Agriculture’s impact on microbial diversity and associated fluxes of carbon dioxide and methane

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Agriculture has marked impacts on the production of carbon dioxide (CO2) and consumption of methane (CH4) by microbial communities in upland soils—Earth’s largest biological sink for atmospheric CH4. To determine whether the diversity of microbes that catalyze the flux of these greenhouse gases is related to the magnitude and stability of these ecosystem-level processes, we conducted molecular surveys of CH4-oxidizing bacteria (methanotrophs) and total bacterial diversity across a range of land uses and measured the in situ flux of CH4 and CO2 at a site in the upper United States Midwest. Conversion of native lands to row-crop agriculture led to a sevenfold reduction in CH4 consumption and a proportionate decrease in methanotroph diversity. Sites with the greatest stability in CH4 consumption harbored the most methanotroph diversity. In fields abandoned from agriculture, the rate of CH4 consumption increased with time along with the diversity of methanotrophs. Conversely, estimates of total bacterial diversity in soil were not related to the rate or stability of CO2 emission. These combined results are consistent with the expectation that microbial diversity is a better predictor of the magnitude and stability of processes catalyzed by organisms with highly specialized metabolisms, like CH4 oxidation, as compared with processes driven by widely distributed metabolic processes, like CO2 production in heterotrophs. The data also suggest that managing lands to conserve or restore methanotroph diversity could mitigate the atmospheric concentrations of this potent greenhouse gas.

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Introduction

Carbon dioxide (CO2) and methane (CH4) are currently responsible for about 80% of the radiative forcing from long-lived greenhouse gases in Earth’s atmosphere (Forster et al., 2007). The atmospheric concentrations of both gases are higher than they have been over the past 650,000 years and continue to rise (IPCC, 2007; Rigby et al., 2008). Land-use change, especially deforestation and row-crop agriculture (Schlesinger and Andrews, 2000; Smith et al., 2000), has an important role in determining the rate of these fluxes and also changes the structure of microbial communities in soil (Borneman and Triplett, 1997; Smith et al., 2000; Zhou et al., 2008).

The linkage between altered trace gas emissions and microbial community structure in soil may be more than associative—contemporary models in ecology suggest a positive relationship between species diversity and the magnitude and stability of ecosystem processes catalyzed by those species (Tilman et al., 2001; Symstad et al., 2003; Fargione et al., 2007; Ives and Carpenter, 2007). Should this relationship also hold for the soil microbial communities responsible for trace gas production and consumption, opportunities may arise for mitigating emissions. For microbes in soil, however, the relationship between species diversity and the magnitude and stability of trace gas fluxes has rarely been tested. Although evidence to date suggests that such a relationship is unlikely for decomposition and other soil processes broadly catalyzed by many microbes (Schimel, 1995; Groffman and Bohlen, 1999), diversity may matter substantially for processes catalyzed by microbial groups more specialized and depauperate (Cavigelli and Robertson, 2000).

We tested the hypothesis that the magnitude and stability of CH4 consumption in well-drained soils, a process catalyzed by relatively few bacteria, is
positively correlated with the diversity of methanotrophs present. We also tested the corollary hypothesis that the magnitude and stability of CO$_2$ production, a process carried out by all heterotrophic bacteria in soil, is not related to the diversity of bacteria present.

We measured in situ fluxes of CO$_2$ and CH$_4$ and assessed microbial diversity across five different land-use intensities at the Kellogg Biological Station Long-Term Ecological Research (KBS LTER) site in southwest Michigan. KBS LTER, with replicated plots of the same soil series (Crum and Collins, 1995) that differ in land use, represents an ideal system for determining how land use impacts microbial communities and the greenhouse gas fluxes they control. Past studies have typically investigated either gas flux or the microbial community at analogous sites, but rarely has the function mediated by the microbes and the microbial community been investigated simultaneously across a land-use gradient. Our intent was not to unravel the root causes of variation in either community structure or greenhouse gas flux, but to show an association that, if directional, could suggest insights into long-term management options for the microbial community and their gas fluxes. To our knowledge, this is the first study to correlate methanotroph and bacterial diversity to both the stability and magnitude of the associated in situ greenhouse gas fluxes across a successional land-use gradient, and to use the results to gain insights into management options for abatement of atmospheric greenhouse gas increases.

