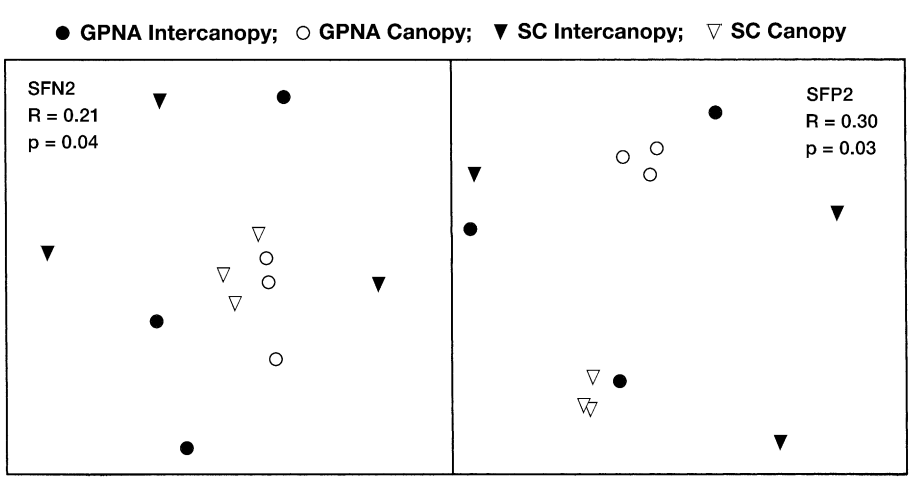

Fig. 5. Non-metric multidimensional ordination of the CLPP generated from different soils and Biolog plate types for fungi.

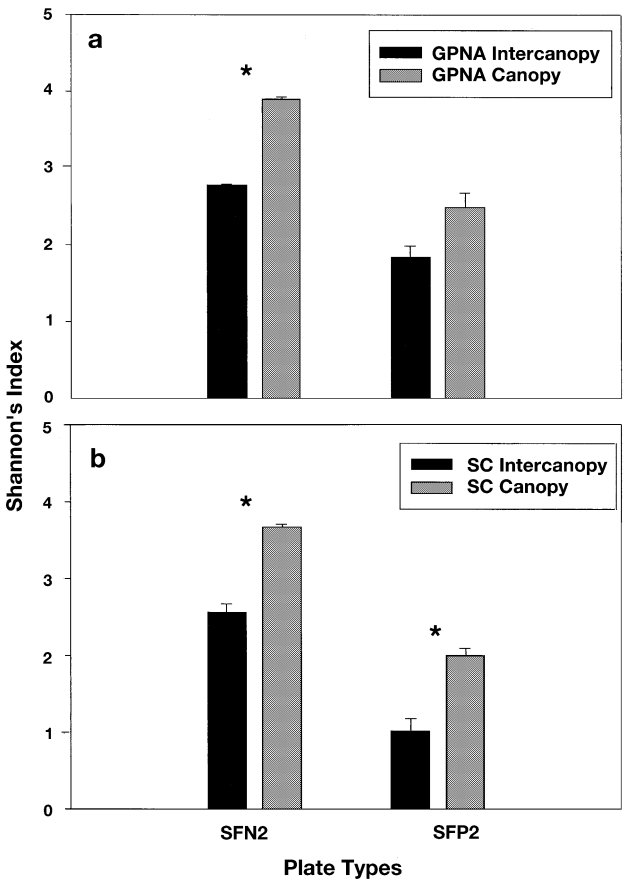

Fig. 6. SDI of carbon substrate use by the fungal community from GPNA (a) and SC (b) sites generated using two different Biolog plate types. Vertical bars denote one standard error of the mean. Asterisks between bars indicate significant differences between the soils taken from canopy and intercanopy areas within a given plate type.

$R = 0.21$, $P = 0.02$, respectively). Furthermore, fungal plates showed similar patterns in SDI among the various soils (Fig. 6). Both fungal plate types produced significantly higher SDI values in the canopy than in the intercanopy areas at SC, but only the SFN2 plate found a significant difference between the soils from the two canopy areas at GPNA (again, higher in soils under a tree canopy). Overall, SFN2 plates produced higher fungal SDI values than SFP2 plates.

4. Discussion

Although a few studies have used multiple CLPP plate types, [47] including some direct comparisons between types, [26] our study is the first to compare and contrast the CLPPs from all five commercially available Biolog plate types using the same soils and incubation conditions. The vast majority of studies using these CLPP plate types have employed only one, the GN2 plate, for bacterial community analyses, while recently a few researchers have tested SFN2 plates for fungal analyses [5,32].

