

# Comparative Biogeochemical Cycles of Bioenergy Crops Reveal Nitrogen-Fixation and Low Greenhouse Gas Emissions in a *Miscanthus × giganteus* Agro-Ecosystem

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## ABSTRACT

We evaluated the biogeochemical cycling and relative greenhouse gas (GHG) mitigation potential of proposed biofuel feedstock crops by modeling growth dynamics of *Miscanthus × giganteus* Greef et Deuter (miscanthus), *Panicum virgatum* L. (switchgrass), *Zea mays* L. (corn), and a mixed prairie community under identical field conditions. DAYCENT model simulations for miscanthus were parameterized with data from trial plots in Europe and Illinois, USA. Switchgrass, corn, and prairie ecosystems were simulated using parameters

published in the literature. A previously unknown source of nitrogen (N) was necessary to balance the plant nutrient budget in miscanthus crops, leading us to hypothesize that miscanthus growth depends on N-fixation. We tested for nitrogenase activity by acetylene reduction of whole rhizomes and bacteria isolated from the rhizosphere and miscanthus tissue. Our results supported the hypothesis that biological N-fixation contributed to the N demand of miscanthus, a highly productive perennial grass. Corn agro-ecosystems emit 956 to 1899 g CO<sub>2eq</sub> m<sup>-2</sup>y<sup>-1</sup> greater GHGs (including CO<sub>2</sub>, N<sub>2</sub>O, CH<sub>4</sub>) to the atmosphere than the other biofuel crop alternatives because of greater N<sub>2</sub>O emissions from fertilizer additions. Of the feedstock crops evaluated in this study, miscanthus would result in the greatest GHG reduction.

**Key words:** biofuel; bioenergy; carbon sequestration; cellulosic; corn; DAYCENT; ethanol; prairie; switchgrass; soil carbon.

Received 22 August 2009; accepted 29 November 2009;  
published online 22 December 2009

**Author Contributions:** SCD, ADK, and WJP performed the research, contributed new methods and models, analyzed the data, and wrote the paper; FGD, SDG, and CMS contributed new methods and analyzed data; EHD conceived of the study and contributed to data analysis and writing.

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## INTRODUCTION

Cultivation of biofuel crops is expanding because of the increasing disparity between fuel demand and supply (U.S.DOE 2006; EIA 2008), whereas the environmental impacts of bioenergy production remain unknown (Davis and others 2009). Uncertainties about the greenhouse gas (GHG) mitigation of biofuels depend in part on nutrient cycling efficiencies of feedstock crops. Sustainability of biofuel production depends on ecological processes that maintain high-yielding crops with low GHG emissions. The primary ethanol feedstock in the USA, *Zea mays* L. (corn), has been genetically engineered to maximize carbon (C) and nitrogen (N) allocation to grains and requires large applications of fertilizer, resulting in high N<sub>2</sub>O emissions. Reduction of residue removal can offset fertilizer requirements, but second-generation biofuel feedstocks will be harvested for cellulosic biomass and thus will require more residue removal from cropland than is currently required in harvests of first-generation grain feedstock. Here, we analyze the nutrient cycling efficiencies of potential feedstock crops to determine the relative environmental impacts of bioenergy crop alternatives. Although there are many stages of biofuel production that incur environmental costs, the focus of this analysis was limited to only terrestrial ecosystem processes that occur on a bioenergy farm.

Nutrients are removed from an agro-ecosystem every time biomass is harvested. These nutrients otherwise would be returned to the soil as litter in unmanaged ecosystems or in conventional crops with limited residue removal (Anderson-Teixeira and others 2009). Loss of organic matter represents a management challenge because fertilizer applications incur a high energy and GHG cost (Crutzen and others 2008; Field and others 2007; Davis and others 2009). Nutrient cycling efficiencies that dictate fertilizer requirements vary among crop species, so direct comparisons of yields and nutrient balances that differ among candidate biofuel crops are needed.

Plant species with high nutrient-use efficiency may be the most viable options for sustainable biofuel crops because GHG emissions associated with fertilizers would be minimal. *Miscanthus × giganteus* Greef et Deuter (miscanthus) was recently introduced in the USA as such a species (Heaton and others 2008; Miguez and others 2008); because, it is a perennial grass that has been grown in experimental trials in temperate regions of Europe with no nutrient additions (Beale and Long 1997; Clifton-Brown and Lewandowski 2000; Cosentino and others 2007; Christian and others 2008).

Miscanthus maintains high photosynthetic rates over a large temperature range (Beale and others 1996), exploits a longer-than-average growing season (Heaton and others 2008), and yields more than two times the biomass of other candidate biofuel grass crops (that is, *Panicum virgatum* L.; Heaton and others 2004). Litter accumulates prior to winter harvests of aboveground biomass, contributing to soil organic matter (SOM) (Heaton and others 2008). There is also evidence of high N retranslocation prior to senescence of aboveground tissues of miscanthus (Beale and Long 1997; Beuch and others 2000). Even with these advantages, harvest inevitably removes some nutrients from any crop ecosystem, and it is still undetermined if there is a biofuel crop that can withstand such losses without soil amendments. Miscanthus appears sustainable in the short-term and less N-limited than *P. virgatum* L. (switchgrass) or corn (Cosentino and others 2007; Danalatos and others 2007; Miguez and others 2008), but the long-term ecological consequences of harvesting a crop-like miscanthus remain unknown.

Ecosystem process models are valuable tools for synthesizing biogeochemical cycles and generating new hypotheses about nutrient cycling. The synthesis provided by ecosystem models can expose specific mechanisms that are the most influential for changing terrestrial C and N budgets (for example, Parton and others 2007). Modeled projections of ecosystem processes can be integrated with experimental trials of biofuel crops to determine ways of reducing energy costs and GHG emissions incurred by various cultivation practices. In this study, we employed the DAYCENT model (Parton and others 1998; Del Grosso and others 2005) to simulate C and N cycling of agro-ecosystems that were converted from annual row crops to continuous corn or perennial biofuel crops. The perennial biofuel crops included in this study were miscanthus, switchgrass, and a mixed prairie community because these have been proposed as cellulosic feedstocks that might replace corn in the Midwestern USA.

