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Authors
DeAngelis, Kristen M
Fortney, Julian L
Borglin, Sharon
et al.

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Anaerobic Decomposition of Switchgrass by Tropical Soil-Derived Feedstock-Adapted Consortia

Kristen M. DeAngelis,a,b Julian L. Fortney,c Sharon Borglin,c Whendee L. Silver,c,d Blake A. Simmons,b,e and Terry C. Hazenb,c,f,g

Microbiology Department, University of Massachusetts, Amherst, Massachusetts, USAa; Microbial Communities Group, Joint BioEnergy Institute, Emeryville, California, USAa; Ecology Department, Earth Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, California, USAa; Department of Environmental Science, Policy and Management, University of California—Berkeley, Berkeley, California, USAa; Biomass Sciences and Conversion Technology Department, Sandia National Laboratories, Livermore, California, USAa; Department of Civil and Environmental Engineering, University of Tennessee, Knoxville, Tennessee, USAa; and Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee, USAa

ABSTRACT Tropical forest soils decompose litter rapidly with frequent episodes of anoxic conditions, making it likely that bacteria using alternate terminal electron acceptors (TEAs) play a large role in decomposition. This makes these soils useful templates for improving biofuel production. To investigate how TEAs affect decomposition, we cultivated feedstock-adapted consortia (FACs) derived from two tropical forest soils collected from the ends of a rainfall gradient: organic matter-rich tropical cloud forest (CF) soils, which experience sustained low redox, and iron-rich tropical rain forest (RF) soils, which experience rapidly fluctuating redox. Communities were anaerobically passed through three transfers of 10 weeks each with switchgrass as a sole carbon (C) source; FACs were then amended with nitrate, sulfate, or iron oxide. C mineralization and cellulase activities were higher in CF-FACs than in RF-FACs. Pyrosequencing of the small-subunit rRNA revealed members of the Firmicutes, Bacteroidetes, and Alphaproteobacteria as dominant. RF- and CF-FAC communities were not different in microbial diversity or biomass. The RF-FACs, derived from fluctuating redox soils, were the most responsive to the addition of TEAs, while the CF-FACs were overall more efficient and productive, both on a per-gram switchgrass and a per-cell biomass basis. These results suggest that decomposing microbial communities in fluctuating redox environments are adapted to the presence of a diversity of TEAs and ready to take advantage of them. More importantly, these data highlight the role of local environmental conditions in shaping microbial community function that may be separate from phylogenetic structure.

RESEARCH ARTICLE

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L iquid biofuels derived from nonfood crops, such as switchgrass, are a promising replacement for nonrenewable petroleum-based fuels. The U.S. Department of Energy has identified cellulosic biofuels as integral to meeting our current and future energy needs, partly due to a large renewable reservoir of plant biomass (1, 2). Although corn- and sugarcane-derived ethanol production is already under way on a significant commercial scale, cellulosic ethanol production is inefficient and costly; enzymes derived from fungi are expensive to produce and still require feedstocks to be pretreated before fermentation. Alternative biofuels do not compete with food sources, require less energy input, and produce fewer greenhouse gases than corn ethanol (3, 4). Biofuel production from switchgrass and other perennial grasses has the potential to provide more than one-third of the energy for transportation fuels domestically (1, 5) and produce 540% more renewable energy than that consumed for production, with 94% lower greenhouse gas emissions than for gasoline (6). While switchgrass presents a viable solution to improving biofuel cost and energy efficiency, conversion of lignocellulose to sugars stands to be improved through microbial processing.

Tropical forest ecosystems have very high litter and root decomposition rates (7, 8), and these soils have demonstrated the potential to efficiently deconstruct switchgrass into simple compounds (9). High rates of decomposition are fueled by a high
microbial biomass and consistent warm and humid conditions (7, 10). It has been hypothesized that iron cycling fuels considerable decomposition; tropical forest soils experience frequent episodes of anoxic conditions (11, 12), so direct or indirect iron reduction could be an avenue of growth, with biotic or abiotic regeneration of this terminal electron acceptor under oxic conditions. Fluctuating redox potential conditions create an environment where anaerobic and facultative bacterial metabolisms play a large role in leaf litter decomposition.

