Soil Microbial Community Composition in a Restored Calcareous Subtropical Wetland

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Extreme restoration mechanisms can have important implications in ecosystem development. Complete soil removal during restoration of a freshwater wetland permitted the study of microbial and biogeochemical changes occurring during early development of soils in restored sites of different ages (2-, 5-, 8-,16-yr old). We examined whether the soil microbial community composition (using phospholipid fatty acid analysis, PLFA) was related to the biogeochemical factors across the restored and the undisturbed native site. We observed (i) with accretion of organic matter there was a general shift from N limitation in younger sites to P limitation in the older sites, (ii) soil microbial communities in restored sites were different from that in the native vegetation site, (iii) seasonal variation (dry vs. wet) in microbial community composition in younger restored sites was greater than relatively older sites. Restored sites were characterized by higher relative abundance of fungal biomarkers and higher ratios of gram negative to gram positive compared to the undisturbed native site. Biomarkers for actinomycetes were positively correlated with P concentrations in soils. There did not appear to be any association between fungal biomarkers and soil P. Our results indicate that extreme restoration processes may influence the ecosystem development processes by affecting the soil microbial community composition.

Abbreviations: HID, Hole-in-the-Donut; MUF, methyl umbelliferone; NMDS, nonmetric multidimensional scaling; NV, native vegetation; PLFA, phospholipid fatty acid; TP, total phosphorus.

Primary productivity in the early stages of ecosystem development is greatly influenced by the biogeochemical cycling of nutrients, and is often limited by nutrient availability especially N (Chapin et al., 1994). Many processes that regulate nutrient availability (such as soil organic matter decomposition, mineralization, and biological nitrogen fixation) are microbially driven and therefore, microbial communities form an integral part of the ecosystem development. Development and establishment of microbial community composition is also dependant on the quality and bioavailability of substrates in a system (Ohtonen and Vare, 1998). McKinley et al. (2005) have shown that changes in vegetation type can affect the rhizosphere C quality, which in turn affects the microbial community structure. Alteration in microbial community structure has also been associated with anthropogenic activities and land use change, soil type, fertility, and farming practices (Fraterrigo et al., 2006, Bossio et al., 1998; Kourtev et al., 2003; Lundquist et al., 1999). These studies have shown associations between microbial community structure and the ecosystem processes they mediate in soils. Changes in other factors like water table depth, soil depth, temperature, and seasonal variation also influence the size and activity of microbial communities (Fierer et al., 2003; Keith-Roach et al., 2002; Zelles, 1999). Therefore, microbial community composition may serve as a sensitive indicator of the soil biogeochemical processes.

Extreme restoration processes, like the clearing of soil to bed rock level (Dalrymple et al., 2003) cause great disturbance in biogeochemical processes owing to changes in nutrient levels and microbial community composition. In early development of soils, due to limited availability of nutrients, turnover rates of nutrients are rapid. With time,

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as organic matter accumulates, soil microbial biomass increases resulting in microbial community shifts to organisms that have the ability to recycle recalcitrant pools of nutrients and transform them into bioavailable pools. Effects of change in available soil nutrients has been shown to shift soil bacterial community composition from gram negative to gram positive bacterial community (Bossio and Scow, 1998).

Phospholipid fatty acid analysis has been used in several studies to reflect a broad measure of active biomass, and to elucidate biomass of various functional groups of soil microorganisms (Frostegård and Bååth, 1996; Zak et al., 1994) and has demonstrated the potential as a sensitive biomarker to reveal changes in biomass estimates of microbial community composition (Vestal and White, 1989). In this study we used PLFA to determine changes in microbial community structure in a calcareous subtropical wetland during early stages of soil development (Dalrymple et al., 2003). Natural development of soils after complete removal of soil to bedrock allowed us to determine changes in microbial community composition as they occur during early stages of soil development. We also correlated the biogeochemical parameters of developing soils in restored sites with corresponding PLFA profiles from same sites to investigate associations between them. Most studies have focused on changes in microbial community structure along a long-term chronosequence (Moore et al., 2010), and few studies have shown changes in microbial communities during primary succession of soil.

The objectives of this study were: (ii) to characterize the native microbial community composition in calcareous wetlands with newly developed soils across a short-term (2–16 yr) chronosequence, (ii) to elucidate the influence of wet and dry periods on microbial community composition and structure at different stages of soil development, and (iii) to relate the microbial assemblages to the biogeochemical cycling of nutrients in native and restored wetland sites.

MATERIAL AND METHODS

Site Description

Study sites are located in the Hole-in-the-Donut (HID) region of the Everglades National Park, Florida (Fig. 1). This region is characterized as drained wetlands that were used as farmlands for more than 50 yr (Dalrymple et al., 2003). Once these agricultural lands were abandoned, they were invaded by Brazilian pepper (*Schinus terebinthifolius*), an invasive species (Bancroft, 1973). This region had been dominated with a monoculture of Brazilian pepper for more than 30 yr. In an effort to restore the natural ecology of these wetlands, approximately 2400 ha of HID region was mechanically scraped to bedrock to completely remove the soil, including Brazilian pepper seed bank and to allow revegetation of the natural wetland native plants. Our study site is unique for the "extreme restoration" method of complete soil removal employed to restore it. The soil clearing has been done in specific areas, at different times, over a period of 16 yr. Sites chosen for this study were cleared in years 2003 (C03), 2000(C00), 1997(C97), and 1989(C89). This allowed us to follow the microbial community changes in soils as they develop across a short-term chronosequence of 2, 5, 8, and 16 yr. Because the clearing was only done at one site during each time period, we were aware that absence of another independent replicate site for that time period would result in samples collected from within one site as "pseudoreplication". However, with no alternative to have another independent replicate, and to best represent each site, the soils were randomly sampled and three composite samples were prepared and results were used as statistical replicates. In addition to the cleared sites, an undisturbed native vegetation (NV) site was also identified. This site represents the "reference" point in HID region because it was neither farmed nor invaded by Brazilian pepper.