The main objective of our study was to examine modeled differences in C and N cycling among four bioenergy crops; miscanthus, switchgrass, corn, and mixed species prairie. The acquisition and retention of C and N are key determinants of GHG fluxes that would be associated with bioenergy crops. Model syntheses of biogeochemical cycling included estimates of plant C productivity, soil C accumulation, and ecosystem N balances, and allowed us to estimate net GHG fluxes (that is, CO<sub>2</sub>, N<sub>2</sub>O, and CH<sub>4</sub>) for each crop. Because DAYCENT

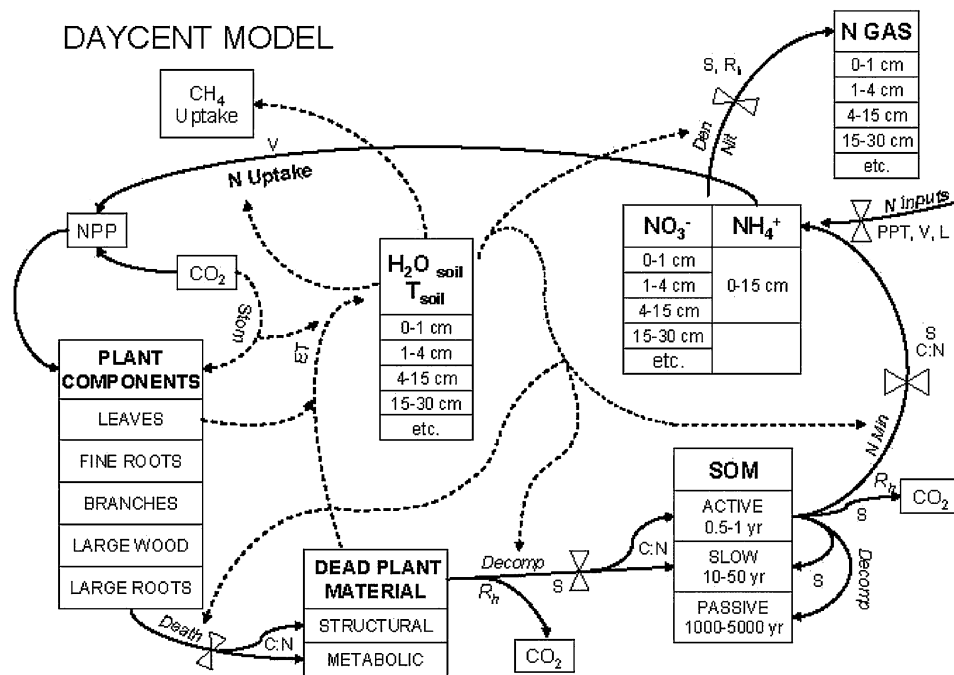
was not yet validated for miscanthus, we calibrated the model for this species and tested the validity of model simulations against experimental observations. After verifying the accuracy of model simulations, we used the DAYCENT model to test the null hypothesis that there were no differences in GHG emissions among bioenergy crop ecosystems. Model calibration exercises indicated that N-fixation was an important component of the N budget for miscanthus. We tested this secondary hypothesis by first screening bacteria isolated from miscanthus tissue for *nifH*, the gene that encodes dinitrogenase reductase, and then measuring nitrogenase activity of both field grown rhizomes of miscanthus and the *nifH*-positive bacteria identified in the plant.

## METHODS

### The DAYCENT Model

DAYCENT (Parton and others 1998; Kelly and others 2000; Del Grosso and others 2001) is based on the widely used biogeochemical model, CENTURY (Parton and others 1994), but differs by implementing a daily time-step instead of a

monthly time-step and therefore has improved descriptions of microbial-mediated N and C fluxes. DAYCENT has been used to simulate the impact of grassland and agricultural management practices on soil C, trace gas fluxes ( $\text{CH}_4$ ,  $\text{NO}_x$ , and  $\text{N}_2\text{O}$ ),  $\text{NO}_3^-$  leaching, and crop yields and has been used to project regional impacts of changes in agricultural land-use practices at local, national, and global scales (for example, Kelly and others 2000; Parton and others 2001; Del Grosso and others 2005, 2009). DAYCENT simulates atmosphere, plant, and soil nutrient exchanges with detailed process descriptions of SOM decomposition and external additions. Soil texture, site-specific climate variables, plant phenology, and management events are the inputs to the model that inform submodels of plant production, organic matter decomposition, soil moisture, and temperature at variable depths, trace gas fluxes, and N transformations (Figure 1). Using DAYCENT, Del Grosso and others (2002) accurately simulated biomass yields, SOM, and gas fluxes in a variety of managed crops, and Adler and others (2007) recently used the model to predict yields for corn and switchgrass that were managed as biofuel feedstocks.



**Figure 1.** Diagram for the DAYCENT ecosystem model showing C and N flows ( $\rightarrow$ ); feedbacks and information flows ( $\dashrightarrow$ ); control on process ( $\bowtie$ ); soil water content ( $\text{H}_2\text{O}_{\text{soil}}$ ); soil temperature ( $T_{\text{soil}}$ ); soil texture (S); C:N ratio of material (C:N); vegetation type (V); SOM; land use (L); heterotrophic respiration ( $R_h$ ); N GAS ( $\text{N}_2\text{O}$ ,  $\text{NO}_x$ , and  $\text{N}_2$ ); net primary production (NPP); precipitation (PPT). Processes are designated by *italics*: *Stom* stomatal conductance; *Death* plant component death; *Decomp* decomposition; *N inputs* N-fixation, deposition, and fertilization; *Nit* nitrification; *Den* denitrification; *Nmin* N mineralization; *ET* evapotranspiration. More detailed model descriptions can be found in Parton and others (1998), Kelly and others (2000) and Del Grosso and others (2001).