Under anaerobic conditions, a complex microbial community comprised of fermenters, anaerobic respirers using a range of terminal electron acceptors (TEAs), and syntrophic and aceticlastic methanogens likely contributes to decomposition (13, 14). This is distinct from temperate systems where fungi dominate litter decomposition through the release of phenol oxidase and peroxidase, enzymes that require oxygen \( (O_2) \) to create free radicals (15). Anaerobic bacteria capable of depolymerizing lignin use different enzymes, such as the beta-aryl etherase produced by *Sphingomonas paucimobilis* (16). There is a large unexplored diversity of bacteria involved in anaerobic decomposition, likely representing novel approaches to lignin depolymerization in particular that address the challenges of biomass conversion. This would be advantageous for large-scale plant biomass conversion, since the production and metabolic engineering of bacteria are easier than those for fungi (17). Understanding the mechanisms of decomposition in these naturally occurring microbial communities will improve development of lignocellulosic biofuels.

We explored patterns in enzymatic activity and microbial diversity between soil types at the extreme ends of an established elevation, rainfall, and soil redox gradient in the Luquillo Experimental Forest, Puerto Rico (12). The two sites were a low-elevation rain forest site with rapidly fluctuating redox (RF) and a high-elevation forest site with low and slowly fluctuating redox (CF). Decomposition is fast at both sites, but degradation of leaf litter *in situ* was nearly an order of magnitude faster in the lower-elevation sites than in the high-elevation sites (7; W. L. Silver, R. Ostertag and A. Thompson, unpublished observation). Both sites have measurable methane production, although net methane production is considerably higher at the upper-elevation site (18). Both soils are rich in poorly crystalline Fe oxide minerals, which are insoluble but somewhat unstable and available for microbial redox. In soils that boast up to \( 10^{13} \) cells per g, iron reducers are found at up to \( 10^9 \) cells per g soil, or 1% of total microbial abundance (10). Iron cycling, which itself is driven both by high Fe abundance and frequent fluctuations in soil redox potential (12, 19, 20), can oxidize C equivalent to 44% of annual litter production (7). Dissimilatory Fe(III)-reducing bacteria compete effectively for limited C with methanogens (21), and there is excellent correlation between CO\(_2\) production and Fe(II) production (10). These soils also cycle N and S rapidly (22–24), which suggests that the identity and abundance of TEAs may have a significant impact on the microbial community and their associated decomposition enzymes.

To understand the role of different TEAs in decomposition and investigate the possibility for exogenous TEAs to change either the microbial communities or the decomposition rates under controlled laboratory conditions, we set up anaerobic feedstock-adapted consortia (FAC) derived from the rain forest site (RF) and cloud forest site (CF). After three transfers, the richness of consortia was about one-tenth that of the original soil inocula, as previously described (9). Once these three replicate consortia were established on switchgrass under anaerobic conditions, we used them to seed new consortia (each with three biological replicates) and measured switchgrass decomposition under fermentative conditions (switchgrass alone) and also with potential denitrification (nitrate added), Fe reduction [iron(III) added], and sulfate reduction (sulfate added). We then examined how exogenously added TEAs affect switchgrass C mineralization as well as microbial community function and structure.

**RESULTS**

**Microbial activity.** To estimate the C mineralization associated with switchgrass degradation, we measured the amount of CO\(_2\) and CH\(_4\) emitted; controls with no switchgrass added had no measurable emissions of CO\(_2\) or CH\(_4\). Carbon mineralization was significantly higher for the cloud forest (CF)-derived FACs than for the rain forest (RF)-derived FACs, regardless of TEAs added, in terms of cumulative CO\(_2\) production \( (P < 0.01) \) and CH\(_4\) production \( (P < 0.001) \) per gram switchgrass added. TEA addition had significant effects on CO\(_2\) in the RF \( (P < 0.0001) \) and CF \( (P < 0.0001) \) and on CH\(_4\) in the RF \( (P < 0.05) \) and CF \( (P < 0.0001) \) FACs (Fig. 1). The nitrate amendment had the highest CO\(_2\) production in the RF-FACs but the lowest CO\(_2\) production in CF-FACs. Iron significantly increased the CO\(_2\) above the background level in the RF-FACs. CO\(_2\) and CH\(_4\) production was suppressed following TEA amendment in the CF-FACs.