We chose the soils used to conduct this study based on previous research using contrasting methodologies that showed large differences in the structure and function of microbial communities in soils taken from tree canopies and intercanopy spaces at each of these sites. For instance, Boyle [39] found that the activities of eight different enzymes and abundance of autotrophic nitrifiers were all higher in soils sampled from intercanopy spaces than under old-growth ponderosa pine trees at the GPNA site. Additionally, Kuske et al. [40] found large differences in the relative abundances of fluorescent pseudomonad and heterotrophic bacteria, humate and chitin degrading actinomycetes, and heterotrophic fungi functional groups between soils taken from intercanopy and canopy areas at SC. Our results using CLPPs appear to corroborate the findings of these previous studies, showing a clear separation between the soil microbial communities present under tree canopies from those found in the intercanopy areas (Figs. 4 and 6).

Overall, our results did not support our hypothesis that incubation temperature influences CLPPs. This result held true whether all the individual substrates were analyzed separately in the multivariate analysis, or if substrate groups were analyzed. Our results contrast with those of Derry et al. [3], who utilized GN2 plates to assess bacterial CLPPs in three arctic soils. In their study, CLPPs varied with incubation temperature, and incubation temperatures more similar to field temperatures increased the contrast among the soils.

We offer several possible reasons for the conflicting results of these two studies. First of all, Derry et al. [3] analyzed data after arbitrarily extending the incubation times for the plates incubated at lower temperatures. Longer incubation periods were used to reduce the possibility of false negatives in these plates. In our study, we believed that normalizing the color (GN2, GP2, ECO) or turbidity (SFN2, SFP2) development in each well by the total development of the plate adequately removed the direct effect of temperature kinetics (i.e., Q_{10} effect) on color development in the different temperature treatments. Hence, the different conclusions from these two studies may be due, in part, to the confounding effect of incubation length on the CLPPs observed at different incubation temperatures. Another important difference between these two studies is that we corrected for the influence of microbial growth on the bacterial plates through turbidity corrections (750 nm read), while Derry et al. [3] did not. Thus, it is unclear whether changes in optical density detected by Derry et al. [3] were due to color development during substrate utilization or turbidity from bacterial growth within the wells over the longer incubation period. Finally, the incubation temperatures applied for our study were all within the natural range of variability experienced by the soils used. In the study by Derry et al. [3], the 30°C incubation temperature was far outside of the natural range of temperatures experienced by microbial communities in

their arctic soils. Hence, the difference in the relative extremes of the temperatures used in each study may also explain the differences in our conclusions.

We also hypothesized that an incubation temperature regime that emulated the diel fluctuation experienced by the microbial communities in the field would differ from the patterns produced from plates incubated at a constant temperature with the same average. Although the constant temperature regime tended to produce higher total color or turbidity development than the associated fluctuating temperature regime, the CLPPs generated from these contrasting regimes were relatively similar for all plates tested. This result further supports our general finding that the CLPPs produced from these plates are fairly insensitive to incubation conditions.

Finally, we hypothesized that the various types of CLPP plates would differ in their ability to distinguish among the bacterial and fungal communities of contrasting soils. This hypothesis also was not supported by our data. Both GN2 and GP2 plates had similar CLPPs among the soils tested. The ECO plates generated similar CLPPs as the other bacterial plates, but due to the smaller number of substrates tested, the ECO plate showed lower overall SDI values. Likewise, both fungal plates showed similar differences between the soil types in both CLPPs and SDI values.

Choi and Dobbs [26] evaluated the relative abilities of the GN2 and ECO plate types to distinguish among the bacterial communities of aquatic samples. They also found that both plate types established similar differences among the CLPPs of the water samples assessed. However, they still recommended the use of the ECO plates because the substrates in this plate type are more 'ecologically relevant' than those on the GN2 plate type [48]. We feel that the greatest advantage of the ECO plate is that this plate type includes three replications of each substrate within a single plate, increasing the likelihood that the CLPP generated is representative of the soil sample assessed. Although the total number of substrates tested is reduced, our results and those of others [26,48], suggest that the number and diversity of substrates contained in the ECO plate are sufficient to delineate between microbial communities found in contrasting environmental samples.

Although we found that the various CLPP plate types used to separately assess bacterial and fungal communities provided similar patterns for a given soil, bacterial plates distinguished among the soil communities differently than did fungal plates. This result suggests that using differences in bacterial CLPPs alone to gauge microbial responses to environmental stresses or to compare soil microbial communities from divergent environments, as have most previous studies employing the CLPP technique, may provide a misleading picture of the response of the soil microfloral community as a whole. We strongly recommend that both bacterial and fungal CLPPs be assessed if the investigator chooses to use CLPPs to compare microbial communities among soils. Finally, we concur with Garland et al. [49], Smalla et al. [50], and McCaig et al. [51] that, while CLPP methods can discriminate between different soil microbial communities, CLPPs may provide little insight about the function of the community in situ. We stress that the CLPPs have the greatest utility when they are combined with other microbial methods that do not rely on the culturing of the soil microflora.

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