Sample Collection

Soil samples were collected from each of the four cleared sites (C03, C00, C97, and C89), and undisturbed NV site (Fig. 1) during the dry

Fig. 1. Sampling sites in the Hole-in the-Donut area in Everglades National Park, FL showing the restored 2-yr old (C03), 5-yr old (C00), 8-yr old (C97), 16-yr old (C89), and undisturbed native (NV) > 100-yr-old sites.

(May 2005) and wet periods (November 2005). Plots of 20 by 20 m were established at all five sites, and within each plot 81 points were marked by laying a grid of 2 by 2 m^2. All 81 internal nodes were surveyed to determine spatial variability in parameters such as relative elevation, soil depth, and water depth. As a result of mechanical scraping and complete removal of soils from the "restored area" soil depth at the C03 site was minimal. Soil depth was measured at several points (n = 81) in a 20 by 20 m^2 plot to obtain a representative number for soil depth. From the elevation survey nine sampling nodes of relative elevations (deep, medium, and shallow) were randomly identified, sampled, and composited to get three soil samples for each site. Sample preparation was as explained by Smith et al. (2007). The three composite samples were used for various biogeochemical and enzyme analyses. For PLFA, two of the composite samples were frozen immediately in the field in dry ice and transported on dry ice before they were lyophilized in the lab. Samples for nutrient and enzyme analyses were transported on ice to Wetlands Biogeochemistry Laboratory, University of Florida, Gainesville, FL and were stored at 4°C.

Nutrient Analyses and Microbial Biomass

Total C and N content were determined using Costech Model 4010 Elemental Analyzer (Costech Analytical Industries, Inc., Valencia,CA). Loss on ignition (LOI) was determined by ashing the samples at 550°C (3–4 h). Soil total phosphorus (TP) and inorganic P was determined using methods described in Wright and Reddy (2001). Organic P was calculated as the difference between TP and inorganic P. Subsamples of soils were oven dried at 70°C for 72 h to determine moisture content. Soil pH was measured on water diluted soil solution (1:1). Samples were allowed to equilibrate for 30 min before the pH measurement was recorded. Microbial biomass carbon (MBC) was determined using the chloroform fumigation-extraction technique as described in Wright and Reddy (2001).

Enzyme Analysis

Phosphatase and β glucosidase enzyme activities were measured fluorometrically as previously described (Marx et al., 2001). Soil samples were homogenized evenly to remove any aggregations. Appropriate dilutions of all soil samples were incubated with fluorescently labeled substrate methyl umbelliferone (MUF)-phosphate and MUF-glucoside to determine the phosphatase and glucosidase activity, respectively. Final concentration of the substrate added was 200 μM. Formation of the fluorescent product MUF was measured at excitation/emission wavelength of 360/460 in a fluorometer (Biotek, Winooski, VT). Quenching curves were prepared for each soil sample to account for any quenching of the fluorescent product by the soil matrix. Soil enzyme activities were normalized by the microbial biomass C and reported as kg MUF released kg^{-1} microbial biomass C h^{-1}.

Phospholipid Fatty Acid Analysis

Lyophilized samples were extracted for PLFA using a modified Bligh and Dyer method (White et al., 1979). Samples were extracted using one-phase chloroform-methanol-buffer extractant, recovered in chloroform, and fractionated on disposable silicic acid columns into neutral-, glyco-, and polar-lipid fractions. The polar lipid fraction was transesterified with mild alkali to recover the PLFA as methyl esters in hexane. The PLFA were analyzed by gas chromatography with peak confirmation performed by electron impact mass spectrometry (GC/MS). We found a total of 34 PLFAs in all soil samples. Four reported studies (Kourtev et al., 2003; Findlay and Dobbs, 1993; Tunlid et al., 1989; Federle, 1986) were used to assign fatty acids of bacterial origin (i14:0, a15:0, i15:0, i16:0, i17:0, a17:0, 16:1w9c, 16:1w7c, cy 17:0, cy19:0, i171w7c, 14:0, and 15:0) and fungal origin (18:2w6, 18:1w9, and 16:1w5c). Sum of bacterial gram positive biomarkers included i14:0, a15:0, i15:0, i16:0, i17:0, and a17:0 and bacterial gram negative biomarkers included 16:1w9c, 16:1w7c, cy!7:0, 18:1w7c, 18:1w5c, cy19:0, 17:1w7c, 14:0, and 15:0. Diagnostic groups of fatty acids were used to calculate ratios of fungal/bacterial and bacterial gram $^{+}$/gram $^{-}$ biomarkers. Microbial indices were calculated using the biomarkers described by Bossio and Scow, (1998) and microbial diversity was estimated by counting the total number of PLFAs. The PLFA nomenclature follows the pattern of A/BωC. The "A" position identifies the total number of carbon atoms in the fatty acid. Position B is the number of double bonds from the aliphatic (ω) end of the molecule. Position "C" designates the carbon atom from the aliphatic end before the double bond. This is followed by a "c" for *cis* or a "t" for *trans* configuration. The prefix "i" and "a" stand for *iso* and *anteiso* branching. Mid-chain branching is noted by "me," and cyclopropyl fatty acids are designated as "cy". Example: 18:1ω7c is 18 carbons long with one double bond occurring at the seventh carbon atom from the ω end, and the hydrogen molecules attached to the doubly bonded carbon molecules are in the *cis* conformation.