Changes in the concentrations of NO\(_3^-\), NO\(_2^-\), Fe(II), and SO\(_4^{2-}\) were measured at the beginning and end of the incubation to estimate redox sensitivity of the microbial communities (Table 1). Net reduction of added NO\(_3^-\) to NO\(_2^-\) was detected in the RF-FACs but not the CF-FACs. Net Fe reduction occurred in both the RF- and CF-FACs that received Fe oxide, as evidenced by the generation of high concentrations of Fe(II) at the end of the incubation. A small but significant amount of net sulfate was reduced in the sulfate-amended RF-FACs.

Beta-galactosidase and cellobiohydrolase activities were significantly higher in CF-derived FACs than in RF-FACs (4-methylumbelliferyl \( \beta \)-d-glucopyranoside (MUB) \[BG\], \( P < 0.001; \) \( \beta \)-d-cellobioside-MUB \[CBH\], \( P < 0.05) \), but there was no difference in activity between sites for N-acetylglucosidase or xylanase activities. There were significant treatment effects in RF-FACs for BG \( (P < 0.05) \), CBH \( (P < 0.01) \), N-acetylglucosamine-MUB \[NAG\] \( (P < 0.0001) \), and \( \beta \)-d-xylopyranoside-MUB \[XYL\] \( (P < 0.0001) \). The scale of the effect of TEAs on enzyme activities was relatively small, with the largest differences being 31% (BG), 56% (CBH), 234% (NAG), and 61% (XYL). There were weakly significant treatment effects in CF-FACs for NAG \( (P = 0.081) \) and XYL \( (P = 0.078) \) and no effect for BG or CBH (Fig. 2).

**Microbial community growth and structure.** Higher C mineralization rates observed in CF-FACs than in RF-FACs did not translate to cell abundance or biomass. There was no significant difference between inocula based on total lipids extracted or direct counts, whether comparing final cell counts or fold increase in cell growth (final count divided by initial count) (see Fig. S1 in the supplemental material). There was a significant treatment effect of TEAs on cell growth in RF-FACs \( (P < 0.0001) \) but not in CF-FACs. Among the RF-FACs, the sulfate-amended incubations had the most growth, followed by the switchgrass and iron-amended incubations, with the nitrate-amended incubations having the lowest growth (see Fig. S1A). When biomass was measured by
lipids extracted, there was no treatment effect for either inoculum type (see Fig. S1B).

Pyrosequencing resulted in 1,310 unique taxa resolved at 100% nucleotide identity. There were no differences between RF- and CF-FAC inocula in richness or diversity (by Shannon’s H or Simpson’s D index). Treatment significantly affected richness and diversity, where the nitrate treatment was lower in diversity compared to the baseline switchgrass, iron-amended, and sulfate-amended FACs (see Table S1 in the supplemental material). Overall, the three replicate microcosms were surprisingly similar considering that they had been separately transferred by 10% serial dilution into fresh media three times.

Dominant taxa were defined as taxa with a relative abundance of at least 2% in at least one sample; 23 taxa in RF-FACs and 14 taxa in CF-FACs fit this criterion. Most dominant taxa were members of the Proteobacteria, and rare species in the RF-FACs (see Fig. S2 and Table S1). The stress biomarker lipid 16:1w7t was significantly elevated in unamended FACs. Total lipids did not vary significantly by TEA treatment.

**FIG 1** Net CO2 (A) or CH4 (B), expressed as μg C per gram switchgrass accumulated in the FACs over the course of the incubation. Values shown are means plus or minus standard errors (n = 3). Compared to the RF-FACs, the CF-FACs had significantly more CO2 (P < 0.01) and CH4 emitted (P < 0.001). Within each FAC, the treatment effect was assessed; treatment had a significant effect on FACs in all cases. Lowercase letters denote treatments that are statistically indistinguishable within each set (n = 3), as determined by Tukey’s HSD test at a cutoff of P < 0.05.