## Model Parameterization

For this study, the DAYCENT model was parameterized to simulate corn, switchgrass, miscanthus, and native tallgrass prairie. No calibration was required for corn, switchgrass, and tallgrass prairie simulations because the submodels for these species have been verified previously. The parameters for the switchgrass submodel were based on the values from Adler and others (2007), but plant C:N ratio boundaries were modified according to Vogel and others (2002) to more accurately reflect plant N uptake and yield for switchgrass grown with variable N fertilization in nearby Iowa and Nebraska. The improvements of the switchgrass submodel reflect more accurate nutrient allocation under variable field conditions. The plant growth parameters for corn were based on the values used for the DAYCENT model in David and others (2009), whereas the parameters for native tallgrass prairie were based on Pepper and others (2005). Our simulations included N fertilizer only for corn; N was applied in November and May at a rate of  $9.5 \text{ g N m}^{-2}$  ( $19 \text{ g N m}^{-2}$  annually). The corn simulations also included a tandem disk plow treatment before planting each year, whereas the perennial grasses only required plowing in the initial year of planting. For validation against field measurements, corn crops were simulated in rotation with soybean crops as this was the most common agricultural practice for which we have ample yield data in our region. The parameterization for corn–soybean rotation included two separate sets of crop parameters, but only the corn parameterization was used for the final model projections of continuous corn.

In the miscanthus submodel, the plant growth parameters were based on data from experiments conducted in Europe (Beale and Long 1995; Clifton-Brown and Lewandowski 2000; Cosentino and others 2007) and in Champaign, Illinois (Heaton and others 2004, 2008). Input parameters for miscanthus were compiled using literature estimates from multiple sites in Europe and Illinois, as referenced above, and then model outputs were compared to measurements at the Champaign, Illinois site. Calibration of the miscanthus submodel, described in the following section, was required because the initial parameterization did not accurately simulate measured rates of biomass accumulation.

## Calibration of Miscanthus Submodel

Our initial simulations of miscanthus biomass accumulation underestimated by more than 50%

values observed in the field by Heaton and others (2008). We first attempted to calibrate the plant stress responses so that biomass allocation was less sensitive to nutrient limitation. Even with no response to nutrient stress, simulated plant growth was limited because there was not enough N available in the soil to support observed rates of biomass accumulation. By maximizing biomass allocation to roots, thereby simulating root access to N in deeper soil layers, the supply rate of N from the soil was insufficient to simulate observed rates of growth for miscanthus in field trials. The only way to configure the model to reproduce observed rates of biomass accumulation was to increase the supply of N in the soil from a source currently not identified in the literature for this species.

## Rationale for N-Fixation Hypothesis

We hypothesized that N-fixation supported the N budget of miscanthus only after testing all other potential sources of biological N. Plant N generally is obtained from the soil ( $\text{NH}_4^+$  or  $\text{NO}_3^-$ ) or the atmosphere ( $\text{N}_2$ ). N can be recycled from dead plant tissue and reused in subsequent growing seasons, but this recycled plant N is greatly reduced by harvests that remove aboveground litter. Fertilizer is applied in agricultural systems to compensate for this loss but was not added to miscanthus simulated in this study. Plants also can conserve N by retranslocating it from leaf and stem tissues to the roots at the end of a growing season. There was substantial retranslocation of aboveground N to rhizomes between August and December (as measured by Heaton and others 2009), and this was included in the parameterization of the miscanthus submodel. N immobilized in the soil microbial community can be transformed and released to plants as  $\text{NO}_3^-$  or  $\text{NH}_4^+$ , but our simulations of miscanthus result in greater C allocated to soil microbes that responded by growing in numbers and incurring a greater N sink in the immobilized soil N pool. The addition of atmospheric N was  $0.6 \text{ g N m}^{-2} \text{ y}^{-1}$  (National Atmospheric Deposition Program), but this was offset by  $0.6 \text{ g N m}^{-2} \text{ y}^{-1}$  that leached from the soil (M. David unpublished results). After accounting for N retranslocation efficiency, N additions from atmospheric deposition, and root allocation to deep N pools, we determined that these were inadequate to meet miscanthus N requirements.

We conducted a sensitivity analysis of the effect of N added to soil (incrementally increasing N additions) through free-living associative N-fixation and through endophytic N-fixation where N

would be more immediately and directly available to plants. These differing N-fixation pathways resulted in different proportional N allocations to plant tissue, soil-mineralized N, and gaseous N volatilization. Based on DAYCENT simulations, we hypothesized that miscanthus hosts N-fixing organisms that may provide up to  $25 \text{ g N m}^{-2} \text{ y}^{-1}$  to meet annual plant demands for N.

## Model Verification

Crop growth simulations were verified against previously published measurements. We used plant production to evaluate how well the model simulated ecosystem dynamics for switchgrass crops (cv. Cave-in-Rock) in Nebraska and Iowa (Vogel and others 2002) and two Illinois sites where both switchgrass (cv. Cave-in-Rock) and miscanthus were grown (Heaton and others 2008). At the Iowa and Nebraska sites, switchgrass was grown using different fertilizer levels, providing a test of model accuracy under variable nutrient conditions. Switchgrass and miscanthus were grown side-by-side at the two Illinois sites without fertilizer additions, providing a test of model accuracy in simulating crop differences. Although the miscanthus submodel was calibrated against one site in Illinois (Champaign), the second site (in Dixon Springs) allowed an independent validation of the accuracy of the new miscanthus submodel. Detailed descriptions of these sites and management histories are provided in Heaton and others (2008). Observed crop yield data for corn–soybean rotation crops in Champaign, Illinois (David and others 2009) were compared with DAYCENT model results also. Corn–soybean was used only to verify model prediction against measurements. The model projections discussed in a subsequent section included only the corn parameterization to simulate continuous corn. Soil and climate characteristics for the plots in Nebraska, Iowa, and Illinois were based on locally appropriate data sets (publicly available from [www.ncdc.noaa.gov](http://www.ncdc.noaa.gov)). We did not have measurements of productivity of tallgrass prairie locally and relied solely on results from Pepper and others (2005).

To verify model performance, regression analysis was used to compare model predictions to annual biomass measurements of miscanthus, switchgrass, and corn–soybean rotation crops. The miscanthus and switchgrass model predictions also were compared to monthly aboveground plant C and N measured in trial plots at two locations (previously described) from 2004 through 2006. A two-way ANOVA was used to test differences between

modeled and measured estimates and whether differences varied by time through the growing season (SAS-JMP software).

## Model Projections

To compare GHG emissions among the four biofuel crops, we used local soil and climate data from Urbana, Illinois, to simulate growth dynamics under identical field conditions. We used daily weather data from the last 7 years (2002–2008) to construct climate files for projections of ecosystem dynamics of the four crops over the next 10 years (2009–2019). Continuous corn was simulated (using parameterization of the corn submodel from previously verified corn–soybean routines) in the 10 year projections to assess the relative GHG emissions associated with this crop if grown without soybean rotation. We assumed N-fixation supplied additional N to the miscanthus ecosystem. Because, we could not distinguish the proportion of this activity that was the result of endophytic or free-living soil bacteria, miscanthus growth was simulated twice. The first simulation of miscanthus growth assumed endophytic N-fixation was the primary source of N, and the second simulation assumed associative N-fixation in soil surrounding the plant was the primary source of N.

