

DISSERTATION

SOIL HETEROGENEITY IN AGRICULTURAL AND NATURAL ECOSYSTEMS:  
RELATIONSHIPS BETWEEN ANAEROBIC ACTIVITY, ORGANIC MATTER,  
NUTRIENTS, AND GREENHOUSE GASES

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## ABSTRACT

### SOIL HETEROGENEITY IN AGRICULTURAL AND NATURAL ECOSYSTEMS: RELATIONSHIPS BETWEEN ANAEROBIC ACTIVITY, ORGANIC MATTER, NUTRIENTS, AND GREENHOUSE GASES

Many soil biogeochemical processes are difficult to predict, in part, due to the spatial heterogeneity of physical, chemical, and biological components of soil. Understanding how heterogeneity forms and affects biogeochemical processes is important because of the ultimate impacts on nutrient availability, carbon storage, and climate change. Oxygen and soil organic matter are two key components of soil microbial habitat, so I performed research to determine how the heterogeneity of each affect ecosystem functions.

Oxygen can be absent in soil aggregates, litter patches, rhizospheres, and the guts of soil fauna, and when this occurs in unsaturated soils with oxic pore air these areas are referred to as anoxic microsites. The formation, persistence and impact of anoxic microsites are poorly characterized because these microsites are difficult to measure, especially across large areas that define ecosystem level processes. I studied what factors cause them to form and persist and how they affect C and N cycling and GHG fluxes.

I performed focused, mechanistic laboratory studies of natural and agricultural soils, as well as field-scale studies of anoxic microsite effects in agricultural systems. In multiple studies, I circumvented the limitations and problems related to measuring soil oxygen or reduction-oxidation (redox) potentials at sub-millimeter scales instead by using gross CH<sub>4</sub> production as an indicator of anoxic microsite presence and activity. I used two relatively recent methodological approaches to make gross CH<sub>4</sub> measurements, CH<sub>4</sub> stable isotope pool dilution for laboratory measurements and a

CH<sub>4</sub> process and transport model for field studies. I found that methanogenesis correlated with respiration, soil moisture, plant presence, and agricultural practice both in laboratory and field studies, indicating that the distribution of anoxic microsites is altered by climatic and land use factors in ways that are similar to the large-scale anoxic zones of wetlands. Methanogenesis was associated with elevated NH<sub>4</sub><sup>+</sup> concentrations and N<sub>2</sub>O flux, but lower NO<sub>3</sub><sup>-</sup> concentrations. These relationships are consistent with slower nitrification and greater denitrification, so measurements of methanogenesis may be a useful proxy for other anaerobic processes. I also found evidence that consistent upland methanogenesis may stimulate methanotrophy (i.e., gross CH<sub>4</sub> consumption) over the course of years, counterintuitively leading to an increase in net CH<sub>4</sub> uptake. Finally, redox potential was not as strong an indicator of methanogenesis as expected, so I join others in concluding that redox potential may not be a desirable method for quantifying anoxic microsites.

I also studied the effects of the spatial distribution of soil organic matter in the form of litter patches in soil. In a laboratory incubation, I manipulated the size and number of litter patches and soil moisture in a uniform mineral soil matrix. I found that dry soils with litter that was aggregated into larger patches exhibited greater rates of decomposition and nutrient availability, but that in wetter soils there were few effects of litter distribution. This complements my studies of anoxic microsites by showing that not only the presence of soil microsites, but variation in their size and distribution can also alter soil processes.

In summary, my dissertation research concentrated on the causes and biogeochemical consequences of anoxic microsites and heterogeneity of organic matter in agricultural and natural ecosystems. My findings have increased our understanding of soil heterogeneity and the potential for it to cause significant changes in nutrient availability, decomposition, and greenhouse gas fluxes.

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## DEDICATION

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## LIST OF KEYWORDS

CH<sub>4</sub> biogeochemistry, soil anoxic microsites, methanogenesis, methanotrophy, isotope pool dilution, agricultural greenhouse gas inventory, tillage, nutrient cycling, anaerobic soil microbiology, plant litter decomposition, resource heterogeneity

## 1 – Introduction

Soils are the foundation of all terrestrial ecosystems and human society depends on them in innumerable ways. Soils produce and store plant nutrients (Paul, 2014), buffer atmospheric greenhouse gases (Smith et al., 2003), and house billions of animals and arthropods, but it is the soil microbes that are responsible for most soil processes (Chorover et al., 2007; Chapin et al., 2011). Civilization would not exist without the food and clean water produced by our soils, however despite their importance our ability to predict soil processes remains limited (Conant et al., 2011; Butterbach-Bahl et al., 2013; Cotrufo et al., 2013).

Humans have sought to understand soil processes for millennia with the goals of having a consistent food supply, avoiding the loss of property, and simply understanding the natural world (Virgil 29 BCE; Liebig, 1840). However, soil presents multiple challenges to a researcher: it is opaque, it is structured, and it contains incredible heterogeneity at scales ranging from nanometers to kilometers (Ettema and Wardle, 2002; Lehmann et al., 2007; Paul, 2014). It has become clear that understanding that heterogeneity is critical for predicting the processes that ecosystems and society rely on (Ettema and Wardle, 2002; Totsche et al., 2010; Ebrahimi and Or, 2015).

Soil is physically composed of mineral particles that are bound by organic and inorganic compounds into aggregates and clods (Oades, 1993). Soil pore structure forms around and within aggregates, resulting in a complex web of connectivity (Currie, 1965; Jasinska et al., 2006; Ebrahimi and Or, 2015). Organic matter is deposited in soils through plant litter fall, rhizosphere exudates and root death, and translocation of material and compounds by soil fauna and fungi (Chapin et al., 2011). Organic matter is then cycled, sometimes for thousands of years (Marschner et al., 2008), through interactions with microbes and soil particles (Dungait et al., 2012; Cotrufo et al., 2015). Beyond organic matter, pH (Rudolph et al., 2013), nitrogen (Schimel and Bennett, 2004; Xi et al.,

2014), and oxygen (Sexstone et al., 1985; Zhu et al., 2014; Liptzin and Silver, 2015) can all vary at small scales. Microbial communities form within the spatially complex habitat, adding another layer of heterogeneity to the physicochemical structures (Young and Crawford, 2004; Ruamps et al., 2011). Soil microbes are in a constant balancing act, maximizing access to optimal electron donors and acceptors while minimizing risk of predation and physiological stress (Fenchel and Finlay, 2008; Paul, 2014). This, together with the soil physico-chemical structure, results in soil processes that are affected by soils' complex spatial structure (Ettema and Wardle, 2002; Mangalassery et al., 2013).

Due to our growing human population, we demand more from soil than ever before, but the immense variability of soils (Jenny, 1941) makes the task of effectively managing or improving soil function very difficult without understanding the fundamental controls. Empirical relationships of bulk soil properties and soil processes have been developed and incorporated into process based models to predict carbon storage (Parton et al., 1998), nutrient availability (Stehfest et al., 2007), and greenhouse gas fluxes (Li, 2000; Del Grosso et al., 2000). However, these have limited accuracy (Wang and Chen, 2012), indicating models of a complex system like soil may require a more mechanistic model to capture interactions and non-linearities adequately (Cotrufo et al., 2013; Wieder et al., 2014). Improved understanding of relationships between soil heterogeneity and processes would provide the information needed to build mechanistic models and advance our ability to manage soils sustainably.

Modern studies of soil heterogeneity have been driven by research in soil carbon and nitrogen cycling (Jansson and Clark, 1952; Six et al., 2004), and more recently we have learned that many other soil processes can be affected by heterogeneity. Soil organic matter is heterogeneously distributed in soils (Six et al., 2000; Totsche et al., 2010) and the demand for oxygen by microbes consuming it can create anoxic microsites across a soil (Sexstone et al., 1985; Zhu et al., 2014; Keiluweit et al., 2016). The availability of oxygen and other terminal electron acceptors alters the

availability and retention of plant nutrients (Chacon et al., 2006; Giles et al., 2012; Husson, 2012), greenhouse gas production and consumption (Smith, 1980; Parkin, 1987; Hojberg et al., 2004; von Fischer et al., 2007; Kammann et al., 2009; Zhu et al., 2014), and heavy metal mobility (Tokunaga, 2010; Kausch and Pallud, 2013; Ying et al., 2013). Heterogeneous soil properties can ultimately affect crop productivity (Loecke and Robertson, 2009b), water quality (Ying et al., 2013), and soil biodiversity (Nielsen et al., 2010; Beare et al., 1992).

Performing soil research in an agricultural setting has many benefits, notably, 1) soil structure can be partially controlled and consistency of heterogeneous distributions increased within study units through typical management operations, and, 2) findings in this setting can be directly applied to the 1.5 billion hectares of cropland worldwide (FAO, 2011). Greater consistency among samples and plots decreases the variability of processes, making it more likely researchers will detect meaningful relationships. Diverse agricultural practices provide an array of treatments that can be leveraged for studies of processes important in both agricultural and natural soils. In part because agricultural soils dominate many landscapes, choices regarding their management have the potential to decrease atmospheric greenhouse gas concentrations (Smith et al., 2008; Lal et al., 2003) and alleviate eutrophication of waterways and marine ecosystems (Elliot and Coleman, 1988; Sebilo et al., 2013; Rabotyagov et al., 2010). This ability to impact major environmental problems through agriculture soil management provides a direct link from research in these soils to major societal benefits.

Here I describe work that addresses relationships between heterogeneous soil properties and microbial activity in agricultural and unmanaged soils. In two experiments I use methanogenesis as an indicator of anoxia that can be measured non-destructively to characterize how heterogeneity in soil oxygen is caused and how it affects other biogeochemical cycles. In the third experiment I investigate the decomposition and nutrient cycling effects of differing the spatial distribution of

plant litter across moistures. I also explore the implications of my findings for natural and agricultural soils and their uses.

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## 2 - Mechanisms underlying tillage effects on the soil CH<sub>4</sub> sink in semi-arid agroecosystems

### **2.1 - Introduction**

Soils hold great potential for mitigating global warming with recent estimates showing that appropriate management could decrease annual greenhouse gas emissions by 15% (Paustian et al., 2016), much of which would occur through decreasing cropland GHG fluxes to the atmosphere (Smith et al., 2008) and increasing soil carbon (C) content (Lal, 2004). Predicting C content and CO<sub>2</sub> fluxes of agricultural soils is becoming more tractable and these metrics are now common elements of climate models, however CH<sub>4</sub> and N<sub>2</sub>O, which have 34 times and 298 times the warming potential of CO<sub>2</sub> over a 100 year timespan, respectively (IPCC 2014), exhibit high variability that is difficult to explain over the wide ranges of soil types, climates, crops, and agronomic practices. The variation of surface CH<sub>4</sub> and N<sub>2</sub>O fluxes may be difficult to predict, in part, because they are the result of multiple subsurface fluxes that include both gross production and gross consumption. Developing our knowledge of the drivers of gross fluxes offers great potential for improving surface flux predictions, but gross fluxes are rarely measured.

Two common agricultural practices, no-till crop production (NT) and fallow rotations, can increase surface CH<sub>4</sub> uptake compared to conventionally tilled (CT) and planted fields, but despite the many studies of net surface CH<sub>4</sub> fluxes from these treatments it is unclear what drives the increased uptake and why it is sometimes absent (Mosier et al., 1991; Kessavalou et al., 1998; Six et al., 2004b; Abdalla et al., 2013; Plaza-Bonilla et al., 2014;). This knowledge gap can be addressed by separating the net surface CH<sub>4</sub> flux into its three components and studying their distinct dynamics and drivers. The three components of surface CH<sub>4</sub> flux (fig. 2.1) are gross CH<sub>4</sub> production and consumption, that occur in the soil profile and determine the concentration of CH<sub>4</sub> in the soil air, and soil diffusivity (i.e., capacity to conduct gas movement by diffusion), which controls the

movement of CH<sub>4</sub> between soil air and the atmosphere (von Fischer et al., 2009). In upland soils gross CH<sub>4</sub> production, methanogenesis, occurs in sub-surface layers of saturated soil or anoxic microsites because it is performed by obligate anaerobic Archaea that depend on other anaerobic products (acetate and H<sub>2</sub>) to produce CH<sub>4</sub>. Gross CH<sub>4</sub> consumption, methanotrophy, occurs throughout oxic regions of the soil, performed by aerobic bacteria that benefit from access to atmospheric CH<sub>4</sub> and O<sub>2</sub>. Diffusivity of a soil is determined by pore shapes, sizes, and connectivity.

The greatest challenge to quantifying gross CH<sub>4</sub> gas fluxes in the field is disentangling methanogenesis, methanotrophy and diffusivity, because each simultaneously affects the surface CH<sub>4</sub> flux, but a new soil CH<sub>4</sub> process and transport model, the MASMC model (Butters et al., in preparation), allows measurements to be interpreted in a way that the component fluxes can be separated. The model uses data of surface CH<sub>4</sub> fluxes and gradients of soil CH<sub>4</sub> with depth, fits them to formulas based on first principles of CH<sub>4</sub> diffusion, production, and consumption, and calculates values for the three components. Other published techniques (Shcherbak and Robertson, 2014; Yang and Silver, 2016) for measuring gross fluxes in the field require additions of isotopically labelled and/or inert gases that may move through the soil in unexpected ways and can necessitate additional equipment for measurements. The MASMC model offers an approach that is methodologically simpler than those techniques (only pre-existing CH<sub>4</sub> is measured) and requires no assumptions about added gases, making it an approach that can be easily deployed by many researchers using existing resources.

Within agricultural soils, variation in methanotrophy, methanogenesis and soil diffusivity arises from both natural and management-related effects on soil properties. Diffusivity decreases as soil moisture increases and AFPS decreases, but it is also strongly affected by pore geometry, which is difficult to quantify for most soils (Werner et al., 2004; Shcherbak and Robertson, 2014).

Methanotrophy is greatest in soils with moderate moisture (van den Pol-van Dasselaar et al., 1998)

and with abundant soil aggregates and pore channels, whose surfaces are primary habitat for upland methanotrophs (Stiehl-Braun et al., 2011a; Stiehl-Braun et al., 2011b). Persistent elevated concentrations of CH<sub>4</sub> have also enriched laboratory cultures of high-affinity methanotrophs, increasing their consumptive capacity over the course of months (Hatamoto et al., 2011) but not when incubated for shorter periods (Malghani et al., 2016). Methanogenesis in upland soils occurs in anoxic microsites (von Fischer and Hedin, 2002; Keiluweit et al 2016), sub-centimeter regions of soil that are void of O<sub>2</sub> due to high rates of O<sub>2</sub> consumption and/or low O<sub>2</sub> diffusion. They can be located in soil aggregates (Sexstone et al., 1985; Wang et al., in preparation), decomposing plant litter (van der Lee et al., 1999), rhizospheres with high rates of exudation or root death (Fimmen et al., 2008), and the guts and feces of soil macrofauna (Lemke et al, 2003; Kammann et al., 2009). Wetter soils are expected to increase the strength of most anoxic microsite controls, increasing their size and/or prevalence.

We hypothesize that the increased CH<sub>4</sub> uptake of NT and fallow soils is due to an increase in diffusivity or methanotrophy. There are indications that NT soils may host greater methanotrophy (Hütsch, 1998) and have greater diffusivity (Prajapati and Jacinthe, 2014), however, the variation in CH<sub>4</sub> uptake following conversion from CT to NT indicates that the cause of greater uptake may not be simple. Increased uptake in NT soils is typical of plots that were converted to NT more than 15 years ago, while those converted to NT less than 7 years ago often have similar uptake to CT plots or even less (see Appendix). We would expect dynamics like these if there is a gradual change in net flux components after NT is adopted. The increases in soil aggregation and expansion of pore networks (Six et al., 2004a) that follow conversion to NT could cause gradual increases in diffusivity over time. Methanotrophy could increase slowly following conversion because populations of high-affinity methanotrophs (i.e., those that depend on low, atmospheric CH<sub>4</sub> concentrations) grow slowly (Maxfield et al., 2006) and prefer to colonize large pores that are destroyed by tillage



(Stiehl-Braun et al., 2011a; Stiehl-Braun et al., 2011b). The long-term increase in CH<sub>4</sub> uptake of NT soils could also be explained by declines in methanogenesis, but the primary soil changes that occur after conversion to NT are actually more likely to increase methanogenesis, such as increases in soil moisture (Halvorson et al., 1996) and soil carbon (Lal, 2004). Although fallowing fields has been shown to induce greater CH<sub>4</sub> uptake than planting the fields (Mosier et al., 1991; Kessavalou et al., 1998; Liebig et al., 2010), the sparse data on this practice make its likely relationships with CH<sub>4</sub> components less clear. Plots under fallow treatments typically do not receive fertilization and related decreases in NH<sub>4</sub><sup>+</sup> could release methanotrophs from metabolic inhibition that can occur at high NH<sub>4</sub><sup>+</sup> concentrations (Aronson and Helliker, 2010).