**TABLE 1** Concentrations of added TEAs at the beginning and end of FAC lab incubations

| Process and consortia | Added TEA | Tinit | Tfinal | P valuea |
|-----------------------|-----------|-------|--------|----------|
| **Denitrification**   |           |       |        |          |
| Rain forest FACs      | NO3−      | 11.4 ± 0.07 | 0.01 ± 0.01 | ***      |
|                       | NO2−      | 0.01 ± 0.01 | 3.66 ± 0.44 | **       |
| Cloud forest FACs     | NO3−      | 11.6 ± 0.13 | 10.08 ± 1.59 |           |
|                       | NO2−      | 0.03 ± 0   | 0.02 ± 0  |           |
| **Iron reduction**    |           |       |        |          |
| Rain forest FACs      | Fe(II)    | 0.44 ± 0.06 | 6.87 ± 0.07 | ***      |
| Cloud forest FACs     | Fe(II)    | 0.71 ± 0.06 | 6.4 ± 0.26 | ***      |
| **Sulfate reduction** |           |       |        |          |
| Rain forest FACs      | SO4−      | 7.89 ± 0.32 | 6.15 ± 0.3  | *         |
| Cloud forest FACs     | SO4−      | 7.85 ± 0.15 | 7.91 ± 0.13 |           |

a ANOVAs were evaluated at P value cutoffs of <0.05 (*), <0.01 (**), and <0.001 (***)(n = 6).
The cloud forest feedstock-adapted consortia (CF-FACs) were more efficient at decomposing switchgrass; higher enzyme activity and rates of C mineralization of CF-FACs were accompanied by similar numbers of cells and amounts of biomass compared to findings for RF-FACs. The greater efficiency of biomass conversion of the CF-FACs could be explained by the different life histories of soil microbes from the tropical cloud forest compared to those from the rain forest. The higher-elevation cloud forest soils experience frequent low-redox conditions, while the lower-elevation rain forest soils experience higher overall redox potential and so are adapted to more-frequent O₂ availability. In general, tropical forest soils are likely to be adapted to fluctuating redox; compared to static oxic or anoxic conditions, fluctuating redox resulted in the highest number of active microbes (25, 26). Based on these studies, we would hypothesize that the higher-elevation, lower-O₂ soils should have lower field decomposition rates; indeed, this has been shown under field conditions (7). However, under anoxic laboratory conditions the lower-redox CF-derived FACs performed better than the RF-derived FACs. The increased C mineralization of CF-FACs over that of RF-FACs is apparently not due to increased biomass, since these two inocula were indistinguishable by direct counts and by lipid biomass, suggesting that an equivalent number or mass of microbes was able to more efficiently deconstruct the feedstock. Either the planktonic nature of

Through cultivation of feedstock-adapted consortia (FACs) derived from tropical rain forest (RF) and cloud forest (CF) soils, we found that the RF-FACs were able to make use of a broader suite of TEAs than CF-FACs, while the CF-FACs were more productive than RF-FACs. In each case, productivity was indicated by respiration, cell biomass, and enzyme activity during deconstruction of switchgrass under static anoxic conditions. The RF-FACs mostly responded positively to added TEAs. In comparison, the CF-FACs were unresponsive to TEAs and had overall higher CO₂ and CH₄ accumulation and higher enzyme activities per gram switchgrass. This is particularly surprising given the more rapid decomposition rates of the rain forest site under field conditions (Silver et al., unpublished). The reduction of Fe(III), SO₄²⁻, and NO₃⁻ by tropical soil communities has the potential to drive considerable C mineralization (10, 22, 24, 25), so these experiments were designed to explore the extent to which anaerobic switchgrass decomposition would be affected by TEA additions in culture. We suggest that a combination of the differential abundances of specific organisms (such as dominance of acetate-utilizing Methanosaeta sarcina in the CF-FACs) and the life history of shared microbial populations were responsible for the differences in ability to use different TEAs and in C mineralization efficiency between the two tropical forest soil inocula.