**Materials and methods**

**Gas-flux measurements**

*In situ* rates of CH$_4$ consumption and CO$_2$ production were measured using closed-cover flux chambers (Robertson et al., 2000) across a gradient of land uses at the KBS LTER site located in Hickory Corners, Michigan. Land-use treatments range from conventional row-crop agriculture (AG) (wheat–corn–soybean rotation, chisel plowed, conventional fertilizer and pesticide inputs) to intact, late-successional deciduous forest never converted to agriculture (DF), as well as three types of replicated successional communities: an early successional deciduous forest never converted to agriculture (SF), a never-plowed never-planting community managed as an annually mowed grassland followed by limited inputs to new grassland (MG), and a mid-successional community abandoned in the 1980s (ES), and are well-drained Typic hapludalfs (fine or coarse loamy, mixed) (Crum and Collins, 1995)—but with land-use associated differences in net primary production, soil organic matter (Robertson et al., 2000), aggregation (Grandy and Robertson, 2007), and derived properties, such as moisture content and diffusion. Additional site information is available at [http://lter.kbs.msu.edu](http://lter.kbs.msu.edu). Gas-flux measurements were typically made twice monthly between March and December: detailed protocols, field logs and data are available at [http://lter.kbs.msu.edu/datasets](http://lter.kbs.msu.edu/datasets). Gas-flux data reported in this study are from 1993–2008 (KBS013-001), and soil moisture and soil temperature measures associated with the gas-flux data from 2003–2008 (KBS013_003 and KBS013_004) were downloaded on 2 March 2010. Data for treatments MG and SF are available from 1993–1997 and 2008. Averages incorporate all monthly measurements for the reported time period.

**Assessment of total bacterial diversity**

Five soil cores (2.5 x 10 cm) were collected and pooled from each of two replicate plots in December 2008 representing the five land uses. Plant litter and roots were removed by sieving (4 mm sieve), which also served to homogenize the cores. The composited samples were flash frozen in liquid N$_2$, transported to the laboratory on ice and stored at −80°C. In all, 5-g subsamples were ground in sterile sand and liquid N$_2$ with a mortar and pestle. Microbes were lysed enzymatically and chemically, followed by isolation of the released DNA using acetyltrimethylammonium bromide-based method (Zhou et al., 1996) and further purification on cesium chloride gradients (Sambrook and Russell, 2001). Sequence tags for the V6 region of the 16S rRNA-encoding gene were generated (Sogin et al., 2006) and sequenced using Life Science’s 454 GS-FLX pyrosequencing chemistry (Huse et al., 2008) at the Woods Hole Marine Biological Laboratory Bay Paul Center. An average of 15 800 sequence tags were generated for each sample. Membership in an operational taxonomic unit (OTU) was defined as sequences sharing 97% nucleotide identity using the VAMPS MBL Bay Paul Center pipeline (Huse et al., 2008).

**Methanotroph census**

The diversity of methanotrophs across the KBS LTER landscapes was assessed via PCR amplification of the β-subunit of particulate methane mono-xygenase (pmoA). Template DNA for the amplification reactions was purified with the MoBio (Carlsbad, CA, USA) PowerSoil DNA Isolation Kit from 0.25 g subsamples from each of 75 soil cores collected in June 2006: 5 cores from each of 3 replicates of the 5 land-use treatments. pmoA was amplified from 45 ng of template DNA with primers A189 (5′-GGNGACTGGGACCTTCTGG-3′) and A682 (5′-GAACGCNGAGAAGAAASGC-3′; labeled with 6-carboxyfluorescein) (Holmes et al., 1993). The products of technical replicates of PCR reactions were pooled to minimize amplification bias, then purified and
digested sequentially with three restriction endonucleases for terminal restriction fragment length polymorphisms analysis. See Supplementary Information for detailed protocols.

Clone libraries of pmoA amplicons were created from a selection of soil samples and amplification conditions (Supplementary Table S1) to provide a molecular phylogeny of methanotrophs. Clone libraries were sampled until rarefaction curves were asymptotic (Supplementary Figure S5). Sequences from clone libraries were binned using DOTUR (Schloss and Handelsman, 2005), if they shared 94% average nucleotide sequence similarity—a species-level designation for functional genes (Konstantinidis et al., 2006). Representative pmoA sequences were deposited in GenBank under accession numbers FJ529724–FJ529808, and GQ219582 and GQ219583.