The goals of this study were, (a) to determine if gross fluxes and diffusivity explain variation in the surface CH<sub>4</sub> fluxes across agricultural treatments, and (b) to characterize the environmental drivers of gross fluxes in cropland soils. We worked in semi-arid, dryland wheat-fallow rotation plots established 46 years prior. Over two summers we measured surface gas flux, belowground soil gas concentrations, and soil properties likely to translate effects of agronomy onto surface flux components. We measured CO<sub>2</sub> and N<sub>2</sub>O fluxes since these gases indicate soil O<sub>2</sub> distribution and, thus, have bearing on anoxic microsite presence. We combined field CH<sub>4</sub> data with the MASMC model to estimate methanogenesis and methanotrophy in the soil profile as well as soil diffusivity – making this the first published study to make these measurements simultaneously across agricultural treatments in the field. Finally, we fit regressions of gas fluxes and microbial activities with soil and climate data to determine how soil properties and agricultural practices alter the activity of CH<sub>4</sub>-cycling microbes.

## 2.2 - Methods

### 2.2.1 - Site info and field design

This study was conducted at the USDA-ARS Central Great Plains Research Station (Akron, Colorado, USA) in plots under two tillage treatments: conventional tillage (mold-board plowing) with chemical fertilizers (CT) and no-till with chemical fertilizers (NT). The NT plots were established in 1967 and CT plots in 1989. The plots were annually rotated between wheat and fallow, all plots were planted with winter wheat in 2013 and were fallowed in 2014. Wheat was physiologically mature on 6/28/13 and was harvested on 7/8/13. Consistent with the long-term fertilization regime, in October 2012 all plots received 13.6 kg ha<sup>-1</sup> of urea (46-0-0) just prior to planting the winter wheat. CT fallow plots were tilled in the spring of 2014 and had sweep operations throughout the summer to control weeds, fallow NT had herbicidal weed control. Further details of the long-term plot management at this site can be found in Mikha et al. (2013) and Halvorson et al. (1996). Studies at this site have found that CT and NT soils contain 6.55 g kg<sup>-1</sup> and 7.55 g kg<sup>-1</sup> total carbon, respectively (0-20cm; Mikha et al. 2013). Three plots from each treatment were used in this study (n=6 plots), plot replicates 2, 3, and 4 by the site researchers' replicate numbering. The plots were 11 x 30 m and soil and gas sampling was dispersed across each plot.

### 2.2.2 - Soil bulk density, temperature, water content, and air-filled porespace

Soil temperature was measured hourly at 10cm depth at a weather station located between study plots. Daily averages (calculated from hourly measurements) were used in the statistical analyses described below. Soil volumetric water content (VWC) for 2013 and 2014 gas sampling dates was estimated for each treatment from a set of three overlapping VWC data sets from 2013, 2014 and 2015, in conjunction with daily precipitation data. VWC data was collected with a Trase System 1 (model 6050X1) time-domain reflectometry system (Soil Moisture Equipment

Corporation, Santa Barbara, CA, USA) and Decagon 5TE probes (Decagon Inc., Pullman, WA, USA). Bulk density of soils was calculated from soil samples taken June 2014. These samples were taken by slowly inserting beveled poly-vinyl chloride (PVC) pipe pieces to 14-16cm (6.35cm ID, 7.62cm OD), into the soil surface and then excavating them. The core soil volume was measured, the soil removed and sieved to 4mm, and the total dry mass calculated with gravimetric moisture measurements from soil sub-samples. Air-filled porespace (AFPS) was calculated as  $1 - (\text{VWC} / \text{total porespace})$  for each sampling date and treatment.

### 2.2.3 - Field gas installation and hardware:

In each field plot, three 20cm diameter PVC chamber anchors were installed for the duration of the season. At the same plot, we installed a set of soil air probes to sample gas concentrations as a function of depth (10cm, 20cm, 30cm, 50cm, and 80cm). Soil gas sampling probe design followed Butters et al. (in preparation). Briefly, 3.18cm OD PVC pipe was cut to the desired depth of the probe, 0.64cm OD polypropylene tubing was cut and inserted into the length of the pipe and affixed to each end through black rubber stoppers; these stoppers prevented the PVC pipe from venting the soil air to the atmosphere. A plastic Luer-lok connector valve was affixed to the 3cm tubing protruding beyond the stopper at the top of the probe. A PVC pipe cage 6cm long was attached to the bottom of the probe with a PVC coupler, protecting the 1cm tubing that protruded from the bottom stopper and creating a volume of soil air that could be sampled. All couplers, stoppers, and tubes were affixed with epoxy resin and/or silicon caulk to prevent leakage. Probes were constructed at lengths 10cm, 20cm, 30cm, 50cm, and 80cm, as previous simulations indicated this spacing would optimally characterize the soil air profile to fit the gas flux model (Dr. Gregory Butters, unpublished). Installation of the probes in soil was accomplished by first taking a soil core from the appropriate depth with a PN150 Environmentalist's Sub-Soil Probe (JMC Soil/Clements

Associates Inc., Iowa, USA), widening the hole with a 3.5 cm diameter thin-walled metal pipe when necessary, then inserting the probe into the hole and sealing around the PVC pipe with soil. These probes were installed in a line with approximately 20cm between adjacent probes.

Surface flux anchors and chambers were constructed from 20cm diameter PVC pipe. Anchors were 10cm long, with a beveled bottom edge; chambers were composed of three components, PVC pipe section (5cm long), a 3.3 liter stainless steel mixing bowl (20cm mouth diameter) glued in an inverted position on the PVC section, and a 60 cm long vent hose reaching from a port in the bowl to a double-plate vent (Xu et al., 2006). This chamber was designed to minimize pressure differences between chamber headspace, soil air, and atmosphere and is similar to LI-8100-103 chambers (LI-COR Inc., Lincoln, NE, USA). The exterior bowl portion of the chambers was covered in ~2cm of hard spray-on foam to reduce solar heating of headspace gas. Anchors and chambers had complimentary milled lips to provide gas-tight seals. Flux anchors were installed by placing them between rows of wheat and striking them with a 25cm x 25cm square steel tamper until <5cm of anchor wall remained above the soil surface.

#### 2.2.4 - Field gas measurements

Field gas measurements were made at two to three week intervals, with six dates in 2013 (May 24 and 30, June 13 and 28, July 11 and 31) and five dates in 2014 (June 4 and 19, July 1, 17 and 31). This frequency of samplings was designed to both capture distinct environmental states likely to affect microbial activity and reduce temporal auto-correlation of measurements. We gathered n=17 and 16 plot samplings in 2013 for NT and CT, respectively; and in the 2014 sampling n=14 and 15 for NT and CT respectively. Chamber and soil air measurements were taken on the same day for each plot. Chambers were placed on anchors to create a headspace from which gas samples were taken. Gas samples of 30ml were taken in 20ml glass vials at regular intervals. In 2013 the sampling

occurred at 1, 11, 21, and 31 minutes after chamber closure, in 2014 the samplings occurred at 6, 14, 22, 30, and 38 minutes after chamber closure. Headspace gas was mixed by pumping a syringe five times before pulling the gas sample. Soil air probes were sampled once on each measurement date. First, 5ml of gas was removed from the probe to prime the probe's gas tubing with pure soil air. Next, the syringe was pulled to 30ml and the pressure was gradually allowed to equilibrate to fill the syringe with 30ml of soil air. In the case of surface gas samples, the sample was taken 5cm above soil surface. All gas samples and standards were stored in evacuated 30mL vials with aluminum crimp tops and grey butyl septa.

Gas concentrations ( $\text{CO}_2$ ,  $\text{CH}_4$ , and  $\text{N}_2\text{O}$ ) were measured on a custom hybrid gas chromatograph/laser absorption spectroscopy system, with  $\text{N}_2\text{O}$  on a Shimadzu 14B gas chromatograph (GC) with electron capture detector, and  $\text{CO}_2$  and  $\text{CH}_4$  on a Los Gatos Greenhouse Gas Analyzer (LG). The GC was plumbed in a standard configuration with on-column injection of sample via injection port into an  $\text{N}_2$  carrier gas stream. In a novel configuration that we developed, air samples drawn from the same vial as for  $\text{N}_2\text{O}$  were injected into a stream of zero air (i.e., 80%  $\text{N}_2$ , 20%  $\text{O}_2$ , no trace gases) via an injection port on the inlet line to the LG. Sample air was drawn by the LG internal pump from the inlet line through an open split. Sample  $\text{CH}_4$  and  $\text{CO}_2$  moved through the LG as pulses over time that were transmitted by the analog-out ports on the LG and integrated by Shimadzu EZstart software. During sample injection, an AOC-5000 Combi-PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) injected 5ml of sample gas into the separate injection ports of the GC and LG. All samples were calibrated against certified lab standard gases.

#### 2.2.5 - Field gas flux model and calculations

Net surface fluxes were calculated for each chamber as linear regressions of chamber headspace gas concentration over time. Diffusivity, methanotroph and methanogen activity were estimated using the "microbial activity soil methane cycling" (MASMC) model (Butters et al., in

preparation), fitting the model to chamber gas and soil air concentrations. Briefly, this is a 1-dimensional fate and transport model that includes the possibility for simultaneous production and consumption of the gas in the soil. It has a spatial upper boundary of the chamber headspace and infinite lower boundary. It assumes the gas being modeled is subject to consumption dynamics that are first-order over gas concentration and production that is zero order. There are also assumptions of uniform soil diffusivity, methanotrophic activity, and methanogenic activity with depth in the soil profile. The model is described by equation 1, where  $D$  is gas diffusivity of the soil,  $\mu$  is methanotrophic activity,  $\gamma$  is methanogenic activity,  $a$  is % v/v of air-filled space in the soil,  $C_g$  is concentration of the gas (i.e., CH<sub>4</sub>),  $t$  is time, and  $x$  is the vertical dimension of the soil profile.

$$\frac{\partial C_g}{\partial t} = \left(\frac{D}{a}\right) \frac{\partial^2 C_g}{\partial x^2} - \mu C_g + \gamma \quad (1)$$

Although the MASMC model is based on an infinite lower boundary, the surface gas flux is only affected by its components down to the depth of net reactivity. This is the depth over which soil gas concentration changes before plateauing to an upper or lower asymptote (e.g., in fig. 2.2 a lower asymptote is approached at 80cm); below this depth, production, consumption, and diffusivity have no effect on surface fluxes. In this study and Butters et al. (in preparation) the typical depth of 95% net reactivity falls above 1m in most soils and above 2m in >95% of soils. Even though the model assumptions of vertical homogeneity of diffusivity and biologic activities are likely frequently violated, a set of analyses have shown that model fits yield results representative of more realistic heterogeneous distributions, with a moderate bias towards the values of diffusivity and biologic activities in the soils closer to the surface (Butters et al., in preparation). Analytical solutions to eq. 1 (Butters et al., in preparation) were implemented in R (see supplementary information for code) to fit the MASMC model, the parameters were optimized using `GenSA::GenSA()`. Input data for the model fitting were headspace gas concentrations from each replicate chamber, the plot's soil gas data, and the % total air-filled space for the plot. The model fit output the three primary model

components: soil diffusivity ( $\text{cm}^2 \text{min}^{-1}$ ), methanotrophic activity ( $\text{min}^{-1}$ ), and methanogenic activity ( $\text{ppm CH}_4 \text{min}^{-1}$ ). Since methanotrophy is a first order process this methanotrophic activity constant would need to be multiplied by local  $\text{CH}_4$  concentrations to be directly comparable to the methanogenic activity constant. An example fit of the MASMC model to data can be seen in fig. 2.2. Surface fluxes and model components for each chamber were averaged by plot, using a geometric average for the parameters that was later log transformed for statistical analysis.

#### 2.2.6 - Statistical analyses

Statistics were calculated using R, version 3.2.5 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria). The following data were log transformed to make error distributions Gaussian: surface  $\text{CO}_2$  flux, surface  $\text{N}_2\text{O}$  flux, soil diffusivity, methanogenic activity, and methanotrophic activity.

We performed three main analyses of our results: ANOVAs across agronomic treatments, a regression of surface  $\text{CH}_4$  flux as predicted by flux components, and regressions of all surface fluxes and flux components over treatments and environmental covariates.

Our first statistical analysis, which we refer to as the “treatment ANOVAs”, used an ANOVA framework to characterize the effect of treatments on the observed response variables:  $\text{CH}_4$ ,  $\text{CO}_2$  and  $\text{N}_2\text{O}$  fluxes plus the MASMC model estimated surface  $\text{CH}_4$  flux components - diffusivity, methanogenesis and methanotrophy. We constructed the treatment models by nesting the plot # as a random variable within agronomic treatment, these were analyzed in R (version 3.2.5) with the `lme4::lmer()` function. Response variables in these models were first tested for autocorrelation over time using the `nlme::lme()` and `nlme::acf()` functions (Zuur et al., 2009, chapter 6) – none exhibited autocorrelation, allowing us to exclude date as a factor. This statistical design is similar to that used for gross gas flux analysis by Yang and Silver (2016).

Our second and third statistical analyses used an optimal model search linear regression approach to determine which factors best explained in surface gas fluxes ( $\text{CH}_4$ ,  $\text{CO}_2$ , and  $\text{N}_2\text{O}$ ) and in the three surface  $\text{CH}_4$  flux components (diffusivity, methanogenesis and methanotrophy). The second analysis (the “component regression”) regressed surface  $\text{CH}_4$  flux over the three flux components to determine which of those best explained variation in surface  $\text{CH}_4$  fluxes (table 2.2, first model). The third analysis (the “covariate regressions”) individually regressed the three gas surface fluxes and all three  $\text{CH}_4$  flux components over environmental factors that have known direct or indirect mechanistic relationships with the response variable analyzed as well as treatment factors (table 2.2, remaining models). Physical and chemical impacts of agricultural practices are translated through soil properties, to microbial activity, which scales up to biogeochemical processes. These covariate regressions allow us to make the middle connection by determining which factors may control microbial activity. Combining treatments with environmental properties in the factor model pools allows for a comparison of their impacts on response variables: since environmental properties have more direct relationships with microbial activity than treatments, treatments that emerge as significant factors in the regressions most likely had effects on the responses that were not mechanistically translated through the measured environmental properties.

For these regressions all continuous data were centered and normalized prior to regression analysis. The optimal regression models we report were selected from pools of predictor variables which included squared factors and factors in second order interaction. Details on candidate predictor factors can be found in table 2.1. Exhaustive model selection was performed with all possible models from the predictor model pools using `leaps::regsubsets()`. We utilized Bayesian Information Criterion analysis (BIC), an information theoretic approach (Schwarz, 1978), to rank models for the optimal fit. The optimal model was selected from the subset of models within 2 BIC



units of the minimum BIC model (because models within 2 BIC units can be considered similarly good fits); from that subset the optimal model was the one with the highest  $R^2$ .

We also calculated the relative importance of each factor in the optimal model. Relative importance (RI) is a metric calculated for regressions with non-orthogonal covariates and is interpreted as a partial  $R^2$  would be, i.e., it is compared to the regression's total  $R^2$  to represent the portion of total model explanatory power contributed by that factor (Johnson and Lebreton, 2004). To calculate the RI of predictors we analyzed them using `relaimpo::calc.relimp()` with the `lmg` method (Lindeman et al., 1980) which averages sequential sums of squares over all orderings of predictors (Grömping, 2006).

The rotation of wheat and fallow is linked to the years 2013 and 2014, respectively, however patterns in precipitation and air temperature were similar between those years and our analysis minimizes any seasonal climatic influence on statistical results by including soil temperature and moisture as covariates in regression analyses.

## **2.3 - Results**

### 2.3.1 - CH<sub>4</sub> flux components model performance

The MASMC model was able to successfully fit all data sets; a total of 186 chamber data sets (averaged to 62 plot data sets). The MASMC model generally fit the data well (fig. 2.2 is typical of the fits). Methanogenic activity data was truncated at a lower bound of  $e^{-10}$  because values below that limit are not meaningful for process estimates; 41 of 62 methanogenesis estimates were above this threshold.

### 2.3.2 - Soil bulk density, temperature, and water content

CT had lower ( $p < 0.01$ ) bulk density than NT and nearly half the VWC ( $p < 0.001$ ) of NT in both wheat and fallow (fig. 2.3). NT VWC was higher ( $p < 0.01$ ) in the fallow year. AFPS was approximately twice as great ( $p < 0.001$ ) in CT soil than NT because NT's high bulk density and VWC interact to decrease AFPS. Soils did not contain layers of waterlogged soil because near-surface VWC was never close to saturation and soil air samplers were never flooded (down to 80cm).