**FIG 2** Enzyme activities for beta-glucosidase (BG), cellobiohydrodase (CBH), N-acetyl glucosaminidase (NAG), and xylanase (XYL) are shown for the RF-FACs (left) and CF-FACs (right), expressed as nmol substrate converted per gram of biomass per hour. Values shown are means plus or minus standard errors (n = 3), and ANOVAs were performed to test the null hypothesis of no difference between TEA treatments within an enzyme activity rate, with associated P values shown as “*” (P < 0.05), “**” (P < 0.01) and “***” (P < 0.001).

**TABLE 2** Diversity of FACs by TEA treatment, calculated as overall richness, Shannon’s diversity index (H), Simpson’s index (D), and inverse Simpson’s index (invD)

| Statistic | SG | SG + NO₃⁻ | SG + FeOx | SG + SO₄⁻ | P value |
|-----------|----|------------|-----------|-----------|---------|
| Richness  | 172.2 ± 39.1 b | 54.8 ± 57.1 a | 148.8 ± 105.6 ab | 123.8 ± 29.9 ab | * |
| H         | 2.46 ± 0.27 b | 0.75 ± 0.73 a | 2.05 ± 1.04 b | 2.23 ± 0.33 b | ** |
| D         | 0.84 ± 0.04 b | 0.33 ± 0.31 a | 0.71 ± 0.35 b | 0.83 ± 0.05 b | ** |
| invD      | 6.45 ± 1.31 b | 1.83 ± 0.96 a | 5.94 ± 2.89 b | 6.14 ± 1.71 b | ** |

*Lowercase letters denote different levels of significance as determined by Tukey’s HSD test at P < 0.05. SG, switchgrass; FeOx, iron oxide.

*ANOVA's were evaluated at P value cutoffs of <0.05 (*) and <0.01 (**) (n = 6).
Organisms with facultative metabolic capabilities seemed to be preserved throughout the transfers in the RF-FACs but not the CF-FACs, despite the fact that all consortia were adapted without the addition of exogenous TEAs. This was evident in the SO$_4^{2-}$-amended FACs, where addition caused a significant increase in cell growth, as well as a significant increase in xylulose dehydrogenase enzyme activity. Compared to findings for other TEA treatments, the SO$_4^{2-}$-amended RF-FACs had increased numbers of Desulfovibrio bacteria, which are known SO$_4^{2-}$ reducers (28) with innate tolerance for O$_2$ that would aid in survival in fluctuating-redox soils (29). Desulfovibrio was also enriched in the Fe(III)-amended RF-FACs, though to a lesser extent than in the SO$_4^{2-}$-amended FACs. This suggests the possibility of Fe(III)-reducing Desulfovibrio, though members of the genera Clostridium and Aeromonas were also present and were likely reducing Fe(III) (30, 31). Siderophores, humics, or other electron shuttles may permit a range of microbes to indirectly reduce Fe(III), producing Fe(II) (32, 33). Like Fe(III), SO$_4^{2-}$ can be abiotically oxidized in environments where redox potential fluctuates, creating a sulfur cycle of generating terminal electron acceptors to fuel C mineralization (34–36). Both Fe(III) and NO$_3^-$ increased CO$_2$ production, though both decreased cell abundance. Denitrifiers are facultative aerobes that prefer O$_2$ but have the capacity to switch to NO$_3^-$ (or NO$_2^-$) reduction under O$_2$-limited conditions and are phylogenetically diverse (37). Dissimilatory NO$_3^-$ reduction to ammonium (DNRA) has also been shown to constitute as much as 75% of the turnover of the NO$_3^-$ pool in tropical forest soils (22). The organisms performing DNRA in one instance were discovered to consist of spore-forming bacteria (38). The buildup of NO$_2^-$ in the NO$_3^-$-amended FACs could have contributed to the repression of activity and cell growth in the NO$_3^-$-amended RF-FACs and suggests that while DNRA occurs in the field, it was not likely to be occurring in our FACs.