**Statistical analysis**
The effect of land use on CH$_4$ consumption and methanotroph richness was explored through $t$-tests, and a one-way multivariate analysis of variance (SAS Institute Inc., Cary, NC, USA). Linear regressions were used to relate mean greenhouse gas flux and species diversity, as well as the recovery of mean CH$_4$ consumption and mean methanotroph diversity following cessation of agriculture. Multiple regression analysis was used to assess the relative contribution of temperature and soil moisture to gas flux. The Sørenson index was calculated for each pairwise comparison of methanotroph communities in KBS LTER treatments and plotted using two-dimensional non-metric multidimensional scaling (Hammer et al., 2001). A one-way analysis of similarities was used to assess the significance of differences between communities (Hammer et al., 2001).

**Results**

**Greenhouse gas flux and abiotic regulators**
Rates of CH$_4$ consumption varied significantly with land use: the highest average annual rate of consumption was measured in the deciduous forest. Mid-successional fields, either abandoned from agriculture 50 years ago (SF) or never-tilled sites managed as grasslands (MG), consumed CH$_4$ at a significantly decreased rate as compared with the forest (DF), but significantly higher than early successional plots (ES) or agricultural plots (AG) ($t$-tests, $P<0.01$). The lowest rates of CH$_4$ consumption were consistently recorded in plots currently managed for row-crop agriculture (Figure 1a). There was approximately a sevenfold difference between the average annual rate of CH$_4$ consumption in the deciduous forest and the conventional agriculture plots (7.93 and 1.02 g CH$_4$-C ha$^{-1}$ day$^{-1}$, respectively).

CO$_2$ flux from soil also varied with land use. Soils in the early and mid-successional sites emitted 80% more CO$_2$ than the agricultural site, whereas the rate in the deciduous forest was 18% higher than the agricultural site. The highest rates of CO$_2$ emission and CH$_4$ consumption were recorded during the summer months for all treatments (Supplementary Figure S1); seasonal flux patterns were similar for all land uses. Soil temperature and moisture, two master regulators of biological processes in soil, explained portions of both fluxes, but were 10 times more effective in explaining the efflux of CO$_2$ ($r^2 = 0.12$, $P<0.001$) than rates of CH$_4$ consumption ($r^2 = 0.01$, $P = 0.06$).

**Methanotroph diversity and CH$_4$ consumption**
To relate methanotroph diversity to annual averages of CH$_4$ consumption across the gradient of land uses at the KBS LTER, a method employing terminal restriction fragment length polymorphisms was developed to capture all methanotrophs present,
rather than methods that would assess metabolically
active populations of methanotrophs at a particular
time. The pmoA gene was targeted for this assay
because it encodes a subunit of particulate methane
monooxygenase—the first enzyme in the pathway of
CH$_4$ oxidation and the defining enzyme of aerobic
methanotrophs, except for *Methylocella* species
(McDonald et al., 2008; Rahman et al., 2010). The
terminal restriction fragment length polymorphism
assay distinguished 11 OTUs that were confirmed as
*pmoA* gene fragments both through sequence
analysis and comigration of terminal restriction
fragments with those from cloned controls. These
11 OTUs encompassed all of the major clades of
methanotrophs defined by the *pmoA* gene
sequences from clone libraries from KBS LTER
(Supplementary Figure S2).

Methanotroph diversity, like the rates of CH$_4$
consumption, also varied significantly with land
use ($P<0.05$; analysis of variance) (Supplementary
Table S2B). The fewest methanotroph OTUs and
lowest rates of CH$_4$ consumption were found in
the agricultural plots, whereas the deciduous forest
harbored the greatest number of methanotroph
OTUs and the highest rates of CH$_4$ consumption.
Modeling treatment effects on rates of CH$_4$
consumption while excluding methanotroph diversity
as a covariate yielded reduced experimental error
and indicated that methanotroph richness influences
the rate of CH$_4$ consumption (Supplementary
Table S2C).