Statistical Analyses

Means and standard errors were calculated for nutrient parameters and microbial indices at each site. ANOVA was used to analyze data, with age of sites and two sampling periods (wet and dry). Significance of post hoc pairwise comparisons were determined using Tuckey's HSD test. Analyses were done using JMP software and for all tests of significance $\alpha = 0.05$.

Lipid profile patterns were described using mole percentage distribution of individual PLFA. The PLFA mole percent were analyzed using nonmetric multidimensional scaling (NMDS). Lipids were ordinated using NMDS to examine changes in microbial community structure. This multivariate statistical method avoids assumptions of linear relationships among variables and preserves rank order of distances from the dissimilarities within samples (Clarke, 1993). We used the Bray–Curtis dissimilarity matrix before subjecting the data to NMDS. We used 50 runs with the real data and 50 runs with the randomized data to provide the basis for test of significance at each dimensionality tested. Diagnostic scree index indicated that a two dimensional was the best solution. The NMDS was run again after specifying the optimal dimensions and best starting configuration. The final data were recorded with the most optimum stress of 6.2. Ordination scores of sites were used in multivariate analysis to determine if the microbial community composition differed among the sites and between the two sampling seasons. This method allowed the large data set to be compared simultaneously. To aid in the interpretation of the lipid biomarkers summarized by the derived axes, Spearman rank correlation coefficients were computed by correlating ordination axes with corresponding environmental variables. Spearman rank correlation does not assume variables are distributed normally (Sokal and Rohlf, 1995). The biogeochemical variables included total C, total N, total P, inorganic P, microbial biomass C, microbial enzyme activities (expressed as per gram dry soil per hour) and moisture content. Analyses were done using PC-ORD (Version 6. MjM Software, Gleneden Beach, OR) and JMP ver 7.0.2 (2007, SAS Institute Inc).

Table 1. Soil characteristics of calcareous wetland in cleared sites and undisturbed native vegetation (NV) site. Samples were collected during dry season (May) and wet season (November).

Variables	C03 (2-yr-old site)	C00 (5-yr-old site)	C97 (8-yr-old site)	C89 (16-yr-old site)	NV (undisturbed)
Elevation range	**0.65–0.70 m**	**0.55–0.60 m**	**0.50–0.60 m**	**0.45–0.50 m**	**0.60–0.65 m**
Dry period					
Moisture content, %	9.5	7.1	9.0	16.1	14.9
TC, g kg^{-1}	149 ± 7	150 ± 1	164 ± 1	169 ± 5	157 ± 4
TN, g kg^{-1}	6.2 ± 0.4	6.9 ± 0.1	8.2 ± 0.4	9.1 ± 0.4	7.4 ± 0.3
LOI, %	18.7 ± 1.0	18.1 ± 1.4	22.5 ± 2.0	22.5 ± 1.6	13.7 ± 0.3
TP, mg kg^{-1}	1.06 ± 0.07	0.49 ± 0.02	1.15 ± 0.05	0.74 ± 0.05	0.14 ± 0.01
HCl Pi, mg kg^{-1}	0.6 ± 0.08	0.16 ± 0.01	0.65 ± 0.08	0.27 ± 0.03	0.03 ± 0.002
OrgC/N	15.1	13	13.2	12.3	9.3
N/P	5.8	14.1	7.1	12.3	52.8
Microbial biomass C, g kg^{-1}	2.0 ± 0.6	2.0 ± 0.7	3.2 ± 0.03	3.7 ± 0.4	4.1 ± 1.1
Total PLFA, μmol kg^{-1}	91 ± 23	93 ± 12	127 ± 14	126 ± 7	96 ± 32
Wet period					
Moisture content, %	43.5	48.3	58.8	60 ± 2	44 ± 1
TC, g kg^{-1}	158 ± 2	158 ± 3	177 ± 6	181 ± 3	151 ± 1.8
TN, g kg^{-1}	7.3 ± 0.02	7.7 ± 0.3	8.9 ± 0.5	9.9 ± 0.4	6.9 ± 0.2
LOI, %	22.3 ± 1.4	19.6 ± 1.2	22 ± 1.5	22 ± 0.5	12.9 ± 0.6
TP, g kg^{-1}	1.18 ± 0.02	0.48 ± 0.03	0.95 ± 0.05	0.64 ± 0.03	0.14 ± 0.002
HCl Pi, g kg^{-1}	0.72 ± .01	0.17 ± 0.01	0.49 ± 0.03	0.25 ± 0.01	0.03 ± 0.001
OrgC/N	15	13	12	11	9
N/P	6.1	16	9.3	15.4	49
Microbial biomass C, g kg^{-1}	7.1 ± 0.1	7.1 ± 0.6	8.6 ± 0.7	10.6 ± 2.0	5.5 ± 0.6
Total PLFA, μmol kg^{-1}	110 ± 10	130 ± 7	148 ± 10	173 ± 19	161 ± 59

Table entries are means plus/minus standard deviation (n = 3 composite samples).

RESULTS

Soil pH did not vary much across the cleared sites and averaged 7.7 (± 0.12). However, pH values in the NV sites averaged to 8.1(± 0.09). Soil depth increased with the age of the cleared plot with 0 to 5 cm in C03 site to ~ 1 to 15 cm in C89 site. Soil depth in the NV sites ranged from 3 to 21 cm.

Soil total C concentrations and accumulation increased with age of soils (2–16-yr old) in cleared sites (Table 1). Total C in sites C89 and C97 was significantly higher than in C00, C03 and NV sites (p = 0.0002). In the undisturbed NV site, total C values were similar to those observed in C03 and C00, and total C during the wet period (151 ± 2 g kg^{-1}) was lower than that in dry period (157 ± 3 g kg^{-1}). A relative increase in total C content was observed in all cleared sites during the wet period. Organic matter content (expressed as LOI) was 60% greater in the cleared sites than the NV site.