The addition of different TEAs created changes in C mineralization and populations of methanogens in CF-FACs, where CH$_4$ production was appreciable in the unamended CF-FACs as well as the CF-FACs amended with Fe(III) and SO$_4^{2-}$. While CH$_4$ was produced in the Fe(III)-amended CF-FAC microcosms, Fe(III) amendment did not increase CO$_2$ concentrations relative to the background treatment. Iron(III) addition decreased net CH$_4$ production, likely due to competition with dissimilatory Fe reducers for acetate (21). Net CH$_4$ production in Fe(III)-amended CF-FACs was significantly depressed, though not as low as in the other FACs, suggesting that either the Fe(III) reducers were not as efficient as the other community members or that the methanogens competing with the Fe(III) reducers for labile C were not as effective under Fe(III)-reducing conditions. The switchgrass-only, Fe(III)-amended, and NO$_3^-$-amended CF-FAC microcosms contained 7% Methanobacterium species, which are hydrogen- and formate-utilizing methanogens, and [in Fe(III) and switchgrass-only treatments] 3% Methanosarcina species, acetate-utilizing methanogens (27, 39, 40). The SO$_4^{2-}$-amended CF-FACs did not contain members of Methanosarcina, suggesting that SO$_4^{2-}$ was somehow inhibitory. Members of the Clostridia in clusters 8, 3, and 2 were all significantly enriched in SO$_4^{2-}$-amended FACs compared to findings for Fe(III)-amended FACs. There is much phenotypic variation within the Clostridia that enables these organisms to live in a variety of environments (41), and apparently even the subtle selective pressures of different additional TEA amendments is enough to select for a specific set of Clostridia bacteria.

The established feedstock-adapted consortia generally contained a few dominant species with long tails of richness extending into the hundreds of rare species, which may play a role in stabilizing the activity of these communities. The dominant species, defined as having a relative abundance of 2% or greater, comprised a substantial portion of the total richness of these consortia, which were mostly comprised of the Clostridiales, as well as members of the Rhizobiales, Bacteroidales, methanogenic Archaea, and De-
**sulfovibrio.** Taxa in the long tail of the communities were made up of taxa from phyla commonly found in soils but uncultured, with little known about their function. In the RF-FACs these included taxa from the phyla *Gemmatimonadetes* and Op10. CF-FACs had the same diversity but fewer dominant taxa and different rare taxa from those of the RF-FACs. The rare taxa have been selected for their ability to grow in the anaerobic enrichments but may also contribute to the activity. For example, it is known that anaerobic cellulose degradation by *Clostridium straminisolvens* CSK1 is stabilized by the presence of noncellulolytic, aerobic bacteria that are thought to reduce the local redox potential (42). Taxa need not be abundant to play important roles in ecosystem function; it has been shown in bog soils that the sulfate reducer *Desulfovibrio* was present at low (0.006%) abundance but was responsible for the majority of soil SO$_4^{2-}$ reduction (43). Increased richness has been shown to stabilize mixed communities in natural environments, especially when richness is low (below 10 species) (44).

Cooperation among disparate taxa in these mixed communities is possible and will become evident with attempts to isolate contributing members under the same conditions.

**MATERIALS AND METHODS**

**Lab incubations.** Soils were collected from the Luquillo Experimental Forest, Puerto Rico, part of the National Science Foundation (NSF)-sponsored Long Term Ecological Research Program. Soils from the Bisley Research Watershed (rain forest site [RF]) and the Pico del Este Short Could Forest (CF) were used as inoculum for three replicate feedstock-adapted consortia (FAC) each. The fieldwork was conducted and samples collected and transported under USDA permit number P526P-08-00634. Site selection was based on redox states of the soil, which change along an elevation and rainfall gradient. The CF site is in an upper montane tropical cloud forest at approximately 1,050 m above sea level (18°18’N, 65°50’W) and experiences approximately 4 to 5 m rainfall annually and a high frequency of low-redox conditions (45). The RF site is in a lower montane wet tropical forest at approximately 270 m above sea level.
was placed in a 6-mm microscope slide well and allowed to dry. Wells were prepared in 10-fold dilutions with filter-sterilized 1 M phosphate-buffered saline (PBS) (Sigma Chemical Company, St. Louis, MO) for each anion was used to determine measured concentrations. 