## Isolation of N-Fixing Bacteria

To test the hypothesis that miscanthus relies on N-fixation to meet its annual N requirement, roots and rhizomes were harvested from 4- to 5-year-old plots in Champaign, Illinois, in September 2006. Aboveground tissues of two plants from each plot were collected. Two soil cores with intact root and rhizome tissue were collected at the location of each plant sample. Rhizosphere soil was washed from rhizomes using 40-ml sterile deionized water, and collected in a sterile container for characterization of rhizosphere bacterial populations. Rhizosphere soil was used immediately to inoculate N-free semi-solid AcD agar (Burriss 1994) to enrich for potential N-fixing bacteria. To isolate endophytic bacteria, roots and rhizomes were surface sterilized by immersion in 1.3% sodium hypochlorite for 5 min, followed by a second wash in fresh 1.3% sodium hypochlorite for an additional 3 min. Roots and rhizomes were rinsed with five washes of sterile-distilled water and then crushed in distilled water using a sterile mortar and pestle (Chelius and Triplett 2000). An aliquot (50  $\mu\text{l}$ ) of the liquid portion of the homogenized roots and rhizomes was used to inoculate 10 ml of semi-solid N-free AcD medium (Burriss 1994). Cultures were trans-

ferred several times to fresh medium to enrich for diazotrophs before isolation and purification of individual colonies on AcD agar plates.

Miscanthus stems were surface-sterilized by dipping in ethanol, flamed briefly, then centrifuged to isolate the apoplastic fluid (Dong and others 1994). Bacteria in apoplastic fluid of stems were isolated by plating directly on N-free AcD plates.

DNA was extracted from 96 purified bacterial isolates using the FastDNA kit (MP Biomedicals, Solon, Ohio, USA) and screened for the presence of *nifH* (the gene that encodes dinitrogenase reductase) by PCR (Rosch and Bothe 2005). Phylogenetic assignments of potential diazotrophs (indicated by detection of *nifH*) were identified through sequencing of the 16S rRNA genes, and comparison with sequences in the Ribosomal Database Project using the Classifier function (Lane 1991).

## Acetylene Reduction

Bacterial isolates described above were stored in 50% glycerol after purification. Colonies of each of the bacterial isolates with the *nifH* gene were cultured in a test tube using two different N-free media, agar broth and semi-solid AcD medium, and then allowed to grow for 1 week. Each tube was then flushed with air, sealed with a rubber septum, and 10% of the head space was replaced with acetylene. After 60 h of incubation at room temperature, a 500- $\mu$ l sample of air was removed from the head space of each test tube with a syringe and injected into a gas chromatograph (Hewlett Packard; Palo Alto, California, USA) with a flame ionization detector and a GS-Alumina column (Agilent; Santa Clara, California, USA) to detect acetylene reduction to ethylene, which indicated nitrogenase activity.

Three replicate cultures of three genotypes of *Rhizobium leguminosarum* (legume symbionts with known nitrogenase activity) were included in the experimental design as positive controls, and negative controls included three replicate cultures each of *Escherichia coli* (which does not possess genes for nitrogenase), a *nifH*-positive bacteria grown in a high-N medium (which should de-activate nitrogenase), and a *nifH*-positive bacteria with no acetylene treatment. Along with the positive and negative controls, acetylene reduction assays were carried out for five of the *nifH*-positive isolates from March 31 to April 2, 2009 ( $n = 3$  for each bacterial strain) and then all seven *nifH*-positive isolates were assayed for acetylene reduction activity from April 13 to 16, 2009 ( $n = 6$  for each bacterial strain). Results from both dates were combined for

statistical analysis with  $n = 9$  for each type of culture. A one-way ANOVA (SAS-JMP software) was used to resolve differences among isolates and controls.

To test nitrogenase activity in situ, ten additional rhizomes of miscanthus were extracted from trial crops in Champaign, Illinois, in 2009 using a root corer and slide hammer. Soil and rhizome material that was extracted with each core was potted in an  $8 \times 8$  cm<sup>2</sup> pot, watered, and kept in a greenhouse for 5 days. Rhizomes were then gently removed from the soil, rinsed, and placed in 150-ml jars with 10 ml water to maintain moisture. The jars were flushed with air, sealed with septa, and 10% of the head space was replaced with acetylene. After 60 h of incubation in the dark, ethylene production was measured using the gas chromatograph described above.

Three rhizomes were collected from the field on May 23, and acetylene reduction assays were conducted from May 31 to June 2, 2009; seven additional rhizomes were collected on June 7 and assayed for nitrogenase activity from June 12 to 15, 2009. Three rhizomes were sealed in jars without acetylene to serve as negative controls. Although ethylene is produced in small amounts in plant tissue, the negative controls allowed us to account for baseline ethylene production. Results from both dates were combined in statistical analyses ( $n = 10$ ). A *t*-test was used to resolve differences in ethylene produced from rhizomes treated with and without acetylene.

## RESULTS

Nitrogen-fixation was confirmed in whole miscanthus rhizomes by measuring ethylene that was produced from all rhizomes treated with acetylene ( $t = 5.26$ ,  $P < 0.01$ ). No ethylene was detected in the negative controls. Seven bacterial strains isolated from miscanthus possessed the *nifH* gene. Of these *nifH*-positive strains, three were isolated from rhizome tissue, two were from stem tissue, one was from root tissue, and one was isolated from the rhizosphere. DNA sequencing of *nifH* and small subunit rRNA genes indicated that all seven *nifH*-positive isolates were closely related to Enterobacteriaceae species that have been identified in other grasses (Haahtela and others 1981; Chelius and Triplett 2000); members of this genus are not legume symbionts (Haahtela and others 1981; Chen and others 2003). Nitrogenase activity was detected in four of the seven *nifH*-positive bacterial strains grown in N-free culture after isolation from miscanthus; two of these had significantly greater

ethylene produced when acetylene was applied than the ethylene produced from negative controls ( $P < 0.05$ , Figure 2).