There was a strong positive correlation between
average annual rates of CH$_4$ consumption and the
number of *pmoA* OTUs across the KBS LTER
landscapes ($r^2 = 0.52$, $P<0.004$) (Figure 2a). Regression
of average rates of CH$_4$ consumption from 1993–
2008 versus *pmoA* diversity yields a nearly identical
relationship ($r^2 = 0.55$, $P = 0.002$), as does summer
rates of CH$_4$ consumption ($r^2 = 0.52$, $P = 0.003$).

To further explore methanotroph diversity and
search for new *pmoA* genes, 12 *pmoA* clone libraries
were created from DF and AG samples with
additional amplification conditions (Supplementary
Table S1, SI text). All libraries were sampled until
rarefaction curves were asymptotic (Supplementary
Figure S5), in an effort to capture the full sequence
diversity in each library. Although altered ampli-
fication conditions captured some additional *pmoA*
genomes, diversity was always greater in the forested
plots as compared with the agricultural treatment
(Supplementary Figure S3). Clone libraries revealed
a new clade of methanotrophs, KBS1 (Supplemen-
tary Figures S2, S3), in addition to those previously
identified in soils (Horz et al., 2005; Knief et al.,
2005; Lau et al., 2007). Using a species definition of
94% identity in functional genes (Konstantinidis
et al., 2006), 24 methanotrophic species were
identified in cumulative libraries from the forested
plots, but only 7 from the agricultural plots. As has
been described previously, some subunits from
ammonia monooxygenase genes (*amoA*) also
amplified with the *pmoA* primers (Holmes et al.,
1995). The *amoA* clones were recognized by the
presence of diagnostic amino acids (Horz et al., 2005;
Stoecker et al., 2006) and their clustering in phyloge-
netic trees (Supplementary Figure S3). They were
excluded from methanotroph diversity estimates.

**Bacterial diversity and CO$_2$ flux**

We estimated bacterial diversity through analysis of
454 pyrosequencing of the V6 region of the 16S
rRNA encoding gene (Sogin et al., 2006) and
clustering reads at 97% identity (Huse et al.,
2007). An average of 15,800 sequence tags was
analyzed for each of the replicated samples.
Rarefaction curves (Supplementary Figure S6) sug-
gest that we did not capture the full sequence
diversity, and so we used the Chao 1 estimator of
richness (Chao, 1984) to compare diversity. To control for unequal sequencing depths, we estimated richness in each sample at 6600 sequences, the Chao 1 estimate from our lowest sequenced sample. There was no obvious relationship between CO2 production and estimated bacterial diversity (P = 0.522 for linear regression, Figure 2b).

Influence of diversity on stability
To assess variability in the flux of CO2 and CH4 across treatments, we compared the coefficient of variance in the annual fluxes for each land management practice in each year between 2006 and 2008, the years for which molecular data was collected. The variance in CH4 was negatively correlated with methanotroph diversity (P < 0.001): sites with the highest richness had the lowest variance (Figure 3a). Conversely, variation of CO2 flux was not correlated with overall bacterial diversity (Figure 3b).

Recovery from agriculture
Given the relationship between methanotroph diversity and CH4 consumption, changes in the membership of the methanotroph community were assessed following the release of lands from row-crop agriculture. The community of methanotrophs differed following the abandonment of agricultural practices (P < 0.005, analysis of similarity), and soils began to recover in terms of CH4 consumption and methanotroph diversity (Figure 4). The methanotroph species present in the mid-successional plots began to overlap with those in fields that have not been used for agriculture (MG and DF) (Supplementary Figure S4).

There was a linear trajectory for the recovery of both CH4 consumption (r2 = 0.56, P < 0.001) and methanotroph richness (r2 = 0.99, P = 0.020) over time with the slopes of these lines nearly parallel. Extrapolation of the linear relationships suggests that approximately 80 years would be required for full restoration of CH4 consumption and methanotroph diversity to the levels of the current deciduous forest (Figure 4).

Discussion
Species richness is an integral component of community ecology, with the richness of plant species linked to productivity (Tilman et al., 2001) and greater temporal stability in plant production
Our study establishes a definitive correlation between methanotroph richness and CH$_4$ consumption across a successional gradient of landscapes from row-crop agriculture to deciduous forest.