### 2.3.3 - CH<sub>4</sub> surface gas flux

Our treatment ANOVAs show that both treatments exhibited net CH<sub>4</sub> uptake at the soil surface in wheat and fallow, averaged over each year, with the greatest uptake occurring in NT-wheat (fig. 2.4A). NT-wheat had greater uptake than both fallow treatments ( $p < 0.01$ ), and under wheat both tillage treatments had greater uptake than CT-fallow ( $p < 0.05$ ). Uptake in NT-wheat was 40% greater than uptake under CT-wheat, though not significant in the two-way ANOVA. Overall, the agronomic treatment analysis indicates that there is an important interactive effect of tillage and rotation.

The component regression (table 2.2, first model) of surface CH<sub>4</sub> flux over diffusivity, methanogenesis and methanotrophy shows that methanotrophy and diffusivity together explain 80% of the variation in flux. For our regression analyses we cannot use partial R<sup>2</sup> to compare the relative explanatory power of different model factors have in relation to the total explanatory power of regressions, because the predictor factors may be correlated (i.e., non-orthogonal). Instead we utilize “relative importance” (RI), a metric that can be interpreted similar to a partial R<sup>2</sup>, and the RI ratio of RI/model R<sup>2</sup>, which shows the factor's importance compared to total model explanatory power. The component regression indicates that methanotrophy is the most important component

(RI ratio 0.53/0.80) for explained variation in surface CH<sub>4</sub> fluxes across the study. Methanogenesis did not appear in the optimal regression.

When CH<sub>4</sub> flux is analyzed in the covariate regression, over treatment factors and soil properties (table 2.2), it becomes clear that NT plots have greater uptake than CT. This is indicated by the relatively large negative coefficient estimate of NT (-0.192) and the NT RI ratio of 0.36/0.71, representing more than 50% of the explanatory power of the regression. AFPS had the next largest RI ratio (0.20/0.71), though its effect was mainly expressed through interactions where greater uptake is related to high AFPS in combination with either high temperature or NT treatment. Higher VWC was associated with greater uptake as was lower soil temperature.

#### 2.3.4 - CO<sub>2</sub> and N<sub>2</sub>O surface gas fluxes

CO<sub>2</sub> and N<sub>2</sub>O fluxes have very similar patterns across treatments. Emissions are highest from NT-wheat, followed by CT regardless of rotation, and lowest in NT-fallow (fig. 2.4B and 2.4C). The only significant difference found in the CO<sub>2</sub> and N<sub>2</sub>O treatment ANOVAs is that NT-wheat had greater emission than NT-fallow for both gases. The CO<sub>2</sub> covariate regression ( $R^2 = 0.34$ ) shows that higher flux is primarily associated with wheat and NT treatments, and is positively related to VWC and AFPS to a smaller degree (table 2.2). In the N<sub>2</sub>O covariate regression ( $R^2 = 0.57$ ) CO<sub>2</sub> flux is by far the most important predictor (RI ratio 0.42/0.57), with a positive and exponential relationship to N<sub>2</sub>O flux. Soil temperature and VWC both have negative relationships with N<sub>2</sub>O flux and NT had higher rates of N<sub>2</sub>O emission (table 2.2).

#### 2.3.5 - Components of net CH<sub>4</sub> flux over treatment and rotation

Diffusivity, methanogenesis, and methanotrophy vary with both tillage and rotation. The treatment ANOVA shows soil diffusivity was greater in CT than NT soils, mirroring the

measurements of bulk density (fig. 2.3A & 2.4D). On average, CT diffusivity was over three times greater than that of NT soils and diffusivity was greater under wheat than fallow (fig. 2.4D). The covariate regression of diffusivity indicates that soils with greater AFPS also had greater diffusivity. Both methanogenesis and methanotrophy were much greater in NT-wheat than any other treatment, as indicated by the ANOVA results (fig. 2.4E & 2.4F). CT had lower levels of both methanogen and methanotroph activity than NT, but higher activities under wheat than in fallow. NT had higher methanotrophy than CT regardless of rotation, although the difference between tillages was much smaller in fallow. NT methanogenesis also decreased 96% in fallow, falling to the same levels as CT. The covariate regressions show that methanogenesis is higher when soils are in wheat (RI ratio 0.24/0.70) and NT (RI ratio 0.16/0.70), and when the soils have higher VWC, AFPS, and CO<sub>2</sub> fluxes. VWC and CO<sub>2</sub> effects may occur due to their relationships with soil O<sub>2</sub> concentrations but the impacts of rotation and tillage are not as obvious. When methanogenesis is regressed over only AFPS the relationship is negative (slope = -0.34, p=0.004, r<sup>2</sup>=0.13), but the covariate regression shows a positive relationship between them which is counterintuitive considering that higher AFPS will increase O<sub>2</sub> diffusion into soil. This pattern could occur if the impact of an unmeasured factor that increases methanogenesis at higher AFPS only becomes apparent after effects of treatments, VWC, and CO<sub>2</sub> are taken into account. The methanotrophic activity covariate regression shows that tillage is the most important factor (RI ratio 0.31/0.67) but that AFPS, VWC, and soil temperature all help explain the variance in methanotrophy. Since NT is a factor with strong positive relationship with methanotrophy in this regression, the impact of NT apparent in the treatment ANOVA was not fully explained by covariates we measured. Methanotrophy increased with AFPS and VWC but had a quadratic relationship with temperature, peaking in the warmest soils. Even though they are driven by different sets of environmental and management factors, methanogenesis

and methanotrophy have a tight relationship ( $r^2=0.87$  for measurements above detection limit; fig. 2.5).

## 2.4 - Discussion

### 2.4.1 - Surface CH<sub>4</sub> flux and components

We investigated impacts of tillage and fallow rotations on soil CH<sub>4</sub> cycling by using the MASMC model to determine how surface CH<sub>4</sub> flux is controlled by soil diffusivity, gross subsurface CH<sub>4</sub> fluxes, and soil properties. Plots under NT had greater surface CH<sub>4</sub> uptake than CT, as is typical of long-term NT/CT comparisons, however, uptake decreased when the plots were fallowed, which contrasts with the two previous studies of that relationship (Mosier et al. 1991; Kessavalou et al. 1998).

One of our goals was to determine which of the three surface flux components (diffusivity, methanotrophy, and/or methanogenesis) was responsible for the surface flux differences between agronomic treatments. Previous studies have hypothesized that increased methanotrophy or diffusivity cause this difference, but none have measured and compared these components. At our site, greater methanotrophy is unequivocally driving the higher surface uptake of NT over CT plots – diffusivity in NT soils was lower than CT, which would have an opposite effect, decreasing surface uptake. Surprisingly, NT also had much more methanogenesis than CT, but apparently this did not cause NT soils to become surface emitters of CH<sub>4</sub>. It is less clear why fallowed soils had less uptake than soils growing wheat. Methanotrophy and methanogenesis decreased dramatically during the fallow year as did diffusivity. It is likely that a combination of the methanotrophy and diffusivity decreases led to less surface CH<sub>4</sub> uptake under fallow.

Although our regression of surface CH<sub>4</sub> flux over its three components made it clear which of the components were most important for variation in surface flux, the covariate regression over

agronomic treatments and environmental properties provides less clarity regarding the environmental drivers of surface CH<sub>4</sub> flux. That regression confirms that surface CH<sub>4</sub> uptake varies with AFPS, VWC, and soil temperature, but the presence of NT as the most important predictor highlights an inability for gradients of soil properties to explain the surface uptake variation between tillage treatments.

Because surface flux was driven by methanotrophy and diffusivity, analyzing the environmental drivers of those two components independently can provide a clearer picture of how effects of agricultural practices are translated through soil physical and chemical changes. High diffusivity occurs when AFPS is high, as expected, but no other factors were strongly correlated. Methanotrophy's positive relationship with VWC and AFPS and high peak with soil temperature in the covariate regression indicate that soil moisture, temperature and access to atmospheric O<sub>2</sub> and CH<sub>4</sub> may help drive methanotrophy at this site, relationships with methanotrophic activity that have been observed across a range of soils and climates (van den Pol-van Dasselaar et al., 1998; Dunfield, 2007). However, the persistence of NT as an important predictor in that regression shows that agronomic effects on the measured environmental properties do not fully account for the practice's impact on methanotrophy, as was also the case with surface CH<sub>4</sub> flux. One explanation for the effects of NT on methanotrophy lies in the very similar patterns of methanogenesis and methanotrophy across treatments.

#### 2.4.2 - Relationship between methanogenesis and methanotrophy

Methanogenesis and methanotrophy were strongly positively correlated with each other, and this relationship persists across tillage and rotation, indicating it was a widespread phenomenon (fig. 2.4 & 2.5). It is possible that many methanotrophic populations are stimulated to grow in response to higher soil CH<sub>4</sub> concentrations generated by nearby methanogenesis. Very strong

correlations between rates of gross CH<sub>4</sub> production and consumption have been described in at least two other studies. Kammann et al. (2009) measured gross CH<sub>4</sub> production and consumption in grassland soil cores and found that the subset with particularly high methanogenesis also hosted high enough methanotrophy to consume 90%-200% of the produced CH<sub>4</sub>. Likewise, Yang and Silver (2016) observed methanogenesis in all flux samplings and a strong correlation of methanogenesis and methanotrophy ( $r^2=0.67$ ). Kammann et al. (2009) also hypothesized that this phenomenon occurs because of a direct trophic link. We believe the observation of this relationship in three unrelated studies combined with evidence that high-affinity methanotroph populations can grow under consistently elevated CH<sub>4</sub> (Hatamoto et al. 2011; Ho et al., 2013) provides strong support for this mechanistic explanation. Further support of this stimulation explanation is the stronger persistence of methanotrophy versus methanogenesis moving from the wheat to fallow rotation. This pattern would be consistent with a stimulation of methanotrophic populations in NT soils that persists for months or years even when methanogenesis has decreased.

Although the stimulation of methanotrophy by methanogenesis appears highly plausible, an alternative explanation is that soil properties in certain plots or soil cores are simply favorable to both methanotrophs and methanogens. Indeed, von Fischer and Hedin (2007) did not find a strong methanogenesis/methanotrophy relationship in intact soil cores, mainly from forests and a few from wetlands. However their findings may have differed from ours could be that forest soil CH<sub>4</sub> cycling diverges from grassland and cropland cycling or because soil habitat was dominated by either methanogen or methanotroph habitat, with little heterogeneity.

### 2.4.3 - Methanogenesis

The mechanisms of formation and maintenance of anoxic and methanogenic microsites are not well understood, but the general requirement to create this habitat is that a small volume of soil

has a high rate of O<sub>2</sub> consumption and/or a low rate of O<sub>2</sub> diffusion. Although non-destructive direct measurement of small-scale soil anoxia is not possible *in situ* in field settings, variation in soil O<sub>2</sub> was indicated in our study by multiple factors that are directly related to O<sub>2</sub> consumption or diffusion. First, the low gas diffusivity and low AFPS of NT soils indicates that less O<sub>2</sub> was able to diffuse into the soil air. NT-wheat soils' elevated CO<sub>2</sub> fluxes relative to other treatments indicate greater belowground respiration and O<sub>2</sub> demand in NT-wheat. Second, high VWC in NT contributed a dual effect, decreasing O<sub>2</sub> content by driving AFPS lower and respiration higher. Previous studies have suggested that production of N<sub>2</sub>O indicates denitrification and thus anoxic microsites (Parkin, 1987; Horn et al., 2003; Stolk et al., 2011). Surface N<sub>2</sub>O flux has a significant correlation when regressed against only methanogenesis ( $p=0.016$ ,  $r^2=0.10$ ), but not a significantly stronger correlation than surface CO<sub>2</sub> flux over methanogenesis ( $p=0.017$ ,  $r^2=0.092$ ). The presence of anoxic microsites may explain why NT-wheat plots had elevated N<sub>2</sub>O fluxes, however, when combined with other environmental properties CO<sub>2</sub> was a better predictor of methanogenesis than N<sub>2</sub>O.

Rates of methanogenesis were much greater during wheat production, suggesting anoxic microsites were more active in the presence of plants, which could be because root death, exudation, and macrofaunal activity are all more frequent in soils with live plants. Similarly, Yang and Silver (2016) found that both methanogenesis and methanotrophy were greater in crop row than inter-row soil. Root turnover and exudation can stimulate microbial respiration that consumes surrounding O<sub>2</sub> and creates anoxic, methanogenic microsites that are C rich. This relationship is typical in rice paddies where 50% of CH<sub>4</sub> produced contains C from recent photosynthate (Philippot et al., 2008). However, rhizosphere anoxic microsites in upland soils have, to our knowledge, only been directly observed along tree roots in deeper soils (>30cm), where iron reduction, not methanogenesis, was quantified (Fimmen et al., 2008). Exudation is an unlikely source since wheat senescence occurred



half-way through the sampling season, but it is possible that the decomposition of recently dead wheat roots caused the high levels of methanogenesis in NT-wheat. Earthworms and other soil macrofauna are better established hosts of upland anoxic microsites. Kammann et al. (2009) found macrofauna present in 75% of their temperate grassland soil cores that hosted high gross CH<sub>4</sub> production, and determined that *Scarabaeidae* larvae could account for the vast majority of methanogenesis measured. Detailed measurements of dissected *Scarabaeidae* larval guts have revealed high rates of methanogenesis, ~-100mV redox potentials, and the presence of other anaerobic products (Lemke et al., 2003). Earthworms produce large quantities of N<sub>2</sub>O from gut denitrification (Horn et al., 2003) but have not yet been found to host methanogenesis (Šustr and Šimek, 2009). Methanogenesis was higher in NT soils which should also have larger macrofaunal communities because tillage decreases the population of arthropods and earthworms (House and Parmelee 1985; Hendrix et al., 1986). Opportunistic macrofaunal sampling from our soil cores taken for bulk density showed 5 of 6 NT soil contained grubs or earthworms, but none of the 6 CT cores contained these macrofauna. If macrofauna were a primary CH<sub>4</sub> source in our plots, methanogenesis may have decreased in the fallow year because plant related macrofaunal food sources were sparse.

#### 2.4.4 - A framework of land management effects on CH<sub>4</sub> flux

In combining our findings with previous studies, we suggest a new conceptual framework for how agriculture may affect CH<sub>4</sub> flux. This framework helps synthesize existing knowledge, hypothesizes a mechanism of the NT post-conversion effect, and generates new research questions. To our knowledge, such a framework of agricultural management effects does not exist in the scientific literature. In this framework (fig. 2.6), climate and management determine changes in soil environment, which, in turn affects rates of microbial activity. This type of conceptual model is similar to those increasingly used in biogeochemical studies, where the mechanistic role of soil

environment, activity and/or communities explicitly mediates effects of ecosystem properties and disturbance to ecosystem function (Wallenstein and Hall, 2011; Schimel and Schaeffer, 2012; Weider et al., 2015)

Under this framework, the long-term NT increase in CH<sub>4</sub> surface uptake as well as post-conversion dynamics of surface CH<sub>4</sub> flux in NT soils (Brewer dissertation Introduction) are explained by intermediate effects on soil habitat. Immediately following conversion to NT, rhizosphere and/or macrofaunal sources return to the soil increasing gross CH<sub>4</sub> production and the soil's net CH<sub>4</sub> flux. Following this transition, the NT methanotroph population undergoes a slower multi-year enrichment and only overtakes gross CH<sub>4</sub> production from sources after 7-10 years. Because upland methanotrophs rely on low concentrations of CH<sub>4</sub> (even in soils with high gross CH<sub>4</sub> production our soil CH<sub>4</sub> concentrations were never >2.5 ppm), it is plausible their populations would respond on this timescale. Six et al. (2004b) found a similar 10+ year delay for NT soils to decrease N<sub>2</sub>O fluxes relative to CT soils following conversion and attributed this to higher VWC in NT soils, but it's also possible rhizosphere/macrofaunal N<sub>2</sub>O sources and net N<sub>2</sub>O-consuming microbes have a similar relationship. These dynamics would be more complex than CH<sub>4</sub> cycle dynamics since N<sub>2</sub>O-consuming microbes are also capable of N<sub>2</sub>O production.

This conceptual model raises a number of questions for future research, addressing them would probe our proposed relationships and build understanding of the impacts on global greenhouse gas budgets.