Total nitrogen (N) content in soils increased with age of cleared plots with 6.2 ± 0.9 g kg^{-1} in the 2-yr-old site (C03) and 9.1 ± 1.6 g kg^{-1} in the16-yr-old site (C89). Older cleared plots C89 and C97 showed significantly higher total N values as compared to C00, C03, and NV sites(p = 0.0003). A slight increase was observed in total N values during wet period in all cleared sites (Table 1). The organic C/N ratios in cleared sites decreased with site age, and were higher (45 and 37%) than those in NV site during dry and wet season, respectively.

Total P content in cleared sites was significantly higher than NV site (p < 0.0001). Within cleared sites C03, and C97 showed significantly higher TP in sites C89 and C00 (p < 0.0001). In the wet period, total P in site C03 was higher than site C97 and C89 was higher than site C00. There was no significant relationship observed between soil P content and the age of the cleared plots. The HCl-Pi which represents inorganic P fraction showed significant differences between all sites with C97 and C03 being significantly higher than C89, C00, and NV sites (p < 0.0001). Relative proportion of inorganic P was high in C03 site (61% of total P) and C89 site (56%) and low in NV site (18%). Soil N/P ratios in NV site was (~48%) higher than that observed in the cleared sites (<16%).

Higher specific enzyme activities were obtained during the wet season in all sites. (Fig. 2). Phosphatase activity in the C03 site was significantly lower (p > 0.05) than the NV site and all other cleared sites with the exception of C97 during the dry period. However, there was no change in the enzyme activities among all sites during the wet period. Specific β–glucosidase activity in NV site was lower than that observed in all cleared sites. The pattern of this enzyme activity in the dry season varied from that in the wet season especially in the C03 and NV sites. Similar seasonal variation was observed in phosphatase activity in the C03 and NV sites.

Both microbial biomass C and total PLFAs biomass increased with age of cleared sites (Table 1). Microbial biomass C in cleared sites was higher in the wet period and ranged from 7 to 10.6 g kg^{-1} as compared to 2 to 3.7 g kg^{-1} observed in dry period. Although microbial biomass C in NV site were comparable to those observed in cleared sites during dry period, they did not increase in the NV site during the wet period as was observed in cleared sites. Microbial biomass C was significantly higher in C89 (p < 0.003) than C00, C03, and NV site during the wet season. However, during the dry period there were no differences in

microbial biomass C among sites. The microbial biomass C in the NV site (5.5 g kg^{-1}) did not vary during the wet and dry periods.

Total PLFA also showed an increasing trend with the age of cleared sites. Total PLFA biomass (nmol kg^{-1} soil) in cleared sites ranged from 91 to 126 nmol kg^{-1} soils during the dry period and 110 to 173 nmol kg^{-1} soil during the wet season. During the dry season, there was no difference across the age of restored sites. During the wet season, total PLFA in the 16-yr plot were significantly higher than those in the relatively recently cleared 2-yr site. Total PLFA in the 16-yr old (C89) site was higher than NV site during both wet and dry periods. Total PLFA biomass positively correlated with total C ($p = 0.007$), total N ($p = 0.008$), and microbial biomass C ($p = 0.001$). Comparing the two methods, there was a significant positive correlation between microbial biomass C estimated using chloroform fumigation extraction and total phospholipids ($r^2 = 0.45$; $p = 0.002$). Similar correlation has been observed in other studies (e.g., Zelles et al., 1995; Feng et al., 2003).

The PLFA analysis identified 32 different phospholipids, present in all soil samples, ranging from carbon chain length of C14 to C20. The microbial community structure profile during both wet and dry seasons was dominated by monoenoics, normal saturated and tertiary branched saturated phospholipids (Table 2). Monoenoic (monosaturated) PLFAs are characteristic of gram negative bacteria (Wilkinson, 1988) and they accounted for 43 to 52 mol% in the soil microbial community profiles. The abundance of monoenoics was significantly different ($p < 0.001$) among all sites during dry period but, did not vary much during wet period. With the exception of C97 and C89, there was an increase in ($p < 0.05$) in abundance of monoenoics during the wet period within each site. The tertiary branched (iso and anteiso) fatty acids, biomarkers for gram positive bacteria (O'Leary and Wilkinson, 1988) accounted for 13 to 19% of the total PLFA and were significantly greater in NV soils ($p < 0.0039$) than in all cleared sites during the dry season (Table 2). The normal saturates constituted 19.5 to 21.9 mol% of total lipids.