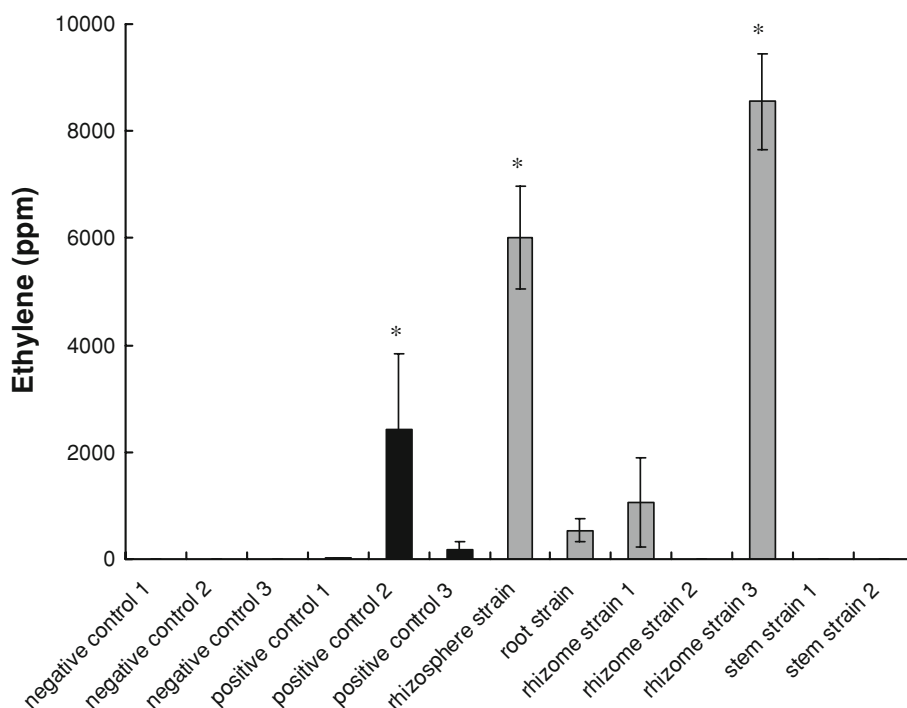
After DAYCENT was calibrated to include N-fixation in miscanthus, simulated biomass and aboveground C yields were similar to observed values (Figure 3A) measured in trial plots established in 2002 by Heaton and others (2008). DAYCENT projections of aboveground plant C in 2004 through 2006 were not significantly different from measurements in June, August, October, or December of those years (Figure 3A;  $F = 0.86$ ,  $P = 0.39$ ). Aboveground C yields of miscanthus were approximately 200% greater than C yields of switchgrass (Figure 3A).

The pool of aboveground N in miscanthus also was accurately simulated by DAYCENT with N-fixation included as a source of plant N (Figure 3B). Monthly estimates of plant N pools from model simulations were not significantly different from measurements ( $F = 0.55$ ,  $P = 0.47$ ). Modeled and measured seasonal dynamics were similar; both plant C and N pools changed throughout the growing season (C:  $F = 8.67$ ,  $P < 0.01$ ; N:  $F = 26.42$ ,  $P < 0.01$ ), and there was a significant

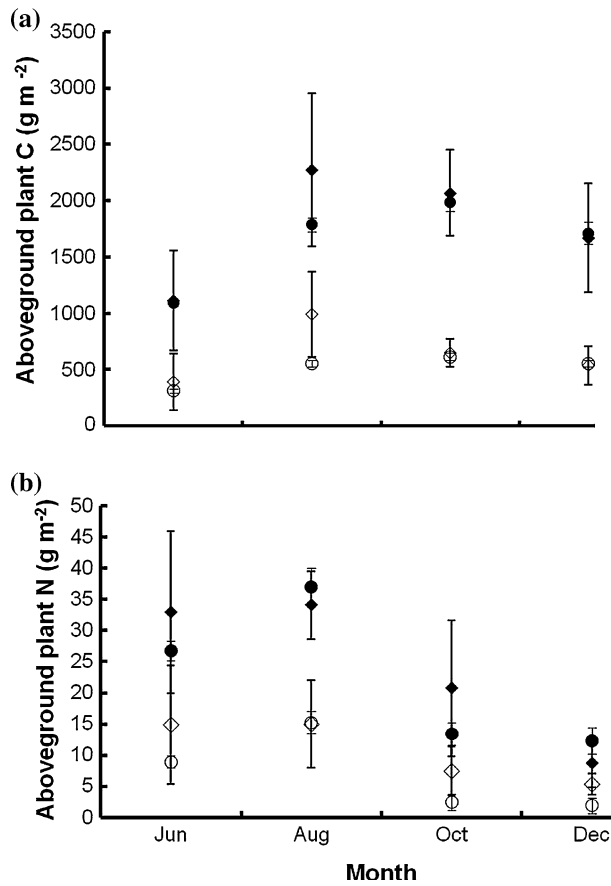
decrease in aboveground N between August and December that was indicative of high rates of N retranslocation in miscanthus. Switchgrass also had significant retranslocation of N between August and December ( $F = 10.8$ ,  $P < 0.01$ ), although changes in aboveground C ( $F = 5.82$ ,  $P < 0.01$ ) were more modest than for miscanthus.

DAYCENT accurately simulated biomass production for all crops (Figure 4). By adding our switchgrass simulations described above to those previously described for other crops in the region (Vogel and others 2002; Adler and others 2007; David and others 2009), we found the model accurately simulated aboveground biomass in corn-soybean rotation and switchgrass field trials across the Midwest ( $R^2 = 0.964$ , slope = 0.99,  $P < 0.01$ ). If N-fixation was assumed for miscanthus, then modeled versus measured estimates of biomass yields for switchgrass, corn, and miscanthus were similarly correlated ( $R^2 = 0.987$ , slope = 1.032,  $P < 0.01$ ) with a near fit to a 1:1 line (Figure 4).