1. *How long does enrichment of high-affinity methanotrophic populations take? How do temperature, moisture, CH<sub>4</sub> concentration, and inhibitory compounds (e.g., NH<sub>4</sub><sup>+</sup>) affect enrichment? How long does elevated surface CH<sub>4</sub> uptake persist after a source of CH<sub>4</sub> is removed?*
2. *What types of soil anoxic microsites are common CH<sub>4</sub> sources and do they all have enrichment effects on methanotrophy?*

3. *How do crop type and rotation effect root exudation, death, and macrofaunal communities? Do different types of tillage and other agricultural practices affect these potential CH<sub>4</sub> sources differently and do those effects translate to surface gas flux?*
4. *What is the size of CH<sub>4</sub> (and N<sub>2</sub>O) production fluxes across macrofaunal taxa (expanding on Šustr and Šimek, 2009)? How are the fluxes affected by organism size and life stage, food source, temperature, and soil type?*
5. *Does tillage affect methanotrophs more by destroying physical habitat (i.e., pores and aggregate surfaces) or decreasing available substrate (i.e., preventing methanogenic sources from forming)?*
6. *Do surface CH<sub>4</sub> fluxes correlate with root exudation, death, or macrofaunal populations at ecosystem scale? Does this explain observed seasonality of surface fluxes?*

#### 2.4.5 - Conclusions and Implications

Our results indicate that the consistent long-term increase in NT CH<sub>4</sub> uptake is due to greater methanotrophy, rather than increases in diffusivity, in NT plots. High methanotrophic rates appear to result from stimulation by methanogenesis within the soil profile, which would mean, counter-intuitively, that upland soils may require high gross CH<sub>4</sub> production to build capacity for higher net surface uptake. Furthermore, our findings add to a growing body of evidence indicating upland methanogenesis is widespread, emphasizing the need for study of the specific causes of this unexpected microbial activity and its biogeochemical implications. Our study suggests the measurement and modelling of gross GHG fluxes and their drivers could be necessary to improve predictions of atmospheric GHG dynamics. Moreover, common agricultural practices have large impacts on microbial activity that may be best understood by using models that explicitly consider management-soil environment-microbe relationships.

Table 2.1: Candidate predictors (i.e., predictor pools) included in optimal model selection of regression models. Candidates were chosen that were ecologically relevant for each response variable.

<b>Surface CH4 flux component model</b>										
	Candidate predictors:		log(diffusivity)	log(methanotrophy)	log(methanogenesis)					
	All of three these were present in interaction with each other and as a squared terms									
<b>Covariate models</b>										
	Candidate predictors:									
Response variable:	Tillage	Rotation	Tillage X Rotation	Tillage X AFPS	AFPS	Temperature	VWC	log(CO2 flux)	log(N2O flux)	log(methanogenesis)
Surface CH4 flux	*	*	*	*	**	**	**	**	NP	NP
Surface CO2 flux	*	*	*	*	**	**	**	NP	NP	NP
Surface N2O flux	*	*	*	*	**	**	**	**	NP	**
Diffusivity	*	*	*	*	**	**	**	NP	NP	NP
Methanotrophy	*	*	*	*	**	**	**	**	NP	NP
Methanogenesis	*	*	*	*	**	**	**	**	**	NP
	* = present									
	** = present in interaction with other "*" predictors and as a squared term									
	NP = not present									

Table 2.2: Regression results with the best linear regression and predictor estimates for each response variable. The predictors of the final models are ranked by relative importance, a value that is a proportion of model total  $R^2$ . Surface  $CH_4$  flux was regressed separately against a pool of MASMC model parameters (upper model) and a pool of treatment and environmental covariates (lower model). VWC = volumetric water content, AFPS = air-filled porespace

Response variable	$R^2$	Predictor	Relative Importance	Coefficient Estimate	P-value
<b>MASMC Model Components</b>					
$CH_4$ flux	0.80	ln(Methanotrophy)	0.53	-0.023	<0.001
		ln(Diffusivity)	0.20	-0.017	<0.001
		ln(Diffusivity) x ln(Methanotrophy)	0.06	-0.010	<0.001
		ln(Methanotrophy) <sup>2</sup>	0.02	-0.003	0.102
<b>Treatments and Soil Properties</b>					
$CH_4$ flux	0.71	No-till	0.36	-0.192	<0.001
		AFPS	0.15	0.000	0.995
		AFPS x Temperature	0.08	-0.010	<0.001
		AFPS <sup>2</sup>	0.05	-0.045	0.035
		VWC	0.04	-0.027	0.026
		No-till x AFPS	0.02	-0.221	0.019
		Temperature	0.01	0.005	0.077
ln( $CO_2$ flux)	0.34	Fallow x No-till	0.11	-1.221	<0.001
		Fallow	0.09	-0.079	0.688
		VWC	0.06	1.286	<0.001
		No-till x AFPS	0.04	-1.704	0.009
		AFPS	0.03	2.246	0.002
		No-till	0.01	2459	<0.001
		ln( $N_2O$ flux)	0.57	ln( $CO_2$ flux)	0.37
Temperature x VWC	0.05			-0.209	0.012
No-till	0.05			0.881	0.024
ln( $CO_2$ flux) <sup>2</sup>	0.05			0.321	0.004
Temperature	0.04			-0.159	0.041
VWC	0.02			-0.344	0.059
ln(Diffusivity)	0.39	AFPS	0.39	0.736	<0.001
ln(Methanotrophy)	0.67	No-till	0.31	6.377	<0.001
		AFPS	0.15	2.831	0.003
		VWC	0.12	1.063	0.103
		Temperature <sup>2</sup>	0.08	0.374	0.002
		Temperature	0.01	-0.035	0.815
ln(Methanogenesis)	0.70	Fallow	0.24	-2.220	<0.001
		No-till	0.16	3.706	0.008
		VWC	0.09	2.589	0.002
		AFPS	0.08	3.006	0.003
		ln( $CO_2$ flux) x VWC	0.07	0.723	0.003
		ln( $CO_2$ flux)	0.06	0.031	0.904

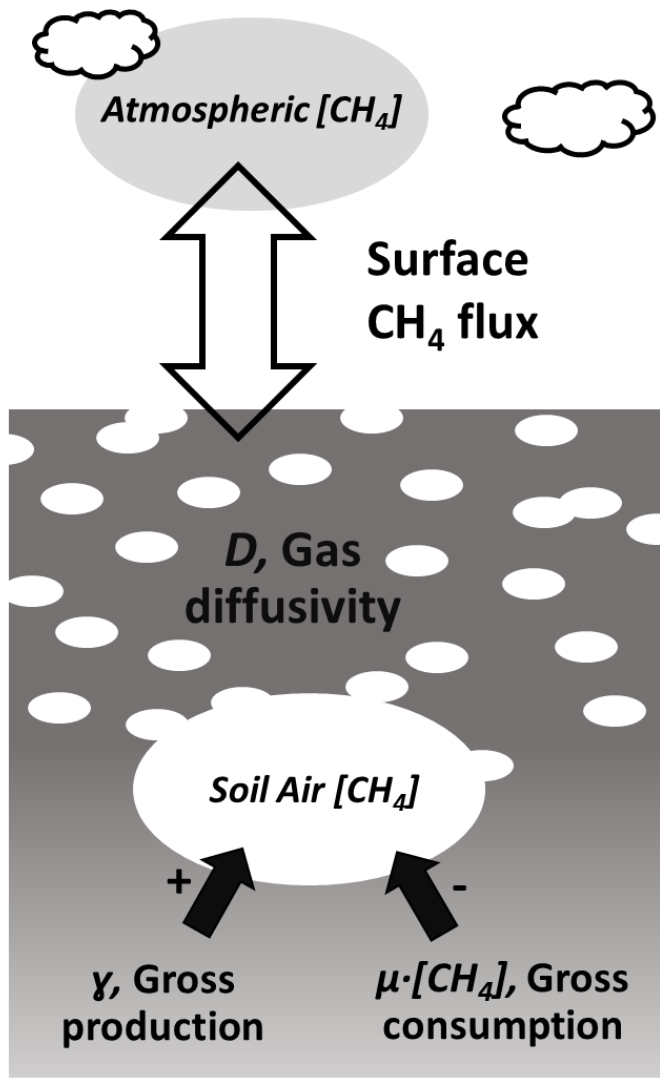


Figure 2.1: Components of surface  $\text{CH}_4$  flux and their MASMIC model terms. Gross  $\text{CH}_4$  production,  $\gamma$ , gross  $\text{CH}_4$  consumption,  $\mu \cdot [\text{CH}_4]$ , and gas diffusivity,  $D$ .

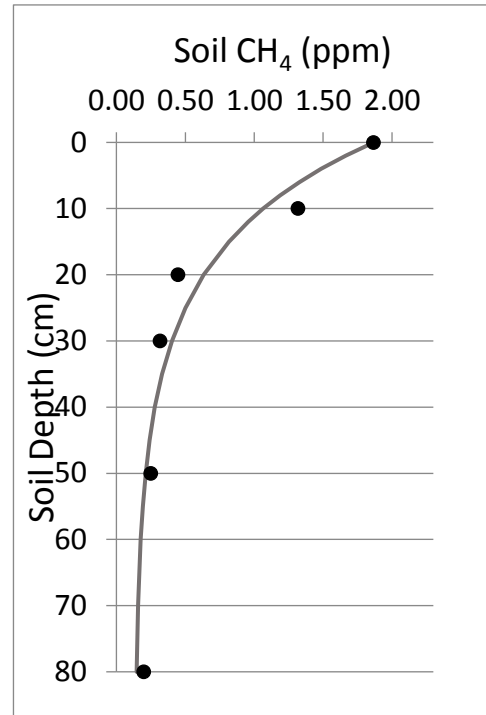
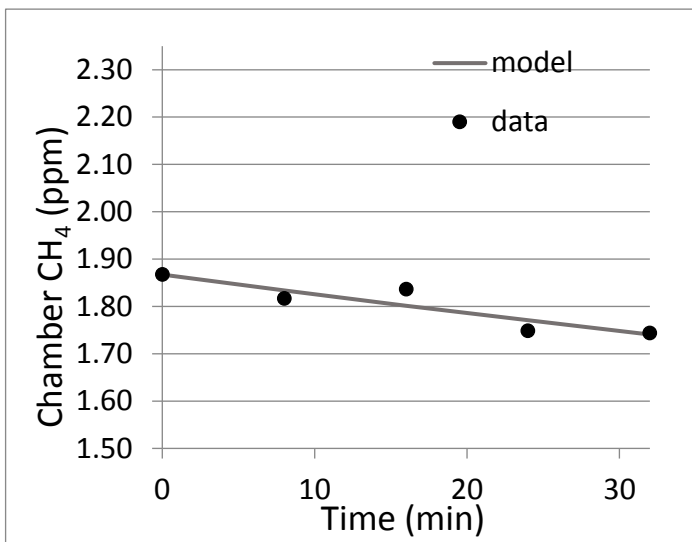


Figure 2.2: An example fit of the MASMC model to CH<sub>4</sub> headspace flux (left) and soil air (right) data. This is data from a plot on 7/31/14.

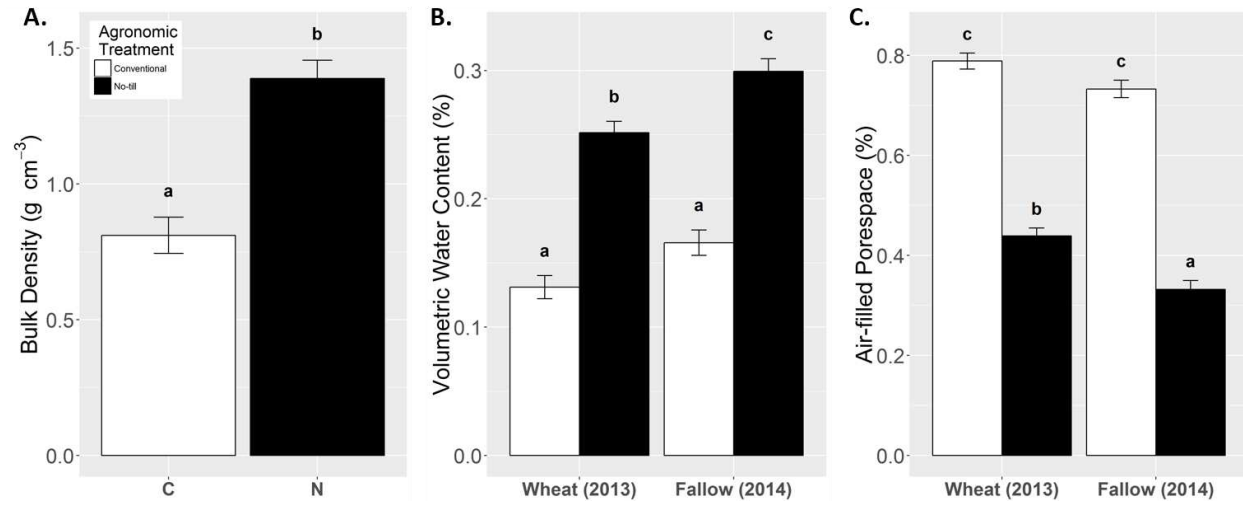


Figure 2.3: Effects of treatment on bulk density (A), treatment and year on soil VWC (B) and air-filled space (C). Wheat was grown in 2013, in 2014 fields were fallowed. Error bars are s.e. White bars are CT, black bars are NT.



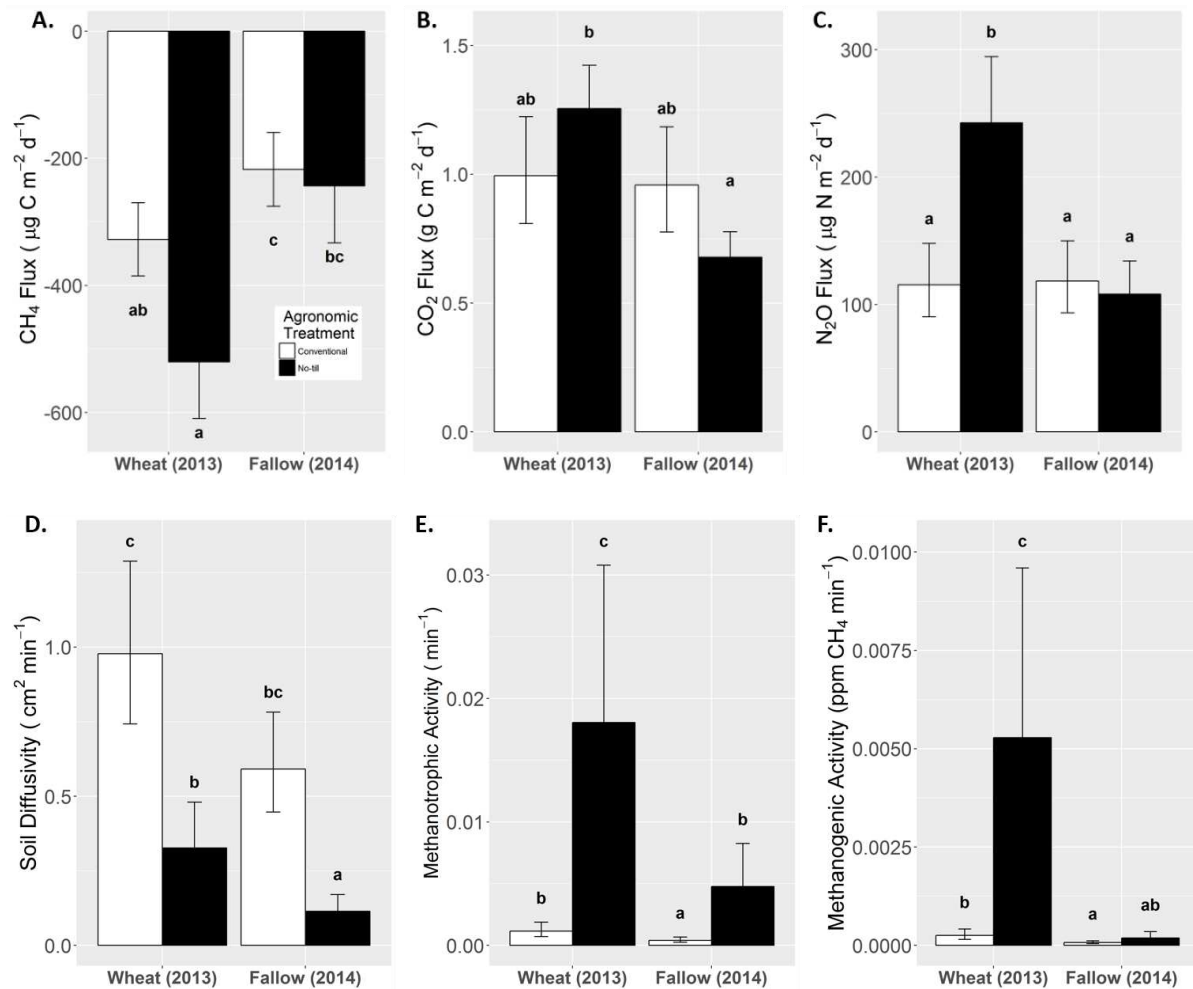


Figure 2.4: Plots of surface net gas fluxes CH<sub>4</sub> (A), CO<sub>2</sub> (B), and N<sub>2</sub>O (C) and MASMC model flux components diffusivity (D), methanotrophic activity (E), and methanogenic activity (F) across treatment and year. Tukey post-hoc multiple comparison connecting letters were calculated with log-transformed data, except for surface CH<sub>4</sub> flux. White bars are CT, black bars are NT. Wheat was grown in 2013, in 2014 fields were fallowed.