Changes in methanotroph diversity and CH$_4$ consumption along this gradient highlight the strong and long-lasting impact of agricultural management. On the basis of recovery of CH$_4$ consumption following abandonment from agriculture, we project that it will require approximately 80 years for methanotroph diversity and CH$_4$ consumption to return to that of a native deciduous forest (Figure 4b). This slow recovery is consistent with worldwide observations that suggest a recovery period of 100 years for CH$_4$ consumption (Smith et al., 2000).

In contrast, we found no observable relationship between bacterial diversity in soil and large differences in the production of CO$_2$ (Figure 2b). Given the extensive microbial diversity in soils and the ubiquitous distribution of CO$_2$-yielding metabolic pathways in heterotrophs, it is perhaps not surprising that there was no discernible relationship between diversity and soil respiration. The lack of a general relationship between bacterial diversity and soil respiration has been documented for other soil types as well (Balser and Firestone, 2005; Wertz et al., 2006). Rather than being related to bacterial diversity, we found that CO$_2$ production from soil was more strongly predicted by soil moisture and temperature—two well-known regulators of soil metabolism (Cook and Orchard, 2008). Respiration of fungi, soil arthropods and plant roots also respond to these master regulators, making it difficult to disentangle the relative contribution from members of this complex community of organisms, but at the KBS LTER, bacterial diversity is not one of the driving factors.

Variability in both CH$_4$ consumption and CO$_2$ production are also consistent with the postulated model (Figure 5). Variation in CH$_4$ consumption was highest in agricultural plots that harbored the lowest diversity, whereas plots with the highest diversity were the most stable. We suggest that increased stability arises from the presence of complementary niches of methanotrophs. As environments vary temporally or spatially across a landscape, the presence of a more diverse assortment of methanotrophs that are able to oxidize CH$_4$ under a broader set of environmental conditions provides for more consistent CH$_4$ consumption. We also recognize that some variability may be contributed by technical limitations associated with the measurement of low rates of CH$_4$ consumption. Variability in CO$_2$ flux was smaller than for CH$_4$ flux, and there was no obvious relationship to bacterial diversity. As predicted by our model, once there is sufficient redundancy in a metabolic process, the gain or loss of species is not expected to influence the overall rate or stability of the process catalyzed by that metabolic group of microbes. It is noteworthy that both aspects of the model are captured in the same

Figure 5 Conceptual model of the relationships between the number of species present in an environment that catalyze a particular ecosystem process (richness) and the magnitude and variability (1 per stability) of that process. Processes catalyzed by microbes with specialized metabolisms that are mediated by a few species are proposed to occur in the shaded area.

(Tilman et al., 2006) across many landscapes (Wacker et al., 2009). Although the mechanisms underling these relationships for macroorganisms are vigorously debated and investigated, microbiologists are just beginning to ask whether functionality, stability and diversity are correlated in microbial communities (Konopka, 2009).

We investigated the relationship between microbial diversity and two ecosystem-level processes that are catalyzed by soil microbes—the consumption of CH$_4$ and the production of CO$_2$. On the basis of ecological models and considerations of biodiversity and microbial metabolism in soil (Schimel, 1995; Groffman and Bohlen, 1999; Zak et al., 2003), we hypothesized that the relationship between microbial diversity and the magnitude and stability of gas fluxes would depend on the total number of species that are present to catalyze each process (Figure 5). If this model is true, the rate and stability of CH$_4$ consumption, which is a specialized metabolic process limited to relatively few species, would be positively correlated with methanotroph richness, whereas CO$_2$ production, the result of general metabolic pathways shared by thousands of heterotrophs, would not be related to bacterial diversity.

As postulated, there was a positive relationship between rates of CH$_4$ consumption and the diversity of methanotrophs across a range of land uses at the KBS LTER (Figure 2a). The observed changes in methanotroph richness are consistent with other studies where methanotroph diversity increased in reforested farmland (Knief et al., 2005; Dorr et al., 2010) and reclaimed pasture lands (Zhou et al., 2008), and where methanotroph richness was linked to CH$_4$ consumption in other sites (Lin et al., 2005; Singh et al., 2007; Degelmann et al., 2010).
sample: a broadly distributed process not linked to ecosystem function and a specialized, narrowly distributed process linked to the magnitude and stability of a process. This suggests that efforts to mediate the impact of agricultural practices on greenhouse gas flux is best focused on organisms and factors contributing to narrowly distributed processes.