We projected the standing aboveground C, soil C, and soil GHG fluxes ( $\text{CO}_2$ ,  $\text{CH}_4$ , and  $\text{N}_2\text{O}$ ) associated with prairie, switchgrass, miscanthus, and

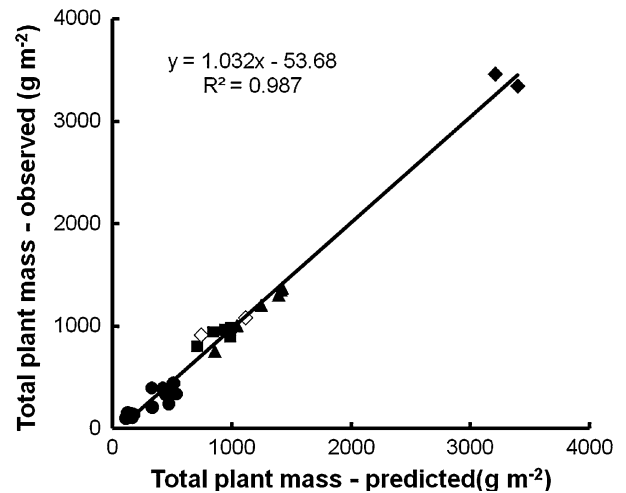


**Figure 2.** Ethylene (ppm) measured as a product of acetylene reduction by *nifH*-positive bacterial cultures isolated from miscanthus. X-axis labels indicate the location of origin of each bacterial strain in the plant. The three negative controls included were cultures of (1) *E. coli*, (2) a strain not treated with acetylene, and (3) a strain with high-N medium. Positive controls included three strains of *R. leguminosarum*: (1) bv. trifolii ANU794, (2) bv. viceae B518, and (3) bv. phaseoli 127K105a. Error bars represent standard error and asterisk indicates a significant difference from the negative control.



**Figure 3.** **A** Three-year averages of modeled (circles) and measured (diamond) aboveground standing plant C in switchgrass (open) and miscanthus (closed) grown in Champaign, Illinois. **B** Modeled (circles) and measured (diamond) plant N in switchgrass (open) and miscanthus (closed) grown in Champaign, Illinois. Measurements are based on data originally published in Heaton and others 2008 and Heaton and others 2009. Each symbol represents the mean value ( $\pm 1$  SD).

corn grown under identical conditions in Urbana, Illinois, over 10 years (Figures 5–7). Simulations revealed that switchgrass and native prairie had similar amounts of aboveground biomass available for harvest in December ( $344\text{--}705\text{ g C m}^{-2}$ ) when harvest is recommended for efficient baling. Aboveground corn yields (harvested in October) ranged from  $405$  to  $717\text{ g C m}^{-2}$ , whereas miscanthus aboveground C ranged from  $1606$  to  $2426\text{ g C m}^{-2}$  (Figure 5). Comparison of simulated levels of soil C for the biofuel crops indicated that miscanthus had the potential to accumulate soil C at a faster rate than other species (Figure 6). The greater soil C in miscanthus resulted from greater litterfall and belowground plant production ( $1041\text{--}1194\text{ g C m}^{-2}\text{ y}^{-1}$ ).



**Figure 4.** Predicted values from DAYCENT of total plant biomass at the end of the growing season (harvest time assigned according to harvest date for each crop and experiment) versus measured values for switchgrass, corn–soybean rotation, and miscanthus. Switchgrass observations were 1995 mean yields from six plots with different fertilization levels in Ames, Iowa (triangle) and Mead, Nebraska (square) (Vogel and others 2002), and 2004–2006 mean switchgrass yields (open diamond) from two sites with no fertilization in Champaign and Dixon Springs, Illinois (Heaton and others 2008); corn–soybean rotation (circle), included only for verification of model (the separate model projection simulated corn only), against observations of plot means over 16 years in Champaign, Illinois, with corn yield in odd years and soybean yield in even years (David and others 2009); and miscanthus (closed diamond) observations were mean yields from 2004 to 2006 in Champaign and Dixon Springs, Illinois (Heaton and others 2008).

Simulated GHG fluxes were converted to  $\text{CO}_{2\text{eq}}$  global warming potentials and included indirect  $\text{N}_2\text{O}$  emissions from soil  $\text{NO}_3^-$  and  $\text{NH}_4^+$  to be consistent with standards established by the Intergovernmental Panel on Climate Change (Forster and others 2007). Switchgrass, corn, and prairie were net sources of GHG ( $\text{CO}_2$ ,  $\text{CH}_4$ , and  $\text{N}_2\text{O}$ ) to the atmosphere, whereas miscanthus was a sink, removing GHG from the atmosphere (Figure 7). If miscanthus does indeed rely on N-fixation to supplement its N requirement, as our data suggested, the net GHG sink averaged over a 5-year period was  $350\text{--}428\text{ g CO}_{2\text{eq}}\text{ m}^{-2}\text{ y}^{-1}$  and was neither a source nor sink of GHG when averaged over a 10-year period (Figure 7). If associative N-fixation was the dominant process rather than endophytic N-fixation, then emissions of  $\text{N}_2\text{O}$  would be greater in miscanthus crops, but the overall GHG flux would still remain lower than that of other biofuel crop options (Figure 7). The corn simulation had the



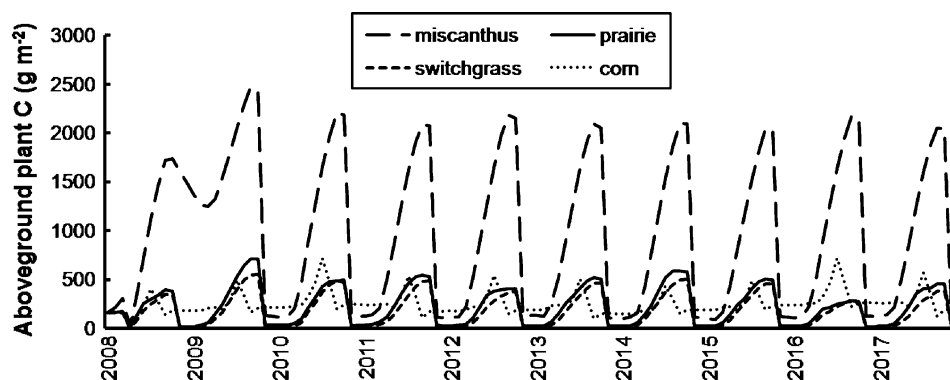


Figure 5. Projected standing aboveground C, a primary metric of interest for bioenergy feedstock producers, of four potential biofuel crops; miscanthus, switchgrass, prairie, and continuous corn, if grown in identical conditions in Urbana, Illinois, over the next 10 years. This projection assumes that the three perennial crops would be harvested in December, when biomass is dry enough for efficient harvest and baling, and that corn would be harvested in October.