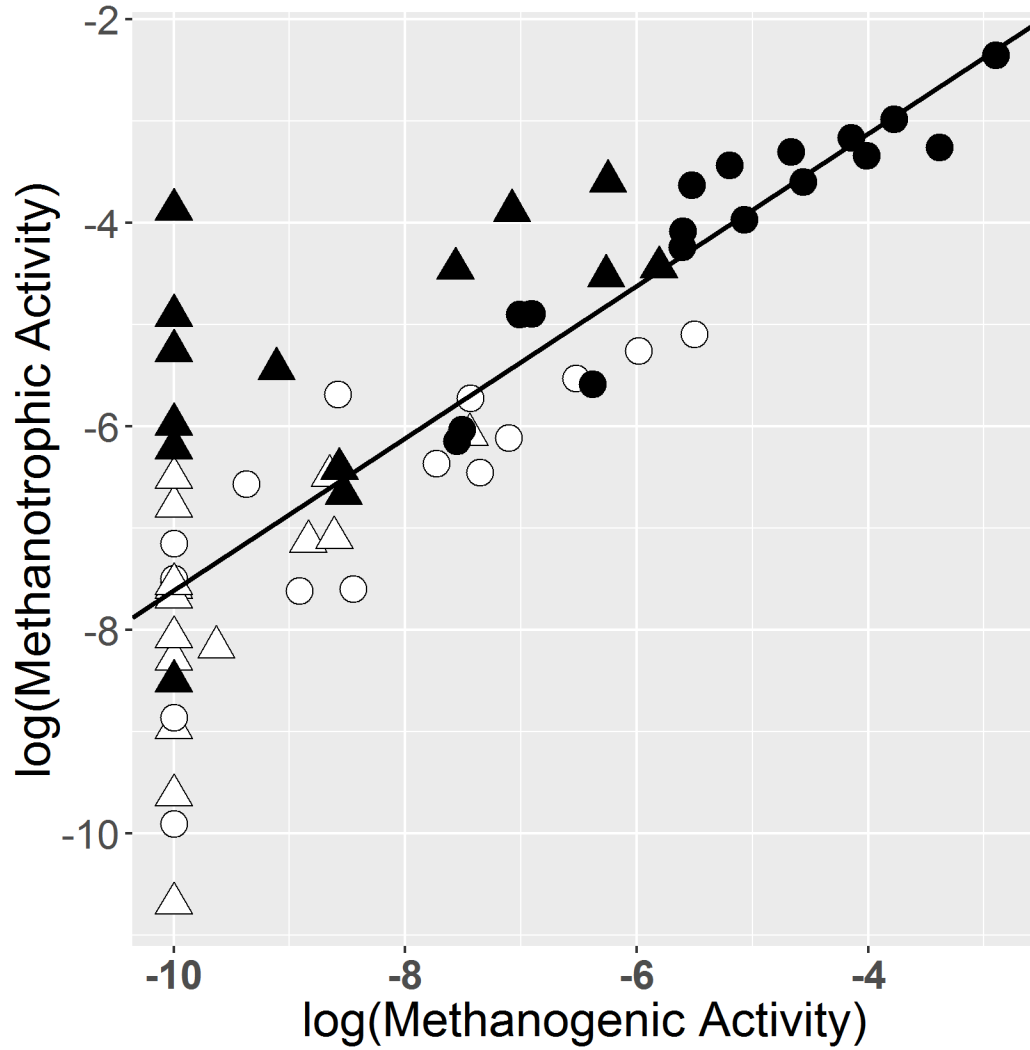


Figure 2.5 Estimates of methanotrophic and methanogenic activity, by agricultural practice; white is CT, black is NT; circles are wheat, triangles are fallow. The solid line indicates the linear fit to all data above detection limits, slope=0.75,  $r^2=0.87$ . Data are natural log transformed, methanogenic activity detection limit was  $e^{-10}$ .

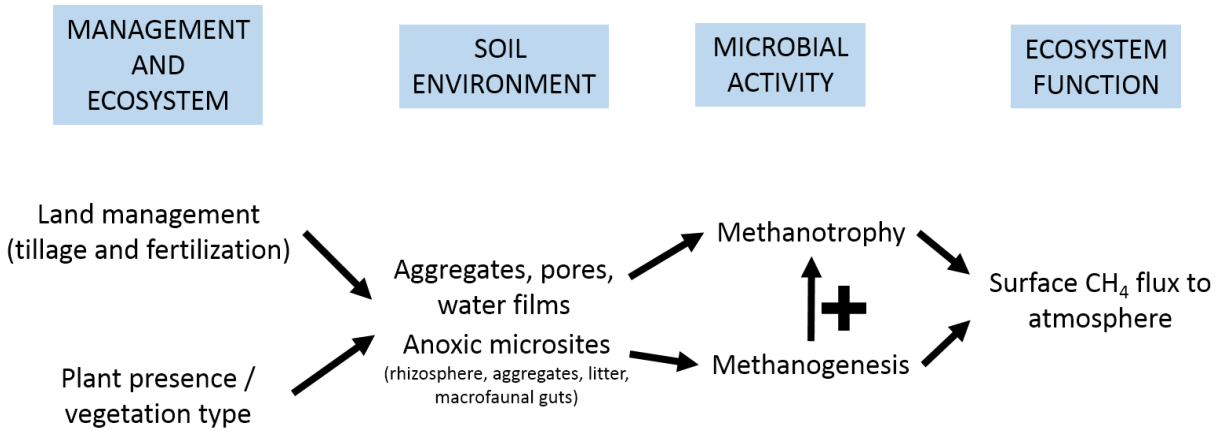


Figure 2.6: Hierarchical framework of relationships between agricultural factors, soil environment, biological CH<sub>4</sub> cycling, and surface CH<sub>4</sub> flux. Black arrows indicate a hierarchical relationship and the arrow from methanogenesis to methanotrophy indicates a positive quantitative relationship.

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### 3 - Impacts of moisture, soil respiration, and agricultural practices on gross CH<sub>4</sub> production measured by stable isotope pool dilution

#### **3.1 - Introduction**

Anoxic microsites occur frequently in upland, unsaturated soils and host a range of anaerobic processes including denitrification, iron reduction, and fermentation. It is not well understood how these reduced regions persist despite the presence of O<sub>2</sub> in the surrounding pores, but even methanogenesis, which only occurs under strongly reducing conditions, has been observed in the relatively well-drained soils of forests, grasslands, deserts, and croplands across an array of climates (von Fischer and Hedin, 2007; Kammann et al., 2009; Angel et al., 2011; Angel et al., 2012; Yang and Silver, 2016). Perhaps because of the very fine scales of heterogeneity associated with this phenomenon (Liptzin and Silver, 2015; Keiluweit, et al. 2016), we lack a practical understanding of the factors that drive their formation and persistence, and we also have little evidence of how other soil processes co-vary with anoxic microsite abundance.

Anoxic regions form and persist when the diffusion of O<sub>2</sub> into a volume of soil is less than the O<sub>2</sub> demand within that volume (Jorgenson, 1977; Sexstone et al., 1985). Thus, relative to nearby oxic soil volumes, anoxia develops at “microsite” scales (here defined as volumes smaller than a typical soil core) due to locally elevated O<sub>2</sub> consumption, decreased O<sub>2</sub> diffusion, or a combination of both. Rates of O<sub>2</sub> consumption can increase with live biomass and energy substrates (i.e., electron donors such as labile organic matter): greater microbial and soil faunal populations and/or more organic matter can increase respiration rates (Keiluweit, et al. 2016). In contrast, rates of O<sub>2</sub> diffusion vary with soil architecture, which defines the pore network available for gas diffusion, and soil water content, which affects the volume of pores open to gaseous diffusion at any given time (Keiluweit, et al. 2016). Studies support the idea that soil water content (Linn and Doran, 1984; Sexstone et al.,

1985; von Fischer and Hedin, 2007; Loecke and Robertson, 2009) and organic matter (van der Lee et al., 1999; Chacon et al., 2006) affect formation and persistence of anoxic microsites, but none have quantified the relationships of these drivers over an array of replicated experimental factors.

In this study, we characterize the extent of anoxic microsite activity by measuring methanogenic activity, i.e., gross CH<sub>4</sub> production. Since methanogenesis is an obligate anaerobic process that occurs in the most reduced environments, its presence in oxic soil indicates that all other anaerobic processes can also be present (Peters and Conrad, 1996; Megonigal et al., 2003). This is a relatively underutilized way to characterize anoxic microsites that has only been used a handful of times (von Fischer and Hedin, 2007; Kammann et al., 2009; Yang and Silver, 2016). In the past, characterization of anoxic microsites was primarily accomplished through measurements with oxygen and redox micro-electrodes which have several drawbacks including high cost, limited sample sizes and repeatability, and restricted operability in field settings. Measurement of methanogenesis is a promising approach because it integrates across the volume of a study soil, measures a function instead of only chemistry, is non-destructive, and can be automated in the lab and extended to the field (*sensu* Brewer, chapter 2).

Our work focuses on anoxic microsites in agricultural systems for two reasons: 1) because different management practices have potential to alter both the biological processes associated with O<sub>2</sub> consumption (e.g., organic amendments) and the physical processes that affect O<sub>2</sub> supply (e.g., tillage); and, 2) because there is strong interest in redox-sensitive properties of agricultural systems, especially inorganic nitrogen speciation and N<sub>2</sub>O emissions. Following from established relationships of environmental properties and wetland methanogenesis, we hypothesize that the presence and magnitude (i.e., flux rates) of methanogenic microsites will be positively correlated with factors related to O<sub>2</sub> consumption (organic matter, soil respiration, microbial biomass) and negatively correlated with O<sub>2</sub> diffusion (WFPS, tillage history). We incubated soils from conventionally tilled, no-till, and

organically fertilized plots over a range of WFPS and measured methanogenesis and CO<sub>2</sub> emission, among other responses. Ultimately, a positive relationship of methanogenesis and CO<sub>2</sub> emission, a measure of biological activity, would indicate O<sub>2</sub> consumption is a control of methanogenic microsite formation, while a positive relationship of methanogenesis with WFPS would indicate O<sub>2</sub> diffusion is a control. These mechanisms are not mutually exclusive and it is likely that both drive methanogenesis, but it is not clear if one is the dominate control of upland methanogenesis

In examining the implications of anoxic microsites, we test the hypothesis that greater methanogenesis will coincide with higher ammonium concentrations and a lower fraction of inorganic nitrogen in the form of nitrate. These patterns have been observed at larger scale studies of wetlands and aquatic sediments (Seitzinger, 1988; Reddy and DeLaune, 2008; Maltais-Landry et al., 2009), but have not been evaluated in upland soils. Given the potential for enhanced coupling of nitrification with denitrification in association with anoxic microsites, we also hypothesize that N<sub>2</sub>O emissions will be positively correlated with methanogenesis.

We also document temporal patterns in methanogenesis because the biological demand for O<sub>2</sub> may change over time. We held the physical factors of soil moisture and structure constant over time while expecting biological activity (measured as soil respiration) to peak in the first two weeks and gradually decrease in the following 15-20 weeks (Cotrufo et al., 1994; Alster et al., 2016). These two time points allow a contrast of early methanogenesis, coinciding with high rates of respiration, with late methanogenesis, occurring well after the peak respiration. Because previous work has shown that even with ample labile C and high WFPS methanogenesis may not begin for 30-80 days following the start of incubation conditions (Peters and Conrad, 1995; Yao et al., 1999) we chose to measure methanogenesis and its covariates at 6 and 21 weeks into the incubation.

Soil macrofauna (e.g., larvae, earthworms) can also host anaerobic processes in their guts (Kamman et al., 2009; Šustr and Šimek, 2009), though these anoxic microsites are primarily under

control of the host as opposed to soil properties. If there are strong correlations between soil faunal activity and methanogenesis, this would indicate that gut interiors may contribute a large portion of soil methanogenic volume.

The goals of this study were to, a) measure effects of agricultural practice, site/soil, and soil moisture on rates of methanogenesis, b) determine how soil properties and treatments affect the formation and persistence of methanogenic microsites, and, c) evaluate relationships between methanogenesis and other redox-sensitive processes including inorganic N speciation, N<sub>2</sub>O emission, and microbial biomass. To achieve these goals, we collected soil cores from fields managed for conventional, no-till, and organic crop production at two long-term agricultural field sites at two field sites with contrasting climates that are common for US croplands: a mesic site in central Michigan and a more xeric site in eastern Colorado. Soils cores were incubated under a range of soil WFPS for five months. During that period we measured, gross CH<sub>4</sub> fluxes and net CO<sub>2</sub>, N<sub>2</sub>O and CH<sub>4</sub> fluxes at 6 weeks and 21 weeks. To quantify gross CH<sub>4</sub> production and consumption we developed and implemented a method of high-throughput, automated isotope pool dilution measurements with dynamic volume headspaces, building on an earlier manual method of static headspace volumes (von Fischer and Hedin, 2002). We characterized soil faunal activity multiple times and at the end of the incubation we measured soil redox potential, inorganic nitrogen, pH, soil carbon, and microbial biomass.

## **3.2 - Methods**

### 3.2.1 - Field sites

Soils were collected from the USDA-ARS Central Great Plains Research Station, Akron, Colorado, USA (40.15°N, 103.15°W, 1384 m elevation), hereafter “Akron”, and the Kellogg Biological Station LTER, Hickory Corners, Michigan, USA (42.4° N, 85.4°W, 288 m), hereafter “KBS”. Mean

annual precipitations are 418mm and 900mm and mean annual temperatures are 9.0°C and 9.7°C for Akron and KBS, respectively (Mikha et al., 2013; Robertson and Hamilton, 2015). Akron soils are Weld silt loam (fine, smectitic, mesic Aridic Paleustolls) with textures ranging from 27-32% sand, 43-50% silt, and 22-28% clay (Mikha et al., 2013) and KBS soils are a mix of Kalamazoo loam (fine-loamy, mixed, mesic Typic Hapludalfs) and Oshtemo sandy loam (coarse-loamy, mixed, mesic Typic Hapludalfs) with textures ranging from 43-59% sand, 27-38% silt, and 14-19% clay (Robertson and Hamilton, 2015).

At Akron, soils were collected from long-term tillage study plots in 2-year wheat/fallow rotation that were under conventional tillage (mold-board plowing) with chemical fertilizers (“Akron Conventional”) and no-till with chemical fertilizers (“Akron No-till”), see Mikha et al. (2013) and Halvorson et al. (1996) for management details. Soils were also collected from recently established (2010) organically managed wheat-fallow plots (“Akron Organic”), see Calderón et al. (submitted) for management details. In October 2012, prior to wheat planting, (20 months prior to soil sampling) Akron Conventional and Akron No-till plots received 13.6 kg ha<sup>-1</sup> of urea (46-0-0) and Akron Organic received 109,000 kg ha<sup>-1</sup> of composted beef feedlot manure. Akron Conventional and No-till soils contain 6.55 g C kgdw<sup>-1</sup> and 7.55 C g kgdw<sup>-1</sup> total carbon, respectively (0-20cm; Mikha et al. 2013) and Organic 19.4 g C kgdw<sup>-1</sup> total carbon (0-30cm, F. Calderón, personal communication). Mikha et al. (2013) also found that Akron No-till soils (0-20cm) have roughly twice the mass of macroaggregates (>250 µm) and less microaggregates and silt and clay fraction compared to Akron Conventional.