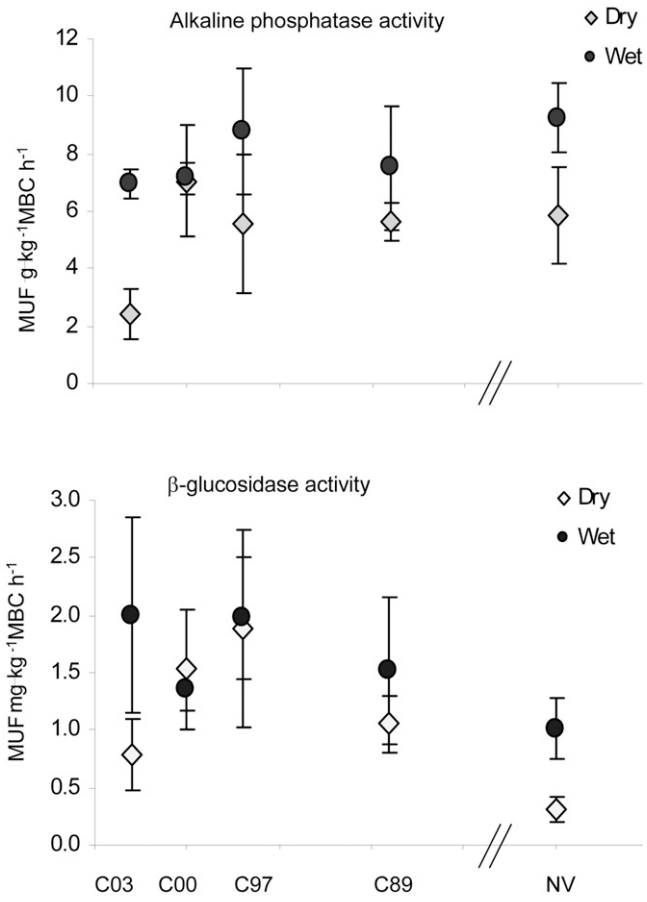

Fig. 2. Patterns of seasonal variation in specific enzyme activities, (top) alkaline phosphatase and (bottom) β-glucosidase (bottom) in Hole-in-the-Donut (HID) soils.

The ratio of bacterial gram negative to gram positive lipid biomarkers did not vary among all sites during the wet period (Fig. 3). However, this was not the case during the dry period, where the ratio of gram negative to positive was ($p = 0.002$) different between sites. Within each site, there was a decrease

Table 2. Groups of phospholipid fatty acid analysis (PLFA) (mol%) present in calcareous wetland soils in cleared sites and undisturbed native vegetation (NV) site. Samples were collected during dry period (May) and wet period (November). Table entries are means of *n* = 2 composite samples.

PLFA	C03 (2-yr-old site)	C00 (5-yr-old site)	C97 (8-yr-old site)	C89 (16-yr-old site)	NV (undisturbed site)
Dry period					
Tertiary branched saturates	16.2	15.6	13.4	14.2	18.8
Monoenoics	43.6	45.4	51.5	47.6	43.1
Branched monoenoics	3.5	3.2	3.3	3.2	2.7
Midbranched saturates	13.1	13.1	12	13	14.8
Normal Saturates	21.9	21.8	19.5	20.2	21.9
Polyenoics	5.2	4.2	3.6	5.1	1.3
Wet period					
Tertiary branched saturates	14.5	14.5	13.9	15.2	15
Monoenoics	48.8	47.6	49.9	46.4	48
Branched monoenoics	2.7	2.8	2.8	2.8	2.3
Midbranched saturates	11.5	12.6	13.8	14.3	15.7
Normal saturates	21.4	21.3	19.7	21.1	20
Polyenoics	3.9	4.1	2.7	3.1	1.3

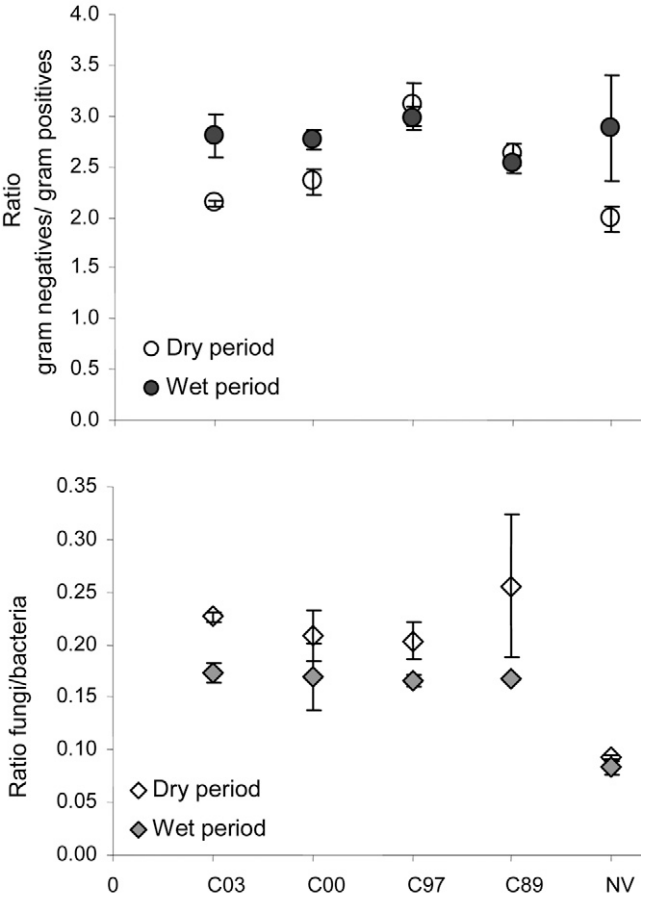

Fig. 3. Ratio of microbial functional groups in calcareous wetland soils in cleared sites and undisturbed native vegetation (NV) site. Samples were collected during dry period (May 2005) and wet period (November 2005). Values represent the ratio of summed average of lipid biomarkers representing (top) Gram negative and Gram positive bacteria and (bottom) of bacteria and fungi.