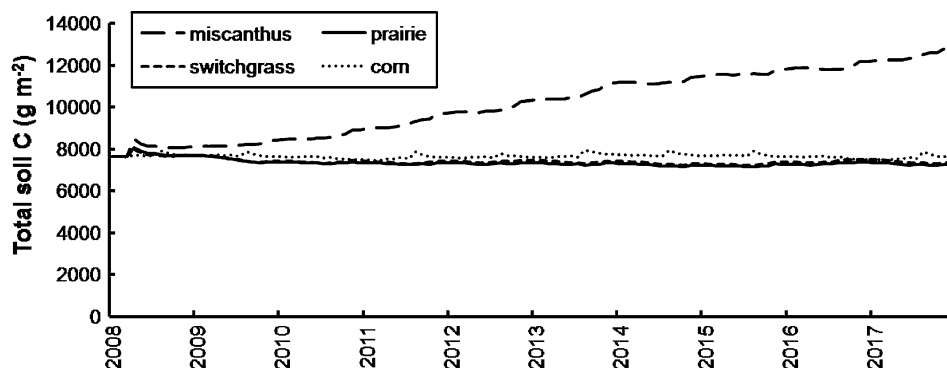


Figure 6. Projected soil C to a depth of 20 cm in four potential biofuel crops; miscanthus, switchgrass, prairie, and continuous corn, if grown in identical conditions in Urbana, Illinois, over the next 10 years.

highest  $N_2O$  fluxes as a result of the addition of fertilizers ( $\sim 19 \text{ g N m}^{-2} \text{ y}^{-1}$ ).

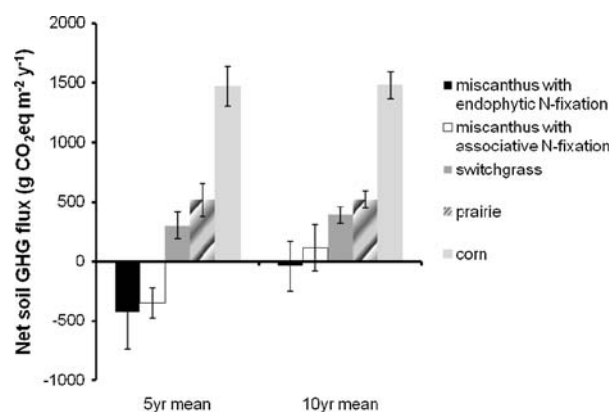
## DISCUSSION

Predictions of GHG emissions varied among the four potential biofuel crop agro-ecosystems. Although model simulations revealed that corn had slightly greater soil C than switchgrass and prairie (Figure 5), corn also had greater GHG emissions than these crops because of the addition of chemical fertilizers (Figure 7). Miscanthus had the lowest GHG emissions of the biofuel crops considered here and produced greater standing aboveground C and soil C than the other crops (Figures 5 and 6). The DAYCENT model successfully simulated biogeochemical dynamics of the different biofuel cropping systems in Illinois only if N-fixation was added to the N budget of miscanthus. The assumption that miscanthus hosts N-fixing bacteria was validated by results from acetylene reduction experiments that confirm nitrogenase (N-fixing) activity in miscanthus rhizomes and in *nifH*-positive bacteria isolated from miscanthus (Figure 2). The ability to host

N-fixing bacteria and to maintain high rates of aboveground biomass production suggests that miscanthus, at least when grown in Illinois, may be unique among biofuel feedstocks in its ability to mitigate GHG emissions and amend soil C.

Nitrogenase activity was variable in the positive controls as well as among experimental cultures of *nifH*-positive bacterial strains (Figure 2). Because there was no variability in ethylene produced among the negative controls, we are confident that the strains with significantly greater ethylene production ( $P < 0.05$ ) had active nitrogenase. However, the strains that did not have significantly greater ethylene production ( $P > 0.05$ ) may have active nitrogenase under conditions not simulated in the cultures. For example, the environment in the host plant may facilitate nitrogenase activity in some of the bacterial strains. All field-grown rhizomes had detectable ethylene production that may have been linked to nitrogenase activity in multiple strains of bacteria.

The amount of N-fixation we estimated for miscanthus was greater than rates reported for other herbaceous ecosystems (for example, Garten and



**Figure 7.** Projected net annual soil GHG flux in CO<sub>2</sub> equivalents (g CO<sub>2</sub>eq m<sup>-2</sup> y<sup>-1</sup>) for biofuel crops grown for 5- and 10-year periods: GHG flux included net CO<sub>2</sub>, direct, and indirect N<sub>2</sub>O, and CH<sub>4</sub> fluxes from the crop ecosystem. Estimates for miscanthus GHG fluxes were calculated two ways: with endophytic N-fixation and with associative N-fixation. Positive fluxes indicate a net source of GHG that would accumulate in the atmosphere and negative fluxes indicate a net sink of GHG that would be sequestered in the agro-ecosystem. Not included in the GHG fluxes was C in aboveground plant biomass that would be harvested or GHG emissions incurred throughout the production chain (farm machinery, conversion processes, use in automobiles, and so on). GHG fluxes represent means (±SE).

others 2008). The ability of miscanthus to support high rates of N-fixation may be explained by the greater efficiency with which miscanthus converts solar energy into biomass relative to other grass species (Dohleman and others 2009). Comparison of field measurements of switchgrass and miscanthus that were grown on the same soils without fertilizer indicated that C yield and plant N uptake for miscanthus were at least two times greater than for switchgrass (Heaton and others 2008, Figure 4). Although N-fixing bacteria may be present in switchgrass ecosystems, model results indicate they did not contribute substantially to the overall N budget. Switchgrass also has less energy (Dohleman and others 2009) to support N-fixation. Endophytic diazotrophs have been identified in corn (Palus and others 1996; Triplett 1996; Montañez and others 2009), but over-fertilization (Mulvaney and others 2005) dilutes any contribution of biological N-fixation to the corn N budget.