Iron analysis. Total iron(II) concentrations were determined by a colorimetric ferrozine assay (52, 53). A 250-μl aliquot was dispensed from each FAC into 5 ml of 0.5 N hydrochloric acid (HCl) and allowed to extract for 20 min. Twenty-five microliters of the HCl solution was then added to 1 ml of ferrozine. Absorbance was immediately recorded at 562 nm on a Beckman DU 640 spectrophotometer (Beckman Coulter, Inc., Brea, CA). A 4-point calibration (0.5, 1, 5, and 15 mM) of iron(II) sulfate was used to quantify sample measurements.

Iron chromatography. Nitrate, nitrite, and sulfate concentrations were measured by iron chromatography (IC) using a model ICS-2000 IC ( Dionex Corporation, Sunnyvale, CA) with an IonPac AG11 guard column (4 by 50 mm), IonPac AS11 analytical column (4 by 250 mm), conductivity suppressor (ASRS Ultra), and eluent generator creating a potassium hydroxide gradient. Samples were filtered with a 0.2-μm nylon membrane and diluted 16-fold in ultrapure Milli-Q water. A 3-point calibration (100, 300, and 600 μM) for each anion was used to determine measured concentrations.

Cell counts. Direct counts were made of each sample based on an acridine orange staining technique with epifluorescence counting (54). A sample was taken from each lab incubation, preserved using 4% formaldehyde, and stored at 4°C. Direct counts were obtained by diluting samples 10-fold with filter-sterilized 1× phosphate-buffered saline (PBS) (Sigma Aldrich Corp., St. Louis, MO). Ten microliters of each dilution was placed in a 6-mm microscope slide well and allowed to dry. Wells were stained with 2.5 mg · ml⁻¹ acridine orange for 2 min in the dark. Unbound acridine orange was rinsed off by dipping slides into deionized water. Cells were imaged using a fluorescein isothiocyanate (FITC) filter on a Zeiss Axioskop microscope (Carl Zeiss, Inc., Germany). Cell counts are expressed as numbers of cells per ml culture, and fold-increase counts are reported as final counts divided by initial counts.

Enzyme assays. Enzyme activity was determined at the end of the lab incubation by adding 100 μl of culture to wells of a 96-well plate containing 100 μl of the substrates 4-methylumbelliferyl β-d-glucopyranoside (BG) for beta-glucosidase or cellulose activity, β-d-cellobioside-MUB (CBH) for cellobiohydrolase or hemicellulose activity, N-acetyl glucosamine-MUB (NAG) for chitinase activity, and β-d-xylopyranoside-MUB (XYL) for xylanase activity. The fluorescence of each well was determined after a 4-h incubation at 27°C using a Fluorolog-3 spectrophuorimeter (Horiba Jobin Yvon Inc., Edison, NJ) with 365-nm excitation and 442-nm emission wavelengths. Rates of substrate degradation were calculated as the difference in moles of MUB produced over time based on MUB standards. Enzyme activities for each treatment or replicate were normalized for the initial mass of biomass added and are reported as pmol MUB h⁻¹ g⁻¹ dry biomass.

PLFA. Samples were extracted by the Bligh-Dyer method (55–57). Briefly, 10 ml of a 10:5:4 mixture of methanol-chloroform-[H]phosphoric acid-buffered saline (PBS) was added to each sample. Fifty microliters of 500-mg/liter 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL) was added as an internal standard. The mixture was vortexed, sonicated for 2 min, and extracted at room temperature in the dark for 3 h. Phases were separated with the addition of 2 ml of chloroform and 2 ml of water, followed by centrifugation at 2,000 rpm for 15 min to separate the organic and aqueous layers. The lower organic layer was removed to a clean tube, and 2 ml additional chloroform was added to the original extract, which was vortexed and centrifuged and combined with the first layer. This combined organic layer was dried under N₂. The dried extracts were separated into neutral, glycerol, and phospholipids on a C₄₅ silica column (Sigma Chemical Company, St. Louis, MO) by sequential elution with chloroform, acetone, and methanol. All collected fractions were dried under N₂.