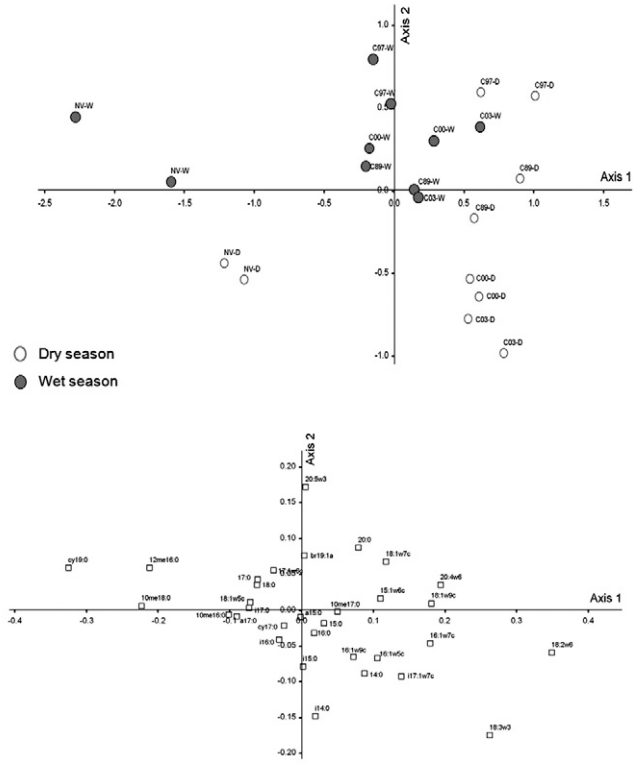

Fig. 4. (top) Scores by age of cleared sites and native vegetation (NV) sites based on nonmetric multidimensional scaling (NMDS) ordination of sites and lipid biomarkers (mol%) and (bottom) association of lipid biomarkers with NMDS ordination axes.

observed in the abundance of gram negative bacteria in C03 ($p <$ 0.004), C00 ($p < 0.02$), and NV ($p < 0.04$) sites (Fig. 1). The ratio of fungal/bacterial lipids did not vary among the cleared sites but was different ($p < 0.05$) from the NV site. Fungal/bacterial abundance increased ($p < 0.05$) in sites C03 and C97 during the dry period. The increase in this ratio was mainly due to increases in fungal biomarkers (18:2w6 and 18:1w9) (Federle, 1986). Another PLFA ratio, cyclopropyl fatty acids to their precursors, indicative of stress was higher in all sites during dry period.

Specific biomarkers that were identified within the PLFA profiles included the actinomycetes lipid biomarker, 10 me18:0 (Kroppenstedt, 1985). Actinomycetes were positively correlated ($p < 0.0001$, $r^2 = 0.93$) with sites age. Lipids 17:1w7c, 10 me16:0, and br19:1a have been used as biomarkers for sulfate reducing bacteria (Oude-Elferink et al., 1998; Kohring et al., 1994; Dowling et al., 1986) and were found in high abundance in soils at all sites. 10me16:0, another common biomarker for sulfate-reducing bacteria, was positively correlated ($p = 0.002$, $r^2 = 0.7$) with site age during the wet period. Phospholipid 17:1w7c was negatively correlated to site age ($r^2 = 0.7$).

Nonmetric multidimensional analyses of PLFA showed that the first two dimensions (axes) accounted for a total of 97% variability in the samples (Fig. 4a). The first axis explained 83% of the variability and separated microbial communities in cleared sites from the NV site ($p < 0.0001$). Microbial community variation during wet and dry periods was evident along NMDS axis 2 (14%). The NMDS analysis also identified phospholipids that were important in explaining the variability in microbial community composition (Fig. 4b). Lipids that contributed ($r >$ 0. 6) to axis 1 were identified as cy19:0, 12me16:0, 10me18:0, 10me16:0, i17:0, a17:0 18:1w7c, 18:1w9c, 16:1w7c, 18:2w6, and 20:4w6c. Several lipids were highly correlated ($r > 0.6$) with NMDS axis 2 including, i15:0, 16:0, 16:1w9c, 14:0, i14:0, br19:1a, 18:1w7c, 20:5w3 and i17:1w7c.

Few environmental variables were significantly correlated with NMDS axes. Axis 1 was negatively correlated with total N, loss on ignition (estimate of organic matter), total P, inorganic P, ratios of C/N, and glucosidase activity. Axis 2 was positively correlated with moisture content, microbial biomass C, and negatively correlated with the two enzyme activities. Total C did not appear to be associated with any of the axes.

DISCUSSION

In this study, PLFA profiles showed that microbial community composition in cleared sites with newly developed soils differed from that in undisturbed soils in NV site. These results were congruent with those reported by DeGrood et al. (2005) and McKinley et al. (2005) who have shown that disturbance to soils can alter the microbial community structure and composition.

During HID restoration, after complete removal of soils although the natural soil development proceeded, the soil environmental conditions like total soil depth, nutrient ratios, and nutrient concentrations in cleared sites differed from the undisturbed NV site. Our results revealed that NV sites were distinguishable by increased abundance of biomarkers for actinomycetes (10me18:0) and gram positive bacteria (i17:0, a17:0). Another lipid whose presence influenced the separation of NV site from cleared sites was 10me16:0, a biomarker for sulfate-reducing bacteria (Findlay and Dobbs, 1993). In contrast, cleared sites were characterized by high abundance of bacterial gram negative biomarkers (16:1w7c, 18:1w7c), and fungal biomarkers (18:2w6, 18:1w9). A possible explanation of such patterns may be that microbial communities in soils are influenced by the nutrient availability. In general, the amount of soil organic matter and nutrients in older systems tends to be low due to leaching that occurs over time (Peet, 1992; Odum, 1969) from the top soils. As suggested by our biogeochemical data, NV sites were characterized with lower organic matter, total P, and inorganic P (Table 1) but high N/P ratios, suggesting that NV site soils were relatively P limited. The HID being a calcareous system would also result in removing P from the bioavailable pool by precipitation with calcium (Lajtha and Schlesinger, 1988). Lower microbial biomass in NV soils as measured by PLFA and chloroform fumigation method further supported this explanation and suggested a decline in nutrient availability which may be indicative of later stages of succession at this site. A similar shift in nutrient availability observed in glacier (Chapin et al., 1994), desert (Lajtha and Schlesinger, 1988), and forest (Lichter, 1998) ecosystems has been suggestive of the late succession periods.