At KBS, soils were collected from the Main Cropping System Experiment plots, treatments one (conventional), two (no-till), and four (organic). The conventional plots are tilled (chisel plow) and receive chemical fertilizers (“KBS Conventional”), no-till plots receive chemical fertilizers (“KBS No-till”), and the organic plots receive chisel tillage with only winter leguminous cover crops as fertilizer (“KBS Organic”), see Syswerda et al. (2011) and Robertson and Hamilton (2015) for

management details. The 3-year rotation for KBS Conventional and KBS No-till is corn/soybean/winter wheat while the rotation for KBS Organic is corn/ryegrass/soybean/winter wheat/red clover, with ryegrass and red clover as winter cover crops. KBS Conventional and KBS No-till plots received 47 kg K<sub>2</sub>O ha<sup>-1</sup> April 18<sup>th</sup>, 2014 and 32.8 kg N ha<sup>-1</sup> and 22.3 kg P<sub>2</sub>O<sub>5</sub> ha<sup>-1</sup> on May 12<sup>th</sup>, 2014. Soil total carbon of these soils (top 25cm) in 2001 was 10.4 g C kgdw<sup>-1</sup>, 11.5 g C kgdw<sup>-1</sup>, and 12.2 g C kgdw<sup>-1</sup> for KBS Conventional, No-till, and Organic plots, respectively (Syswerda et al., 2011). Aggregation varies between KBS treatments with Organic and No-till having more large macroaggregates (2000-8000 μm) than Conventional, No-till having more macroaggregates (250-2000 μm) than the other two treatments, and Conventional having the greatest amounts of the two smallest fractions (53-250 μm, <53 μm; Grandy and Robertson, 2007).

### 3.2.2 - Intact soil core removal and processing

We assayed intact soil cores in this experiment to maintain the soil structures which might affect methanogenesis and anoxia and to ensure our study units were similar to soils under field conditions. At each site, three field (i.e., plot) replicates of each agricultural treatment were visited and six intact soil cores in PVC pipe sleeves were removed from each plot (n=18 plots; n=108 cores extracted). Cores were extracted at Akron on May 21<sup>st</sup> 2014, which was the year of fallow rotation and followed the wheat rotation, and from KBS on June 2<sup>nd</sup>-3<sup>rd</sup> 2014, which was the year of corn rotation and followed the wheat rotation. The cores were collected using 18cm lengths of PVC pipe (7.62 cm i.d.) that had beveled ends to facilitate insertion into soil. During core removal, the PVC pipes were slowly driven into the soil by hand in order to minimize compaction and fracturing of core soils. PVC pipe soil cores were inserted 15±1cm deep and were then excavated out to minimize disturbance to the soil structure, placed in plastic zip-closure bags, and stored on ice in coolers until returned to the lab (<72 hours). The intact cores were then stored at 4°C for less than 14 days until initial soil

processing or the start of the pre-incubation. Only areas of bare soil without plants were chosen for sampling, at KBS these were inter-row areas.

### 3.2.3 - Processing of cores to assess field conditions

A subset of cores from each field plot were processed to obtain measurements of the initial conditions of the soils (n=36). Within 1 week of the field extraction of cores, two of the six cores from each plot were sacrificed to characterize initial conditions; they were sieved (4.25mm), measured for bulk density and subsampled for pH, inorganic nitrogen, soil organic carbon (SOC), and gravimetric moisture. Measurements of bulk density and gravimetric moisture of initial conditions cores was used to determine the percent WFPS at field moisture for each plot and to determine the target water masses for incubation moisture treatment cores. The four remaining cores from each plot were assigned moisture targets across the range of 15-65% water holding capacity. Because each core differed slightly in porosity and because the true porosity of each core was not known until final sacrifice, a relatively uniform distribution of experimental WFPS from 10-95% was imposed.

### 3.2.4 - Incubation preparation and procedures

Cores for the incubation (n=72) were sealed on the bottom (one layer of 5mil HDPE film and two layers of 10mil PVC pipe tape) and placed in a Percival I-35LVL model incubators set to 23<sup>0</sup>C for a 3-7 day pre-incubation. Cores were adjusted to target moistures by air drying or regular moisture additions. Most cores had reached the targets by week 6 of the incubation, although 13 of the 72 cores required more than 15% WFPS change between weeks 6 and 21 to reach targets (10 of these required further drying). Once cores reached target moisture levels the pipe tops were covered with perforated HDPE film to allow gas exchange while avoiding high evaporation. To maintain cores at the target WFPS levels soil moisture was adjusted every 2-5 weeks. Cores were stored in the incubators



at 23<sup>0</sup>C for the duration of the incubation period. The soils did not contain living plants, if any germinated during the incubation they were removed.

### 3.2.5 - End of incubation core processing

At the end of core incubations, we first made gravimetric and redox measurements of the intact cores and then sieved and subsampled the soils. During measurements of soil redox potential, the bottom seal of the PVC pipe was removed and the soil core was placed on a wet, porous mat connected to the reference electrode of the redox array (see below). We measured redox potential at three depths (surface, 3cm, 8cm) with 5 probes (n=15 measurements per core). Fifteen of the soil cores were too dry and hard to pierce with probes so these were not measured. Occasionally probes malfunctioned and that data was excluded. The majority of soil volumes were oxic in each core so we used only the minimum (i.e., most reducing) redox value observed of each core in our analyses since that value should indicate the likelihood of methanogenesis and other anaerobic processes. After final redox potentials were recorded soils were sieved (4.25mm), homogenized, and subsampled. Subsamples were stored at -20<sup>0</sup>C for subsequent pH measurement, maintained at 4<sup>0</sup>C for inorganic N, microbial biomass measures, or, in the case of soil organic carbon content, dried at 55<sup>0</sup>C for 24 hours.

### 3.2.6 - Redox array

Redox measurements were made with an array modified from Rabenhorst (2009): platinum electrodes were connected to a calomel reference electrode with operational amplifiers (MAX406BCSA+), powered by a 9V battery. The reference electrode was connected to the base of the moistened soil cores via a KCl salt bridge. Probes had 5mm long tips made of 0.5mm diameter Pt

wire and potentials were measured with a high precision voltmeter (ThermoScientific Orion 3 Star Benchtop pH meter).

### 3.2.7 - Soil properties

We measured pH in a 1:1 w/w solution of dry soil and deionized H<sub>2</sub>O. Inorganic N was extracted over 24 hours in a solution of 10 gdw soil and 45ml 1M KCl incubated in a 60ml *Whirlpak* bag (Nasco Inc., Fort Atkinson, WI, USA) It was then stored at -20<sup>0</sup>C until NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> were quantified colorimetrically (Alpkem Flow Solution IV, O.I. Analytical, College Station, TX, USA). Microbial biomass C and N were extracted using a chloroform slurry technique adapted from Fierer and Schimel (2003). Our method differs in that jars were shaken for 4 hours at 200 RPM and that cell lysis extracts were sparged with N<sub>2</sub> gas to volatilize any residual chloroform. Microbial biomass extracts were stored at -20<sup>0</sup>C until analysis on a Shimadzu TOC-L (Shimadzu Scientific Instruments Inc., Columbia, MD, USA).

### 3.2.8 - Soil fauna

We documented soil faunal activity throughout the experiment, which primarily amounted to the presence of worm castings on the soil surface or the rare emergence of a coleopteran. Prior to sacrifice, any bioturbation of the soil surface was recorded, and during the sacrifices the presence of earthworms, small worms (i.e., Enchytraeids or nematodes), coleopterae, larvae, and ants was recorded; these soil animals were removed from the sieved soil when possible so their biomass would not impact post-sacrifice chemical measurements. For subsequent statistical analysis, we categorized the impact of soil fauna: category 1, large (>1mm girth) macrofauna found during incubation (i.e., coleopteran emergence) or sacrifice, or evidence of macrofaunal activity (e.g., worm castings) on soil surface; category 2, evidence of small soil fauna/mesofauna or their activity (i.e., sub-millimeter

bioturbation observed on soil surface or macroscopic worms, <1mm girth, observed at sacrifice); category 3, no evidence of soil fauna during incubation or sacrifice.

### 3.2.9 - Net CO<sub>2</sub>, N<sub>2</sub>O, CH<sub>4</sub> fluxes

Measurements of net gas fluxes were made one or two days prior to gross CH<sub>4</sub> measurements and were performed by sealing the cores in plastic containers, elevating headspace [CH<sub>4</sub>] to ~10ppm, taking two headspace gas samples over 4-5 hours, quantifying the change in headspace gas concentrations, and calculating the flux rates. The HDPE containers were 24.5 cm tall with a diameter of 12.4 cm and a volume of 3.05 liters (U.S. Plastics), lids were fitted with black butyl septa for syringe sampling (Geomicrobial Technologies, Ochelata, OK, USA) and sealed with high vacuum grease. After the cores were sealed in the jars concentrated CH<sub>4</sub> to bring the headspace [CH<sub>4</sub>] to ~10ppm, matching the concentrations used in the isotope pool dilution assays. 30ml of room air was also added to the headspace to pressure balance the gas removed at the first sampling. Headspace and soil air were allowed to equilibrate for 45minutes, then the first headspace gas sample was collected. Following this sampling the cores were removed from the jars and returned to the incubator. At each sampling, 30ml of headspace gas was removed and injected into 20ml glass vials sealed with gray butyl septa and stored until measurement.

Headspace gas concentrations (CO<sub>2</sub>, CH<sub>4</sub>, and N<sub>2</sub>O) were measured on a custom hybrid gas chromatograph/laser absorption spectroscopy system; N<sub>2</sub>O was measured on a Shimadzu 14B gas chromatograph (GC) with electron capture detector, while CO<sub>2</sub> and CH<sub>4</sub> were measured on a Los Gatos Research Greenhouse Gas Analyzer (LGR GGA). The GC was plumbed in a standard configuration with on-column injection of sample via injection port into an N<sub>2</sub> carrier gas stream. In a novel configuration that we developed, air samples drawn from the same vial as for N<sub>2</sub>O were injected into a stream of zero air (i.e., 80% N<sub>2</sub>, 20% O<sub>2</sub>, no trace gases) via an injection port on the

inlet line to the LGR GGA. Sample air was drawn by the LGR GGA internal pump from the inlet line through an open split. Sample CH<sub>4</sub> and CO<sub>2</sub> moved through the LGR GGA as pulses over time that were transmitted by the analog-out ports on the LGR GGA and integrated by Shimadzu EZstart software. During sample injection, a AOC-5000 Combi-PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) injected 5ml of sample gas into the separate injection ports of the GC and LG. All samples were calibrated against certified lab standard gases.

### 3.2.10 - Gross CH<sub>4</sub> production

To make measurements of gross CH<sub>4</sub> production (i.e. methanogenesis) in week 6 and 21 we developed and utilized a novel dynamic headspace volume CH<sub>4</sub> isotope pool dilution method. This new method builds on the approach of von Fischer and Hedin (2002) but where that method enclosed soil samples in rigid jars of a static volume, the new method enclosed soil samples in flexible foil-layered polyester bags whose volume decreases with each headspace gas sampling. This dynamic volume approach allows for automated sampling with a laser based spectrometer (MCIA; Methane Carbon Isotope Analyzer, ABB - Los Gatos Research Inc., San Jose, CA, USA) that requires large (i.e. >700ml) gas samples to accurately quantify carbon CH<sub>4</sub> isotope concentrations.

#### 3.2.10.1 - Numerical solution for a dynamic headspace with discrete losses of volume

Because volume was removed in discrete events (e.g., 100ml removed every 5 hours during a 2 minute sampling) the number of moles was re-calculated at the end of each gas removal event (i.e., sampling event). But in the periods between sampling events the headspace can be treated as static. To begin, we adapt an equation for a static headspace adapting from von Fischer and Hedin (2002) eq 4.

We choose to adapt this way to specify the units of  $K$  for clear interpretation of  $K$ 's meaning,

$$\begin{aligned}\frac{dM_v(t)}{dt} &= P - K \cdot C(t) \\ &= P - K \cdot \frac{M_v(t)}{V_S}\end{aligned}$$

Where,

$\frac{dM_v(t)}{dt}$  is the change in the volume of methane per time,

$P$  is the production rate of  $\text{CH}_4$  in volume per time,

$K$  is the consumption rate of  $\text{CH}_4$  in the soil in volume per time per concentration  $\text{CH}_4$ ,

$V_S$  is the total bag headspace volume of all gases (constant during an individual time period),

$C(t) = \Phi(t) = \frac{M_v(t)}{V_S}$  is the concentration of  $\text{CH}_4$  at time  $t$ ;

all concentrations are in vol/vol, not in ppm.

Since the original static headspace solution was,

$$M(t) = \frac{P}{K} - \left(\frac{P}{K} - M_0\right) \cdot e^{-Kt} \quad \text{eq. 5 from von Fischer and Hedin (2002)}$$

and we divided  $K$  of von Fischer and Hedin (2002) eq. 4 by  $V_S$ , we substitute that into the existing solution (i.e., von Fischer and Hedin 2002, eq 5),

$$M(t) = \frac{V_S \cdot P}{K} - \left(\frac{V_S \cdot P}{K} - M_0\right) \cdot e^{-\frac{Kt}{V_S}}$$

The above equation should be used to calculate the change in  $M(t)$  during a single time period (i.e., between two measurements). Then  $M(t)$  and  $V_S$  are adjusted for the next time period by subtracting the methane volume and total bag volume lost during gas sampling.

The solution can be altered slightly to use the initial concentration instead of moles:

$$M(t) = \frac{V_S \cdot P}{K} - \left(V_S \cdot \left(\frac{P}{K} - \Phi_0\right)\right) \cdot e^{-\frac{Kt}{V_S}}$$

Where,

$$\Phi_0 = \frac{M_0}{V_S}, \text{ i.e. the CH}_4 \text{ concentration at } t_0 \text{ in vol/vol.}$$

In order to implement this solution we calculate the expected  $P_{12}$  and  $K_{12}$  for  $\text{CH}_4$  with the model and then calculate the  $^{13}\text{C}$  production and consumption with constants obtained empirically,

$$P_{13} = 0.01055627 \cdot P_{12}$$

$$K_{13} = 0.98 \cdot K_{12}$$

Then the model estimates are compared to the data, goodness of fit term is calculated (e.g., squared error), and that term is minimized through iterative variation of  $P_{12}$  and  $K_{12}$ . See supplemental information for code of the model implementation and optimization in R.

### 3.2.10.2 - Measurement array

The measurement array consists of multiple sample and standard polyester bags connect via high density polyethylene lines to interconnected gas manifolds, with their final output going to the MCIA. The MCIA is set to run constantly and record all data. Gas manifold management was performed by an Arduino MEGA microcontroller (*arduino.cc*, Arduino S.R.L., Italy). This independent microcontroller provided more flexibility of sampling timing as compared to the MCIA internal manifold management software. The system is illustrated in fig. 3.6. The 13 liter polyester foil layered soil sample bags ([www.sorbentsystems.com](http://www.sorbentsystems.com), IMPAK Corp., Los Angeles, California, USA) were fitted with 1/4 inch bulkheads (Swagelok, Solon, Ohio, USA) to enable gastight connection with manifolds.

### 3.2.10.3 - Array operation

First, the MCIA was powered on and allowed to reach a stable temperature. Then soil cores were placed in polyester sample bags that were closed by heat-sealing. Additions of a  $^{12}\text{CH}_4 + ^{13}\text{CH}_4$  mixture brought the bag headspace to  $\sim 10\text{ppm } ^{12}\text{CH}_4$  and  $\sim 1\text{ppm } ^{13}\text{CH}_4$ .  $\text{SF}_6$  was added at the same

time to quantify initial headspace volume. Gas ports were sealed and bags allowed to stand for 4 hours to allow headspace and soil air gases to equilibrate, then a 30ml gas vial sample was taken for later SF<sub>6</sub> quantification via the gas chromatograph. Sample, check standard, and calibration bags were then connected to gas manifolds. Sampling cycles commenced, with gas sampling of the calibration bags, then each soil sample bag; the check standard bag was sampled in between pairs of sample bags to provide information for drift corrections. Sampling cycles were separated by ~11 hours and a total of 6-8 cycles are run. Gas sampling lasted 400 sec for each bag, resulting the removal of 726 ml of gas volume from the bags. Each full cycle with 18 sample bags was ~5 hours in duration. After the final sampling cycle, soil cores were removed from the bags and returned to storage incubators.

#### 3.2.10.4 - Isotope pool dilution data processing and model fit

Data was exported from the MCIA in raw form and a run log of sampling times was calculated according to the sampling schedule. MCIA data and run logs were imported to a database for efficient data handling. Every gas sampling was averaged over the final 150 sec of sampling (the first 250 sec data are discarded) to average over instrument noise and obtain a value of concentration of <sup>12</sup>CH<sub>4</sub> and <sup>13</sup>CH<sub>4</sub> for each sampling time. These initial concentrations were calibrated against the gas calibration standards used at the beginning of the cycle, different calibrations were used for each cycle to correct for inter-cycle instrument drift. Finally, calibrated sample concentrations were corrected for intra-cycle drift, if present, with check standard bag data. The sample bag concentration data and sampling times were exported to model fitting software (e.g., Microsoft Excel or R)

The data were fit to the above derived dynamic headspace volume isotope pool dilution model by iterative optimization (see fig. 3.7 for example fit). The model goodness of fit was assessed via fit statistics (e.g. r<sup>2</sup>) and visually. Poor fits from the same cycle were analyzed for similar trends, if specific sampling cycles showed values that diverged consistently from other cycles or other systematic errors

became apparent drift correction in the data base was revisited to improve corrections or the timepoint(s) in question were removed from the model fit analysis. Gross production (methanogenesis) and consumption (methanotrophy) fluxes were calculated from the model P and K fits.