The methanol fractions containing phospholipids were subjected to a mild alkaline hydrolysis to remove the head group and create fatty acid methyl ester (FAME) compounds by resuspending with 1:1 chloroform-methanol and 1 ml of 11.2-mg/liter KOH in methanol. After vortexing for 2 min, they were incubated in a water bath at 37°C for 60 min. The FAME compounds were then neutralized with 200 μl of 0.1 M acetic acid, extracted three times with 2 ml of hexane, and dried under N₂. Fifty microliters of 46.2-mg/liter methyl undecanoate (Sigma Chemical Company, St. Louis, MO) were added to the dried extracts as an external standard. FAME were detected on an Agilent 6890N gas chromatograph/flame ionization detector (GC/FID) on an HP-60-µ column (0.25-µm inside diameter [ID]) and quantified by comparing to known standards. Peak confirmation was accomplished by using an Agilent 6890 GC/mass spectrometer (MS). Lipid classes were grouped into guilds according to configuration (58–62) as outlined in Table S1 in the supplemental material.

Amplicon pyrosequencing of community SSU rRNA. Samples were taken of liquid medium plus switchgrass from each microcosm, and DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) extraction method as previously described (26). Briefly, frozen samples were added to CTAB extraction buffer and phenol in Lysing Matrix E tubes (Qbiogene) and bead beaten in a FastPrep instrument (Bio101), followed by a chloroform extraction, isopropanol precipitation, and use of the AllPrep DNA/RNA extraction kit (Qiagen). Small subunit (SSU) rRNA gene sequences for high-throughput amplicon pyrosequencing were amplified using the universal primers 926F (5’-aaactYaaaxkgattcgg-3’) and 1392R (5’-gaccgcctggtR tg-3’), where upper-case letters indicate nucleotide redundancies; these primers target the V8 variable region of the 16S rRNA gene from bacteria and archaea as well as the 18S rRNA gene in eukarya (63). The sequences shown do not include adaptor or bar code sequences, and the reverse primer included a 5-bp bar code for multiplexing of samples during sequencing. Emulsion

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PCR and sequencing of the PCR amplicons were performed following the manufacturer’s instructions for the Roche 454 GS FLX titanium technology, with the exception that the final dilution was 1e–8. Sequencing tags were analyzed using the software tool PyroTagger (http://pyrotagger.cgi-psf.org/), which filters by removing low-quality sequences from the set based on the qual file, trims using a 225-bp sequence length threshold, dereplicates, clusters at the operational taxonomic unit (OTU) level based on 97% identity, and then classifies. Eighteen chimeras were detected and removed, leaving 1,310 taxa detected. Classification was based on the greengenes database of rRNA genes (64) for bacterial and archaeal amplicons and the Silva database for eukaryotic amplicons (65).

Statistical analysis. The experimental design included three biological replicates, four treatments, and two soil inoculum types, for a total of 24 samples. Analyses of variance (ANOVA) were performed to a statistical significance level of 0.05, and Tukey’s honestly significant difference (HSD) test was used to determine treatments with statistically indistinguishable measurements. Ordination of the whole community detected by pyrosequencing was performed using nonmetric multidimensional scaling with the Bray-Curtis distance measure based on unifrac distances (66, 67). Joint plots were vectors indicating the direction and magnitude of correlation to individual samples; joint plots were calculated for all measurable variables against the community ordination, and only the factors with significant ($P < 0.05$) correlations are shown. In all results, means and standard errors of the mean are reported for 3 biological replicates unless otherwise indicated. A multivariate permutation procedure (MRPP) was used to determine differences in inoculum or TEA amendment treatment within the microbial community distance matrix. This test yields metric A, which describes the within-group homogeneity and can range from 0 if the groups are completely different to 1 if the groups are identical; for ecological data, it is uncommon to observe A values greater than 0.3 (68). We used Benjamini-Hochberg-adjusted $P$ values in PERMANOVA analysis (69). To calculate richness or diversity (by Shannon’s H or Simpson’s D index), data from CF- and RF-FACs were pooled after determining that they were not significantly different.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00249-11/-/DCSupplemental.

FIG S1, PDF file, 0.1 MB.
FIG S2, PDF file, 0.1 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.1 MB.
Table S3, PDF file, 0.1 MB.
Table S4, PDF file, 0.1 MB.
Table S5, PDF file, 0.1 MB.
Table S6, PDF file, 0.1 MB.

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