We present the first evidence of N-fixation in field-grown rhizomes to support the hypothesis that miscanthus relies on N-fixation to meet its annual N demand. Still, we must rely on modeled predictions of the amount of N fixed in miscanthus crops, a quantity that has proven difficult to mea-

sure in the field (for example, Sevilla and others 2001). High rates of N-fixation by free-living and endophytic bacteria have been documented in sugarcane (Boddey and others 2003; Baldani and Baldani 2005), a tropical relative of miscanthus, but these rates are highly variable (Sevilla and others 2001). Likewise, rates of N-fixation in miscanthus are likely to vary geographically and may contribute to variation in yields of miscanthus at locations outside Illinois. Potential N-fixing bacteria also were isolated from *Miscanthus sinensis*, one of the parental lines of miscanthus (Eckert and others 2001; Miyamoto and others 2004). Other studies found that miscanthus yields were maintained after repeated harvests that removed N (Beale and Long 1997; Christian and others 2008) and that miscanthus had little or no growth response to N-fertilization (Cosentino and others 2007; Danalatos and others 2007; Miguez and others 2008). Although N-fixation may vary geographically, these studies suggest that N-fixation is likely to be important for miscanthus grown in regions beyond Illinois.

GHG fluxes from a miscanthus agro-ecosystem varied with the source of N-fixation (associative or endophytic); the modeled simulation of associative N-fixation in miscanthus resulted in greater N<sub>2</sub>O emissions because of the addition of N to the pool of soil NH<sub>4</sub><sup>+</sup>, which then became susceptible to nitrification and denitrification. In contrast, if endophytic N-fixation was applied to the model, the only N required was the amount needed to meet the internal plant demand and thus N was less susceptible to transformations in the soil. This underscores the importance of quantifying N-fixing activity in miscanthus because this process can mediate the overall GHG budget of the crop and is likely to vary spatially. Also, the 5-year mean annual GHG reduction was greater than the mean GHG reduction after 10 years (Figure 7), suggesting that long-term experiments are essential to accurately estimate GHG fluxes in biofuel feedstocks.

GHG emissions in the prairie simulation were slightly greater than GHG emissions in switchgrass even though these crops had similar yields and soil C storage. A prairie community was comprised of different species, each with a different phenology, resulting in greater mortality and litter production throughout the growing season. Some N was volatilized from fresh litter and during decomposition, contributing to indirect N<sub>2</sub>O fluxes. The addition of small amounts of litter throughout the growing season also stimulates mineralization of N at the soil surface and leads to additional indirect N<sub>2</sub>O

emissions. In contrast, more of the litterfall in switchgrass would occur after the growing season, and most of the standing dead biomass would be harvested before falling to the ground and decomposing.

Aboveground plant C, which is directly correlated with feedstock yields, varied among the four bioenergy crops analyzed here. Miscanthus had the greatest aboveground C as a result of greater N acquisition, with up to four times the biomass of the other crops. Model predictions and initial field data suggested that growth of miscanthus will result in high aboveground biomass and root production that will lead to substantial increases in soil C (Figure 6). Switchgrass and prairie crops had similar aboveground C because of the similarity between the dominant grasses in the prairie community and switchgrass. Tilman and others (2006) found that switchgrass productivity was lower than productivity of a mixed prairie community (that included some N-fixers) if grown on nutrient-poor soils. Our results did not reflect this difference because our projections simulated relatively fertile soils found in Illinois, where switchgrass would not be as nutrient-limited as it would be on degraded marginal lands. Our prairie simulation included the assumption that there was a small number of N-fixing plants in the community, but the contribution of N to support aboveground biomass was offset by the phenology of different species that did not contribute to final standing biomass at the end of the year. The deeper rooting depths of both native prairie and switchgrass relative to corn should result in greater belowground C retention than would occur belowground in corn crops, but corn had greater stover remaining after harvests that contributed more of the aboveground C to soil C storage. The ratio of actual C available for harvest to C residue that accumulates in soil depends strongly on the agricultural practices that are used.

Soil C levels are sensitive to cultivation practices, as indicated by results from previous DAYCENT model simulations of agro-ecosystems in the USA (Del Grosso and others 2005; Adler and others 2007). Soil C accumulation in the corn crops would be greater if a lesser amount of the aboveground corn stover was removed for biofuel production (our simulation included 75% stover removal) or if minimum tillage practices were used to manage the soil (Robertson and others 2000; Del Grosso and others 2005; Adler and others 2007). Corn-soybean rotation agriculture would result in less soil C accumulation than continuous corn, but lower N leaching and GHG emissions from fertilizer additions. Model simulations revealed that replacing

the corn-soybean rotation with continuous corn resulted in a slight increase in soil C over the 10-year projection (Figure 6). Biomass production was two to three times greater for corn than soybean, contributing to greater inputs of C to the soil. Changes in soil C with miscanthus and tallgrass prairie cultivation as predicted by DAYCENT were consistent with results reported by Anderson-Teixeira and others (2009), but predicted changes in soil C of switchgrass crops were lower than those estimated by Anderson-Teixeira and others (2009). The cultivation practices of both switchgrass and corn vary, leading to wide variation in soil C estimates from multiple studies. Geographic variation in soil characteristics and climate also are likely to contribute to variation in soil C accumulation in biofuel crops (Davis and others unpublished). Our projections for soil C cannot be directly extrapolated across the surrounding region without some information about harvest intensity, fertilization rates, soil characteristics, and climate variations.

The results presented here illustrate the relative performance of different biofuel crops on fertile agricultural soils, and the performance of these crops on marginal degraded lands requires further investigation. It is worth noting, however, that N-fixation would be an important benefit for crops grown on nutrient-poor soils. Model syntheses of biogeochemical cycling in biofuel crop ecosystems indicated that large amounts of N were fixed to sustain the observed plant N uptake and soil C storage of miscanthus. Net GHG and N<sub>2</sub>O fluxes from soil depended on the pathway through which N was added to the miscanthus system (greater fluxes result from associative N-fixation relative to endophytic N-fixation). Our projections suggested that the unique N and C cycling of miscanthus crops could provide a greater GHG mitigation potential than other biofuel crops tested in this experiment. All of the perennial grass crops we analyzed would have lower GHG emissions than corn, which is the current biofuel crop in the Midwestern USA.

## ACKNOWLEDGEMENTS

This research was funded by the Energy Biosciences Institute and the Department of Plant Biology, University of Illinois, Urbana-Champaign, Illinois, USA.

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