Two hypotheses have been advanced to explain the underlying basis of the relationship between ecosystem-level processes and diversity: the magnitude of a process can be the result of the combined activities of species present (complementarity hypothesis), or the magnitude can be the result of the presence of one or a few particularly productive species more likely to be present when diversity is high (sampling hypothesis) (Loreau and Hector, 2001; Fargione et al. 2007). Distinguishing between these competing hypotheses would determine whether all methanotrophs or only select species need to be conserved or restored in order to stimulate CH4 consumption where it has been attenuated by change in land use. If cumulative diversity were of primary importance, as suggested in the complementarity hypothesis, strategies that stimulate the growth of all methanotrophs would be most effective. If, on the other hand, certain high-activity species are the primary drivers of CH4 consumption, as suggested by the selection hypothesis, then strategies should be directed towards the establishment of those specific methanotrophs. The concurrent recovery of CH4 oxidation and methanotroph diversity following the abandonment from row-crop agriculture (Figure 4) and the successive accumulation of methanotroph species in addition to those present in the agricultural site (Supplementary Figures S3, S4, Supplementary Table S1) is consistent with the complementarity of methanotrophs leading to increased functionality. Continued monitoring of the recovery of both methanotrophs and CH4 consumption, coupled with functional assays to determine which methanotroph populations are most active throughout the year will provide a rigorous test of these alternative hypotheses.

The slow recovery of CH4 consumption following the abandonment of agricultural management practices suggests that many dimensions of methanotroph niches are disrupted by row-crop agriculture. Recovery of the diversity of methanotroph communities has been observed in sites where plant diversity was increased (Knief et al., 2005; King and Nanba, 2008; Zhou et al., 2008; Degelmann et al., 2010; Dorr et al., 2010), perhaps due to the differential production of monoterpenes (Maurer et al., 2008; Degelmann et al., 2010), suggesting a role for plant diversity in shaping methanotroph communities. Additionally, nitrogen fertilization is known to change the structure of methanotroph communities (Lau et al., 2007; Maxfield et al., 2008), most likely through competitive inhibition of methane monooxygenase by ammonia (Gulledge and Schimel, 1998), although it can stimulate CH4 consumption under conditions where fixed nitrogen is limiting methanotroph metabolism (Bodelier and Laanbroek, 2004). At KBS LTER, ammonia has been shown to cause acute short-term reductions in CH4 consumption rates in DF and SF soils (Suwanwaree and Robertson, 2005). Within the land-use gradient at KBS LTER, differences in gas diffusivity (von Fischer et al., 2009), the disruption of soil aggregates by tillage (Grandy and Robertson, 2007), nitrogen fertilization, as well as documented drivers of microbial diversity (including pH and salinity (Fierer and Jackson, 2006)), are additional factors that might be influencing methanotroph diversity and rates of CH4 consumption. Identifying parameters that influence methanotroph diversity, including the influence of soil type and climate across sites, will be of vital importance if we are to manage lands to conserve or restore methanotroph diversity and enhance the capacity of soil to serve as a stable sink for atmospheric CH4.

Conclusions

One of the most important drivers of species loss in terrestrial ecosystems worldwide is simplification of ecosystem structure that results from intensified land use (Sala et al., 2000). We document a decrease in both methanotroph diversity and CH4 consumption in soils managed for row-crop agriculture compared with native deciduous forests and never-tilled soils managed as grasslands. There was not a similar relationship between soil respiration and bacterial richness, consistent with the prediction that microbial diversity is more likely to be important in the case of specialized metabolic processes rather than broadly distributed types of metabolism and that specialized processes are better targets for microbial mediation. The concomitant recovery of methanotroph diversity and CH4 consumption suggests that all methanotroph species contribute to CH4 consumption as suggested by the complementarity hypothesis. Identifying parameters that impact methanotroph diversity will inform decisions about land management that have the potential to influence the flux of this potent greenhouse gas.

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