### 3.2.11 - Statistics

When the error structures were log-normal, as was the case for  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , microbial biomass, soil organic carbon,  $\text{CO}_2$  emission,  $\text{N}_2\text{O}$  emission, and methanogenesis, we log transformed data prior to statistical analyses. We regressed soil properties and gas emissions over experimental factors (site, agronomic treatment, WFPS) separately to evaluate how they were distributed and compared to methanogenesis. We categorized cores into one of four groups based on the temporal patterns of methanogenesis from week 6 to week 21. This allowed us to test how factors are related to presence or persistence of microsites and how the drivers may change over time. We analyzed the rates, presence and persistence of methanogenesis in two ways, 1) an ANCOVA over experimental factors, 2) single factor linear regression over factors connected to potential mechanisms (i.e., WFPS,  $\text{CO}_2$  flux, soil faunal activity). The ANCOVA allowed us to test how the experimental factors related to agriculture affected methanogenesis. With the regressions we were able to explore the relationships between methanogenesis and soil properties and gas fluxes related to likely mechanisms of methanogenic microsite formation and maintenance. Regressions of persistence used week 21 values for WFPS and  $\text{CO}_2$  flux, methanogenesis regressions used the values aligned with the measurement period. Statistical analyses were performed in R version 3.2.5 and JMP Pro 12.2 (SAS Institute Inc., Cary, NC, USA).



### 3.3 - Results

#### 3.3.1 - Soil properties at sacrifice

Soil physical and chemical properties primarily varied over agronomic treatment and WFPS, and some varied by site. Organic soils had higher  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , and microbial biomass than No-till and Conventional soils, indicating that organic amendments had significantly altered the soil environment (Table 1). No-till soils had lower pH than Conventional and Organic soils. Akron soils had higher pH,  $\text{NO}_3^-$ , and microbial biomass than KBS soils, the latter two differences driven by much higher  $\text{NO}_3^-$  and microbial biomass in Akron's Organic soils. Significant site-based differences in porosity across treatments were driven by porosities around 60% for Akron's Conventional and Organic plots; all other plots had 40-45% mean porosity. Both No-till and Organic cores had 2 to 3 fold more soil faunal activity than Conventional cores.

#### 3.3.2 - $\text{CO}_2$ , and $\text{N}_2\text{O}$ by time and exp. Treatments

Emissions of  $\text{CO}_2$  and  $\text{N}_2\text{O}$  all increased with WFPS and were generally greater in Organic plots than in Conventional and No-till (fig. 3.8). Between weeks 6 and 21,  $\text{CO}_2$  emissions decreased 69% from 7.58 to 2.38 mg C- $\text{CO}_2$   $\text{gdw}^{-1} \text{d}^{-1}$  while  $\text{N}_2\text{O}$  fluxes decreased 57% from 5.64 to 2.42  $\mu\text{g N-N}_2\text{O}$   $\text{gdw}^{-1} \text{d}^{-1}$ . No gas emissions differed significantly between sites.

#### 3.3.3 - Methanogenesis over time and categories

Of the 72 cores that we analyzed, 59 exhibited rates of methanogenesis that were quantifiable (i.e., above the method's detection limit of  $5.2 \times 10^{-5}$   $\mu\text{g C day}^{-1} \text{kgdw}^{-1}$ ). The mean rate of methanogenesis did not differ between weeks 6 and 21, but rates of individual cores did show temporal patterns.

The nature of this data, with both continuous variation in rates of methanogenesis and categorical behavior (i.e., presence/absence and persistence of measurable methanogenesis) enables both continuous and categorical analyses. As illustrated in Figure 3.1, we categorized temporal patterns in methanogenesis by the presence/absence of measurable methanogenesis at 6 and 21 weeks. The categories were: *Consistent* (methanogenesis rates above detection limit at week 6 and 21; n=27 of 72 cores), *Quick* (methanogenesis detected only week 6; n=21), *Slow* (methanogenesis detected only week 21; n=11), and *None* (methanogenesis never detected; n=13). Further, we choose a contrast of Consistent and Quick cores to represent persistence of methanogenesis from week 6 to 21. First we analyzed these three methanogenesis metrics (rates, presence, and persistence) with full factorial ANCOVAs of experimental treatments (site, agronomic treatment, WFPS; table 2, top), then we individually regressed the methanogenesis metrics over factors related to mechanisms of anoxia (CO<sub>2</sub> flux, WFPS, soil faunal activity; table 2, bottom).

### 3.3.3.1 – Week 6 rates and presence

At week 6, the magnitude of methanogenic rates was primarily affected by soil WFPS ( $p=0.007$ ) and secondarily by agronomic treatment ( $p=0.052$ ), while the presence of methanogenesis was instead affected by agronomic treatment ( $p=0.008$ ) and its interaction with site ( $p=0.014$ ; table 2). Rates of methanogenesis increased exponentially with greater WFPS (fig. 3.3B) with the greatest rates occurring at WFPS > 75%. Two previous studies found that anaerobic activities increased dramatically in upland soils with WFPS > 60% (Linn and Doran, 1984; von Fischer and Hedin, 2007), cores falling above this threshold in our experiment were nearly two times more likely to be methanogenic than cores with WFPS < 60%. Organic cores had more than three-fold the rates of methanogenesis that Conventional cores had ( $p=0.052$ ), while No-till rates did not differ from the other agronomic treatments. However, presence of methanogenesis was equally high in both Organic

and No-till cores (present in 19 of 24 cores of both treatments) and much less frequent in Conventional cores (present in 10 of 24 cores;  $p=0.008$ ). Interestingly, this pattern was driven by strong differences among KBS agronomic treatments; in contrast, Akron cores had similar frequencies of measurable methanogenesis across agronomic treatments (fig. 3.9).

In regressions of week 6 methanogenic rates over mechanistic factors, WFPS ( $p=0.002$ ;  $r^2=0.13$ ) and soil respiration ( $p=0.007$ ;  $r^2=0.10$ ) had similar levels of explanatory power (table 2). Combining WFPS, respiration, and fauna in a three factor regression of methanogenic rates resulted in improved explanatory power ( $R^2=0.18$ ) but did not indicate which factor was most important as all were non-significant.

Cores with faunal category 1 (i.e., evidence of large soil fauna) had higher rates of methanogenesis than category 3 (i.e., no evidence of any fauna) that was nearly significant at week 6 ( $p=0.053$ ; table 2). Three of the four cores that had rates of methanogenesis in the range expected from macrofauna (i.e., 1 to 20  $\mu\text{g kg dw}^{-1} \text{ day}^{-1}$ ; Kammann et al., 2009) fell into category 1, the other core with this rate fell into category 2. However, cores in faunal categories 1 and 2 were mostly from Organic and No-till treatments while those from category 3 were mostly Conventional treatments ( $p=0.004$ ), so there is co-variation of SOC and  $\text{CO}_2$  with these soil faunal categories.

### 3.3.3.2 Week 21 rates, presence, and slow-forming methanogenesis

At week 21, WFPS was the primary significant predictor in the experimental ANCOVA ( $p=0.015$ ) and among mechanistic regressions ( $p=0.006$ ). As was the case at week 6, the greatest rates occurred above 75% WFPS (fig. 3.3; fig. 3.10) and during week 21 cores with WFPS > 60% were more than five times more likely to be methanogenic than drier cores. (When combined with week 6 data, cores with WFPS > 60% were 3.15 times as likely to host methanogenesis than cores with WFPS < 60%.) The 3-way interaction was also significant in the ANCOVA ( $p=0.027$ , Table 2), caused by

differences in the regression slope of rate of methanogenesis over WFPS: among the site X agricultural treatment groups there is a significant difference between the strongly positive slope for KBS Organic and the neutral slope for Akron Organic, and all other slopes were neutral or modestly positive (data not shown). The presence of methanogenesis at week 21 was not predicted well by the experimental ANCOVA but greater WFPS and CO<sub>2</sub> flux both correlated with higher likelihood of methanogenesis (Table 2).

In cores where measurable methanogenesis occurred only at week 21 (i.e. Slow category, n=11), most were from Conventional treatments (n=7 of 11). The Slow set of cores did not include any cores with low redox potential, nor did any cores show gleying (i.e., reduced Fe oxides), and did not have higher CO<sub>2</sub> fluxes than any other groups. Although not significantly different, the average week 21 WFPS of Slow cores (54%) was greater than the WFPS of Quick and None cores (39% and 46%, respectively) and less than the average of Consistent cores (62%).

### 3.3.3.3 Persistence

Contrasts between the Quick and Persistent groups may provide insight into the causes of methanogenic persistence. The Consistent group had 50% greater WFPS ( $p=0.001$ ) than the Quick group and twice the CO<sub>2</sub> emission ( $p=0.023$ ) at 21 weeks. The relationship of WFPS with persistence differed by agricultural treatment ( $p= 0.005$ ): Consistent cores had the same WFPS levels as Quick cores in Organic soils, but in No-till soils the Consistent cores fell overwhelmingly (n=10 of 12) above 55% WFPS while the Quick were cores all below 55% WFPS (fig. 3.4). Conventional soils trended towards having greater WFPS in Consistent cores. Compared to all other groups, the Consistent group had the lowest proportion of Conventional soils (18.5%), the highest proportion of low redox measurements (14.8%), contained all the cores with visible gleying (n=7) and had a high proportion of cores (63%) with soil faunal activity (i.e., faunal categories 1 and 2). At 6 weeks the Quick cores'

rates of methanogenesis were lower than the Consistent cores. The 6 to 21 week decline in CO<sub>2</sub> emissions of the Quick cores was not significantly greater than other groups.

#### 3.3.3.4 Consistent absence of methanogenesis

Cores of the None group that never hosted measurable methanogenesis differed from the other groups had lower WFPS prior to the incubation (i.e., field moisture content) than all other groups ( $p=0.006$ ; mean=51% WFPS), but after moisture manipulations were made they did not have different WFPS than other groups ( $p=0.16$ ).

#### 3.3.4 - Relationships of methanogenic microsites with other (redox-sensitive) biogeochemical processes

We present here results of simple logistic and linear regressions between methanogenesis and the other soil properties that we measured. We tested more complex regressions (i.e., multiple regression including combinations of methanogenesis, site, agricultural treatment, WFPS), but these did not yield different inferences or greater understanding than the single-factor regressions provide. A principal components analysis (fig. 3.11) provides an overview of how these factors are related to one another.

##### 3.3.4.1 - N availability

We found clear correlations between methanogenesis and inorganic N across all cores and the various subsets (fig. 3.5). The concentration of NH<sub>4</sub><sup>+</sup> was positively correlated with mean methanogenesis ( $r^2=0.30$ ) while NO<sub>3</sub><sup>-</sup> and % inorganic N as NO<sub>3</sub><sup>-</sup> were negatively correlated ( $r^2=0.17$  and 0.43 respectively). The explanatory power of these fits was much greater than those resulting from regressing inorganic N concentrations over mean CO<sub>2</sub> or N<sub>2</sub>O fluxes ( $r^2 = 0$  to 0.11, not shown).

#### 3.3.4.2 - N<sub>2</sub>O emission

When averaged over weeks 6 and 21, rates of methanogenesis and N<sub>2</sub>O emission were correlated (fig. 3.5). CO<sub>2</sub> emission ( $r^2=0.46$ ) and WFPS ( $r^2=0.31$  and  $0.43$ , for weeks 6 and 21, respectively) were more strongly correlated with N<sub>2</sub>O than methanogenesis is, so in this experiment methanogenesis is an indicator, but not a superior predictor, of N<sub>2</sub>O production.

#### 3.3.4.3 - Microbial biomass

Methanogenesis at 21 weeks was negatively correlated with microbial biomass ( $p=0.039$ ). This relationship is strongest in Organic treatments and not present in Conventional. The highest microbial biomass was found in Organic treatments that had very low or zero methanogenesis at week 21.

#### 3.3.4.4 - Reduction-oxidation potential

Low redox potentials were correlated with elevated rates of methanogenesis at both week 6 ( $r^2=0.30$ ) and week 21 ( $r^2=0.22$ ). Although redox potential does not directly measure any processes it reflects the mean redox state of terminal electron acceptors present, thus in cores with sub-oxic potentials it indicates very different microbial habitats present.

### 3.4 - Discussion

This study addresses fundamental questions of upland methanogenesis formation and persistence, as well as applied questions of agricultural impacts on upland anaerobic processes and their feedback to nutrients. To characterize mechanisms of anoxia in unsaturated upland soils, we measured methanogenesis in soil cores from a range of agricultural treatments, sites, and soil moistures. We found that soil moisture and elevated soil respiration both appeared to play roles in forming and maintaining methanogenesis, and the importance of these drivers shifted over time. Our

results are novel in showing that methanogenesis in upland soils was associated with lower  $\text{NO}_3^-$  and higher  $\text{NH}_4^+$  suggesting an impact on plant nutrition. Soils under no-till and organic managements had a propensity for methanogenesis, especially when wet, but the high variability of methanogenesis across experimental factors suggests that this process may be less dependent on management and environmental factors than other trace gas fluxes (i.e.,  $\text{CO}_2$ ,  $\text{N}_2\text{O}$ , gross  $\text{CH}_4$  uptake).

### 3.4.1 – Relationships of $\text{CO}_2$ flux, WFPS, and macrofauna to methanogenic rates, presence, and persistence

#### 3.4.1.1 - Presence and rates of methanogenesis

We hypothesized that factors closely related to  $\text{O}_2$  consumption and diffusion to microsites,  $\text{CO}_2$  emission and WFPS, respectively, would correlate with methanogenic rates, presence, and persistence. Early in our incubation both  $\text{CO}_2$  flux and WFPS were correlated with methanogenic rates while only WFPS predicted rates and persistence at later times. The coincidence of high  $\text{CO}_2$  flux and high methanogenesis during week 6 is consistent with heavy microbial respiration causing high  $\text{O}_2$  consumption,  $\text{CO}_2$  production, and ultimately anoxic microsites. This conclusion was also suggested in a field study where  $\text{CO}_2$  emission was correlated with methanogenesis ( $r^2=0.17$ ) in a cornfield over the course of a growing season; this relationship was attributed to labile C from roots feeding  $\text{O}_2$  consumption and methanogenesis (Yang and Silver, 2016).

The observed increase in rates and presence of methanogenesis with WFPS in weeks 6 and 21 suggests that soil water may have decreased  $\text{O}_2$  diffusion by closing off pores. Soil moisture also typically increases microbial activity and this dual effect of moisture was likely a factor in week 6 but moisture's effect on  $\text{O}_2$  diffusion appears to have been a clear control of methanogenesis later in the incubation when soil respiration had declined. Our finding that methanogenesis is greatest above 75% WFPS and two to five times more likely to be present above 60% corresponds with previous findings

that anaerobic activity increases sharply above 60% WFPS in agricultural and forest soils (Linn and Doran, 1984; von Fischer and Hedin, 2007). In the above-mentioned cornfield study (Yang and Silver, 2016) and a study of soil cores from a temperate grassland (Kammann et al., 2009), soil moisture was not correlated with methanogenesis. However, both studies compared methanogenesis to gravimetric soil moisture while we used WFPS, a volumetric calculation of soil moisture that characterizes gas diffusivity better and is known to correlate well with anaerobic activity (Linn and Doran, 1984; Castellano et al., 2010). In fact, when our methanogenesis data was regressed over gravimetric moisture we found no significant relationships (not shown).

Kammann et al. (2009) found that *Amphamallin*, *Cetonia* and other arthropod larvae were probably responsible for the majority of methanogenesis in a grassland soil and hypothesized that earthworms have the potential to contribute as well. In our study, cores with soil faunal activity trended towards being more likely to have high early methanogenesis and three-quarters of cores with very high methanogenesis also had macrofaunal activity, however the effect of fauna is difficult to disentangle from effects related to soil respiration and WFPS. Specifically, faunal activity was better maintained in higher WFPS soils and was very uncommon in Conventional soils, which also had low CO<sub>2</sub> emission and soil organic matter content. Earthworms were responsible for most faunal activity in our study, but previous work has not found them to host methanogenesis (Šustr and Šimek, 2009; but see Depkat-Jakob et al. 2012), so it appears unlikely that soil faunal guts hosted meaningful methanogenesis in these soils. Experiments that control and manipulate soil fauna presence over a variety of SOC concentrations may be able to better elucidate the role that larvae and earthworms play in upland methanogenesis.