Under conditions of nutrient limitation, increased presence of biomarkers for gram positive bacteria was observed. Due to their ability to form spores, gram positive bacteria are considered to be more stress tolerant, and therefore, are presumably more prevalent in areas that exhibit environmental stresses such as nutrient limitation (Yao et al., 2000) or extremes in moisture availability and temperature (Borga et al., 1994; Saetre and Baath, 2000). These stress factors would be highest in the very shallow (<2 cm) soils of the cleared HID sites. However, the high gram positive abundance observed at the NV site suggests the microbial community in the NV site to be the most stressed. This observation may be at least partially explained by the extreme nutrient limitation (high NP ratios) and relatively low organic matter content characteristic of these well-developed older soils (Table 1).

At NV sites, compared to the cleared sites, a higher fraction (80%) of total P was present as organic form. Most microorganisms have to enzymatically hydrolyze the organic P to inorganic P to transform it into bioavailable forms (Chróst, 1991). With low bioavailable P, it was expected that the organisms would have a greater P demand but the specific phosphatase activity did not clearly indicate this (Fig. 2). In general, phosphatase activity has been widely used as a potential indicator of P limitation in soil and aquatic systems (Newman et al., 2003; Wright and Reddy, 2001) but C limitation has also been shown to stimulate an increased response of this enzyme (Cotner and Wetzel, 1991). Phosphatase production is an energy consuming process and in a system with low primary productivity and low C it is possible that the organisms were stressed. This explanation further supports the presence of increased gram positive biomarkers at NV site.

Another lipid found in higher abundance in the NV site soils was 10me18:0, a biomarker found in actinomycetes (Kroppenstedt, 1985). This was also present to a much lower extent in the newly formed soils in cleared site. Actinomycetes are gram positive filamentous bacteria that, like other gram positive bacteria, may have an advantage to colonize nutrient-limited systems (Schlegel, 1992). A major characteristic of the newly formed soils in cleared HID sites was an increased abundance of fungal biomarkers (18:2w6 and 18:1w9) relative to the NV site. Others studies have also noted differences in fungal biomass between disturbed and undisturbed soils where disturbance (e.g., by logging or tillage practices) generally correlates with a reduction in fungal biomass either through the removal of carbon sources or disruption of hyphal networks (Fraterrigo et al., 2006; Frey et al., 1999). In the cleared HID sites, soil removal would seem to pose a similar carbon reduction affecting fungal biomass. Despite this hypothesis, total carbon and fungal biomass were both much higher in the recently cleared HID sites than the NV site.

Relatively higher abundance of fungal biomarkers in cleared sites indicates that fungi recovered very quickly in the newly formed soils. Fungal biomass has a high C/N ratio, so it is unclear to what extent the correlation of fungal biomarkers with soil C is determined by substrate carbon availability or the fungal biomass itself (Allison and Vitousek, 2005). Cleared sites showed higher values of C/N ratio. In general, the capacity of organic matter to decompose can be assessed by C/N ratios (Kaye and Hart, 1998). High C/N ratio in soils have been found in systems that have recalcitrant organic matter that decomposes very slowly and N is tightly associated with the organic compounds. Therefore fungi at this site may also be assisting in the breakdown of complex organic compounds (Griffin, 1972). Alternatively, fungi can be inhibited by increased N availability (Bradley et al., 2006). Therefore, another potential factor explaining fungal development in the cleared HID sites could be the presence of more N-limiting conditions (as indicated by lower N/P ratios, Table 1). Increased fungal biomass may also be potentially explained by lower abundance of actinomycetes at the cleared sites as actinomycetes are known to produce antibiotics that inhibit the growth of fungi (Jiménez-Esquilín and Roane, 2005).

Presence of complex C substrates at the cleared sites was also suggested by specific β glucosidase activity. β glucosidase activity in soils has been used as an indicator of carbon cycling in a system (Chróst, 1991). Increased production of this enzyme is stimulated during the presence of polymeric complex C substrates. Heterotrophic bacteria produce these enzymes especially in the absence of usable forms of simple carbon substrates. Specific activity of this enzyme was relatively lower in the NV site suggesting that the cleared sites may have higher complex C substrates.

We observed the presence of lipid biomarkers for the sulfate-reducing bacteria in all the sites. Presence of sulfate-reducing

Table 3. Spearman's correlation coefficients (ρ) for nutrient concentrations in soils regressed with two nonparametric multidimensional scaling (NMDS) axes that explain the greatest proportion of variance. Soils were from restored and native vegetation (NV) sites. Nutrient concentrations are given per unit weight of dry soil. The statistical significances ($P < 0.05$) of correlation coefficients are highlighted in bold.

Variable	Axis1		Axis2	
	Spearman's ρ	$P > \|r\|$	Spearman's ρ	$P > \|r\|$
Soil moisture content	0.17	0.47	0.53	**0.016**
Total C	–0.39	0.081	0.19	0.412
Total N	–0.46	**0.042**	0.21	0.378
LOI†	–0.65	**0.002**	–0.22	0.339
Total P	–0.72	**0.003**	–0.37	0.1023
Inorganic P	–0.69	**0.001**	–0.39	0.083
Microbial biomass C	–0.38	0.099	0.62	**0.004**
C/N	–0.54	**0.014**	0.36	0.123
N/P	0.68	**0.001**	–0.27	0.243
APA	–0.39	0.084	–0.55	**0.011**
BGA	–0.52	**0.02**	–0.58	**0.007**

† LOI: loss on ignition; APA: alkaline phosphatase activity; BGA: β glucosidase activity.

bacteria in similar calcareous wetland system have been previously reported (Castro et al., 2005). In our samples the 10me16:0, a biomarker for *Desulfobacter* (Dowling et al., 1986; Kohring et al., 1994; Taylor and Parkes, 1983), comprised 7 to 10% of the total PLFA. An observed shift of 10me16:0 biomarker across the age gradient of restored sites was positively correlated with increasing average soil depth. Sulfate-reducing bacteria are anaerobes that are likely to inhabit deeper soils that tend to create more anaerobic conditions. Role of some sulfate-reducing bacteria in nitrogen fixation has been demonstrated in several studies (Nazina et al., 1979; Sisler and Zobell, 1951). In HID soils, these bacteria may be involved in the nitrogen fixation. One explanation of increased C/N ratio with age of cleared sites may be due to increase in N content.

Lipid biomarker, 17:1w6c, has been associated with members of genus *Desulfobulbus* (Oude Elfernick et al., 1998; Parkes et al., 1993; Taylor and Parkes, 1983), another sulfate-reducing bacteria. This lipid biomarker is generally accompanied by another biomarker 15:1w6c, another frequently found lipid in this genus. The PLFA profiles of soils in HID region also showed the presence of this marker thereby supporting the presence of members of genus *Desulfobulbus.* Unlike *Desulfobacter* genus, these bacteria did not change in abundance across the age gradient. *Desulfobulbus* spp. are known to be incomplete oxidizers of lactate and propionate (Widdel, 1988), and may be sources of acetate, a C source for bacteria belonging to *Desulfobacter* genus.

Our results indicated greater differences between the soil microbial communities in younger restored sites during the wet and dry seasons. Ordination on the basis of PLFA profiles also separated sites on the basis of wet and dry periods (Fig. 4a). The microbial communities during the dry periods were dominated by gram negative (16:1w9c, 16:1w7c) markers, fungal markers (18:2w6, 16:1w5c); and the lipid biomarker i15:0 which is indicative of gram positive bacteria. The wet season samples were positively associated with 17:1w6c, cy 19:0, 18:0, and 12me16:0. Microbial communities in the NV sites were clearly separated from the restored sites. Lipids that greatly influenced this separation were biomarkers for gram positive bacteria (i17:0, a17:0), fungi, actinomycetes, and sulfate-reducing bacteria.

Although, microbial community structure during primary succession of soils in cleared sites was generally similar, there were differences in relative abundance of some lipid biomarkers during the wet and dry period sampling. During the dry period the younger sites C03 and C00 that were 2- and 5-yr old, respectively, were clearly separated from the older sites C89 and C97 along axis 1 in NMDS analysis. Such a separation between the wet and dry sampling periods, although to a much lower extent was also observed in the NV site. These patterns suggest that younger cleared sites and NV site are similar with respect to shifts in microbial groups while still maintaining a different microbial profile. Lipid biomarkers that appear to influence this differentiation are associated with gram positive bacteria. The older cleared sites, C89 and C97, also showed a shift in microbial community structure during wet and dry period. However, this shift in microbial community structure was less distinct and was along axis 2 which was greatly influenced by lipid biomarkers for fungi and actinomycetes. Soils in dry period were characterized by fungal and bacterial gram positive lipids. Soils in younger sites were extremely shallow and the nutrient storage was lower than that expected in the upper surface of the older deeper soils and therefore, the nutrient exchange was dependent on the external input of nutrients which may be affected by wet and dry periods. However in the older sites, cycling of nutrients may not be as dependant on the external inputs and more dependent on the internal cycling in the soils therefore the microbial communities were not as different. Similarly, microbial biomass also showed a strong dependency on moisture availability, with higher biomass measured during the wet season (Table 1). These results are in agreement with those observed by other studies (Lundquist et al., 1999; Bossio et al., 1998).

Shifts in the ratio of gram negative bacteria to gram positive bacteria were also observed between the two older and two younger cleared sites (Fig. 3). Seasonal variation in microbial community structure during wet and dry period has been observed before (Bossio et al., 1998; Steenwerth et al., 2005).

Phospholipids that strongly influenced the ordination of sites during the NMDS analyses also showed strong correlations with some of the biochemical variables in the sites. Soil moisture content in soils during dry and wet period was significantly different and it appeared to be strongly correlated with lipids that strongly influenced the separation of dry and wet period soils in younger soils C03 and C00 along axis 2 (Table 3). Difference of microbial biomass C in wet and dry soils in C03 and C00 sites was also evident. Nutrient concentrations in soils were mainly correlated with axis 1 that separated the undisturbed NV site from the cleared sites. Significant correlation between concentrations of total P, HClPi, values of N/P ratio and biomarker for actinomycetes suggesting that P may influence actinomycetes negatively. Although other studies have shown a negative association between the fungal biomarkers and soil P availability (Fraterrigo et al., 2006), there did not appear to be any association between the soil P and specific fungal biomarkers in the current study.

This study focused on changes in microbial community composition in native vegetation site in calcareous wetland soils. Based on the biogeochemical data, the process of soil development during primary succession in the HID is characterized by accumulation of organic matter (including C and N), and a gradual conversion of soil inorganic P into organic forms. With these changes, soils began in a more N-limited condition, but became increasingly more limited by P as they developed. In this manner, the chronosequence used in this study offered a unique opportunity to observe changes in the microbial community structure occurring during the early stages of soil formation and ecosystem development in a calcareous wetland system.

Microbial community structure that developed in younger soils was different from the older soils in the HID region. However, during primary succession within a short term of 16 yr, composition of microbial community structure did not vary much. Increases in microbial biomass increased with the age of restored sites, so there is higher fungal presence in the younger sites. Seasonal variation in the soil microbial community structure was more evident during the early stages of soil development. We also provide indication for progress of restoration. Further studies are needed to establish if the trends reported in this study are consistent in other systems especially during stages of early development of soil.

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