#### 3.4.1.2 - Persistence

Both low O<sub>2</sub> diffusion and high O<sub>2</sub> consumption may have contributed to the persistence of methanogenesis since soils that maintained methanogenesis from week 6 to 21, (the Consistent group), had relatively high CO<sub>2</sub> emission and 21 week WFPS. However, the cores whose methanogenesis was lost after week 6 (the Quick group) differed from Consistent cores mainly in having lower WFPS, so soil moisture appears to be the primary factor that determined whether methanogenesis persisted or not. The design of our experiment may also have contributed to the apparent importance of WFPS in persistence because labile organic matter sources were probably largely depleted, and thus CO<sub>2</sub> fluxes much lower, by week 21. Nonetheless, it is plausible that anoxia and methanogenesis can be maintained through consistently high O<sub>2</sub> consumption in soils with continuous labile C inputs (e.g., roots exudates; Fimmen et al. 2008) or large organic matter stores (e.g., litter layers or patches; van der Lee et al., 1999).

#### 3.4.1.3 - Slow and None groups of cores

The Slow group of cores were not significantly different from other groups in any of our measured soil properties. They did have relatively high week 21 WFPS, similar to the Consistent cores, and so higher moisture and slower O<sub>2</sub> diffusion may be important for late formation of methanogenesis as it was for persistence. The *Slow* group may also have required longer to reach measurable methanogenesis because of a lack of biological potential, i.e., there were few methanogens present at start of incubation so it took longer for populations to grow large enough to reach measurable rates. Most Slow cores were from conventionally managed plots and in a field study of the Akron plots the Conventional soils hosted much lower methanogenesis than the No-till plots (Brewer, chapter 2). Compared to no-till soils, tilled soils have lower microbial biomass and microbial communities with altered composition (Acosta-Martinez et al., 2010; Helgason et al., 2010;

Muruganandam et al., 2010) so it is plausible that low biological potential may have delayed the onset of methanogenesis. The absence of methanogenesis in the None cores may also have been the result of low biological potential since those cores had lower WFPS at soil sampling than the other groups and thus may have had lower WFPS and methanogenesis for a long period of time prior to field sampling.

#### 3.4.2 - Agricultural and site effects on methanogenesis

Upland methanogenesis was affected by agricultural practices and site in a variety of ways, some that appear to be related to respiration and WFPS but others that do not. The Organic soils were distinguished by high early rates of methanogenesis and high CO<sub>2</sub> emission, all likely due to higher quantities of soil organic matter. However, the identical frequency of methanogenic activity in Organic and No-till soils, indicates that robust biological activity was not a requirement for methanogenesis to be present. No-till soils had much lower CO<sub>2</sub> emission than Organic at week 6, low enough to be similar to Conventional soils, so the early methanogenesis in No-till soils may have been more dependent on low O<sub>2</sub> diffusion than early methanogenesis in Organic soils. Specifically, the No-till soils we sampled had more large aggregates than the Conventional and Organic soils (Grandy and Roberston, 2007; Mikha et al., 2013), so methanogenesis in No-till may have frequently been hosted in aggregate cores where O<sub>2</sub> is slow to diffuse from inter-aggregate pores. This would have made high organic matter and respiration less important for No-till methanogenesis and WFPS more important. Indeed WFPS was an important factor for persistence of methanogenesis in No-till soils - persistence in No-till soils diverged at 55% WFPS, the wetter soils were persistent while the drier ones lost methanogenesis, but Organic soil persistence appeared to be unaffected by WFPS.

The field site where the soil was from also appears to have affected methanogenesis, both early and late. While early methanogenesis was almost ubiquitous in KBS Organic and No-till and

uncommon in KBS Conventional soils, the presence of methanogenesis in Akron soils did not vary by agricultural treatment. Biological potential may have been greater in the KBS soils that are from a much wetter climate than the Akron soils, although there are many potential causes of this difference. Site also affected late methanogenesis with Organic soils from KBS having 25 fold the rates of methanogenesis compared to Akron Organic soils. The heavy field compost amendments in Akron Organic plots provided substrate for decomposition and the production of much more CO<sub>2</sub> and microbial biomass than KBS. A byproduct of the high decomposition rate in Akron Organic soil was high soil NO<sub>3</sub><sup>-</sup>, which was nearly seven fold greater than in KBS Organic (not shown). It is possible that these high NO<sub>3</sub><sup>-</sup> levels suppressed methanogenesis by allowing denitrifiers and other anaerobes to outcompete methanogens for electron donors or create toxic intermediaries like NO<sub>2</sub><sup>-</sup> (Klüber and Conrad, 1998; Yao et al., 1999; Nazaries et al., 2013).

Agricultural practices can also affect soil porosity and water-holding capacity which, in turn, affects WFPS dynamics. While our study intentionally maintained the WFPS of all agricultural treatments at similar levels and distributions, soil WFPS under field conditions would likely vary among different practices due to differences in soil porosity, rate of evapotranspiration, or other factors. For example, porosity of Akron No-till soil cores was 44% while it was ~59% for Akron's Conventional and Organic cores, so moisture additions varied between these groups to achieve the same WFPS targets. Since this can affect relative rates of N<sub>2</sub>O emission (Linn and Doran, 1984; Castellano et al., 2010) it would likely affect methanogenesis as well so future studies examining anoxic microsites should measure WFPS or matric potential, in addition to other soil moisture metrics.

### 3.4.3 - High variability of methanogenesis

A surprising feature of upland methanogenesis is the low explanatory power of soil properties and processes that predict other soil microbial processes well. In this study, multiple regressions of

methanogenesis with CO<sub>2</sub> flux, WFPS, and/or soil fauna as predictors had a maximum R<sup>2</sup> of 0.18. Previous studies have found similarly weak relationships with these factors: the highest R<sup>2</sup> found regressing methanogenesis over soil moisture, respiration, and other physical or chemical properties was 0.11 for a field study of the same Akron plots (Brewer et al., in preparation) and 0.17 for a grassland (Yang and Silver, 2016). Interestingly, our ANCOVA analyses that included experimental agriculture and site factors explained much more variability (R<sup>2</sup>=0.19 to 0.34) as did a multiple regression of methanogenesis that included agricultural factors in the field study of Akron plots (R<sup>2</sup>=0.70; Brewer et al., in preparation). Experimental ANCOVAs of CO<sub>2</sub>, N<sub>2</sub>O, and gross CH<sub>4</sub> consumption fluxes from our dataset had higher explanatory power, with R<sup>2</sup> ranging from .41 to .90 (not shown). It appears that methanogenesis is more variable than processes contributing to CO<sub>2</sub>, N<sub>2</sub>O, and gross CH<sub>4</sub> consumption fluxes, even in a controlled laboratory setting.

This difference in flux variability may be rooted in the spatial distribution of processes: methanogenesis has a strict set of chemical and biological controls (e.g., highly reduced conditions, presence of methanogens) that co-occur infrequently in upland soils while the other fluxes can occur across most soil areas. Respiration, nitrification, and CH<sub>4</sub> consumption are aerobic activities that require organic matter, NH<sub>4</sub><sup>+</sup>, or CH<sub>4</sub>, which are available throughout most soils. Denitrification requires minimally reduced conditions and NO<sub>3</sub><sup>-</sup>, but these conditions occur frequently in soil aggregates and areas of high respiration (Paul, 2015). Bulk properties such as WFPS or soil organic matter (SOM) content that come from homogenized soil samples may be effective predictors of processes that occur throughout the soil because neither are spatially limited, but methanogenic microsites likely demonstrate high spatial variability and, thus, are poorly predicted by non-spatial measurements of soil properties. If this is the case appropriate metrics of controls, such as the spatial distributions of SOM, aggregate sizes, pore sizes or O<sub>2</sub>, should predict methanogenesis better than bulk properties. Our statistical models that included experimental agronomic and site categories may

have predicted methanogenesis better than CO<sub>2</sub> flux, WFPS, and soil fauna because the experimental categories represent differences in distributions of soil properties or other factors related to drivers of methanogenic stochasticity.

#### 3.4.4 - Methanogenesis and other biogeochemical cycles

While the average rates of methanogenesis were low (0.3 µg C day<sup>-1</sup> kgdw<sup>-1</sup>, or, 0.10 mg C m<sup>-2</sup> d<sup>-1</sup>) and do not indicate major greenhouse gases sources, we also viewed methanogenesis as an indicator of anoxic conditions that provide ideal habitat for other anaerobic processes affecting redox-sensitive soil properties. In a first, we evaluated relationships between upland methanogenesis and soil N availability, gaseous loss via N<sub>2</sub>O, and microbial biomass.

Methanogenesis had a positive correlation with NH<sub>4</sub><sup>+</sup> and negative correlation with NO<sub>3</sub><sup>-</sup> and was a better predictor of these properties than either CO<sub>2</sub> or N<sub>2</sub>O fluxes. This suggests that processes controlling methanogenesis and N-cycling may be closely related. Pools of NH<sub>4</sub><sup>+</sup> may have enlarged in anoxic regions because anaerobic metabolisms require a higher substrate C:N ratio than aerobic metabolisms. Anoxia inhibits nitrification, an aerobic process, so NH<sub>4</sub><sup>+</sup> may have also accumulated in methanogenic soils because it was not being converted to NO<sub>3</sub><sup>-</sup>; this mechanism would explain both the NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> relationships with methanogenesis. Alternatively, the negative relationship between NO<sub>3</sub><sup>-</sup> and methanogenesis may have arisen from denitrifiers out-competing methanogens for C in NO<sub>3</sub><sup>-</sup> rich environments, as mentioned earlier. Finally, dissimilatory reduction of nitrate to ammonium (DNRA) may have converted NO<sub>3</sub><sup>-</sup> to NH<sub>4</sub><sup>+</sup> in soils with greater anoxic volumes (Paul, 2015). The complex relationships between O<sub>2</sub>, N-cycling, and methanogenesis preclude identification of a decisive explanation of our observations.

Correlations between N<sub>2</sub>O fluxes and methanogenesis may have resulted from co-occurring denitrification and methanogenesis since both require anoxia. It is unlikely the two processes would

coincide at micro-scales (i.e.  $<100\ \mu\text{m}$ ) because denitrifiers would out-compete methanogens for SOC (Nazaries et al., 2013), but a large anoxic microsite could have an outer layer where denitrification occurs while the microsite core is deeply reduced and methanogenic. Co-occurrence of  $\text{N}_2\text{O}$  and  $\text{CH}_4$  production has been observed in other studies of upland gross methanogenesis (Kammann et al., 2009) including a field study of these same soils (Brewer, chapter 1). The strength of the correlations from our dataset may be weak ( $r^2=0.07$  to  $0.08$ ) because denitrification can also occur when soils are not reduced enough to host methanogenesis or because  $\text{N}_2\text{O}$  reduction can suppress methanogenesis (Klüber and Conrad, 1998). Alternatively, if nitrification is the dominant source of  $\text{N}_2\text{O}$  instead of denitrification it could be occurring in oxic microsites while methanogenesis occurs in anoxic microsites of soils with high SOC and rates of decomposition.

The negative relationship between methanogenesis and microbial biomass indicate that anoxia may have also slowed the growth of microbial communities and decreased the accumulation of biomass over the incubation period. This appears strongest in the Organic cores where low methanogenic cores have the highest MBC in the experiment while the high methanogenesis cores have some of the lowest MBC measured. This relationship would be expected if anoxic microsites were large or encompassed primary SOC/resource patches since anaerobic metabolisms yield much less energy than aerobic metabolisms. Low microbial biomass in flooded, anoxic soils has been observed by Unger et al. (2009).

### 3.4.5 - Implications

Because anaerobic processes are fundamentally different than aerobic, they can bring unexpected changes to nutrient availability, decomposition, and greenhouse gas fluxes in agricultural and natural soils. Specifically, our findings indicate that upland methanogenesis can indicate the presence of other processes that decrease  $\text{NO}_3^-$ , increase  $\text{NH}_4^+$ , and decrease microbial biomass.

Methanogenesis also co-occurs with N<sub>2</sub>O production. Together, these findings suggest that greater frequency of anoxic microsites and anaerobic activity in upland soils may slow nutrient release or increase gaseous N loss. Further, anoxic microsites and their impacts may form and persist more often in wet soils or high SOC soils, such as those under organic agricultural production.

Table 3.1: Soil property analyses: ANOVA over Agronomic treatments, t-test over site, linear regression over WFPS.  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , MBC were all log transformed prior to analyses, but the ANOVA means are back-transformed to original units. The soil faunal categories are: 1 = large macrofaunal activity, 2 = evidence of mesofaunal activity, 3 = no soil faunal activity observed. For all n=72 except redox potential n=57.

	Agricultural Treatments							Site			WFPS		
	Conventional		No-till		Organic		p	Akron	KBS	p	Sign	p	r <sup>2</sup>
	Mean	Tukey	Mean	Tukey	Mean	Tukey		Mean	Mean				
<b>pH</b>	6.73	A	5.98	B	6.71	A	<0.001	6.76	6.19	<0.001		0.66	0
<b><math>\text{NH}_4^+</math> (<math>\mu\text{g gdw}^{-1}</math>)</b>	1.1	B	1.2	B	2.3	A	0.002	1.5	1.4	0.77	+	0.091	0.04
<b><math>\text{NO}_3^-</math> (<math>\mu\text{g gdw}^{-1}</math>)</b>	28.2	B	30.8	B	69.3	A	0.01	56.1	27.3	0.007	-	0.001	0.14
<b>%N as <math>\text{NO}_3^-</math></b>	91.5		91.9		87.8		0.715	90.8	90.0	0.85	-	<.001	0.20
<b>Redox pot. (mV)</b>	339		305		289		0.586	330	288	0.31	-	0.012	0.11
<b>MBC (<math>\text{mg gdw}^{-1}</math>)</b>	0.32	B	0.27	B	0.81	A	<0.001	0.55	0.30	0.002	-	<0.001	0.16
<b>T<sub>0</sub> Porosity</b>	49.7%	A	42.0%	B	49.7%	A	0.002	54.0%	40.0%	<0.001			

Faunal Cat.	Count		Count		Count	p		Count	Count	p
<b>1</b>	3		9		9	0.004		8	13	0.26
<b>2</b>	2		8		7			11	6	
<b>3</b>	19		7		8			17	17	



Table 3.2: Top; model and predictor statistics for full-factorial experimental factor ANCOVAs of methanogenesis metrics at week 6 and 21: rates, presence, and persistence from week 6 to 21. Models were balanced and complete over Site and Agronomic treatment (model df=12, error df =59, n=72; except Persistence model where model df=12, error df = 36, n=48). Bottom; single factor regressions of methanogenesis metrics over properties related to potential mechanisms,  $r^2$  included where fit is significant.

	<i>Response</i>	<i>Rate</i>	<i>Rate</i>	<i>Presence</i>	<i>Presence</i>	<i>Persistence</i>
		<i>week 6</i>	<i>week 21</i>	<i>week 6</i>	<i>week 21</i>	
<i>Experimental ANCOVAs</i>	<b>Model R<sup>2</sup></b>	0.33	0.34	0.31	0.19	0.39
	<b>Model p</b>	<b>0.012</b>	<b>0.008</b>	<b>0.004</b>	0.090	<b>0.011</b>
<b>Predictor</b>	<b>Df</b>	<b>Factor p</b>				
Intercept	1					
Site	1	0.265	0.196	0.224	0.189	0.999
Ag. treatment	1	<b>0.052</b>	0.753	<b>0.008</b>	0.528	0.289
WFPS	2	<b>0.007</b>	<b>0.015</b>	0.999	0.007	0.992
WFPS <sup>2</sup>	1	0.358	<b>0.054</b>	0.435	0.839	0.221
Site x Ag. treatment	1	0.440	0.227	<b>0.014</b>	0.566	0.903
Site x WFPS	2	0.703	0.775	1.000	0.984	0.998
Ag. treatment x WFPS	1	0.756	0.547	0.941	0.362	<b>0.018</b>
Site x Ag. treatment x WFPS	2	0.640	0.011	0.956	0.436	0.330
<i>Mechanism Regressions</i>		Model p; $r^2$ where significant				
WFPS	2	<b>0.002; 0.13</b>	<b>0.006; 0.10</b>	0.135	<b>0.001; 0.10</b>	<b>0.001; 0.16</b>
CO <sub>2</sub> Flux	2	<b>0.007; 0.10</b>	0.110	0.223	<b>0.009; 0.07</b>	<b>0.022; 0.08</b>
Soil faunal activity	3	<b>0.053; 0.08</b>	0.44	0.379	0.489	0.651

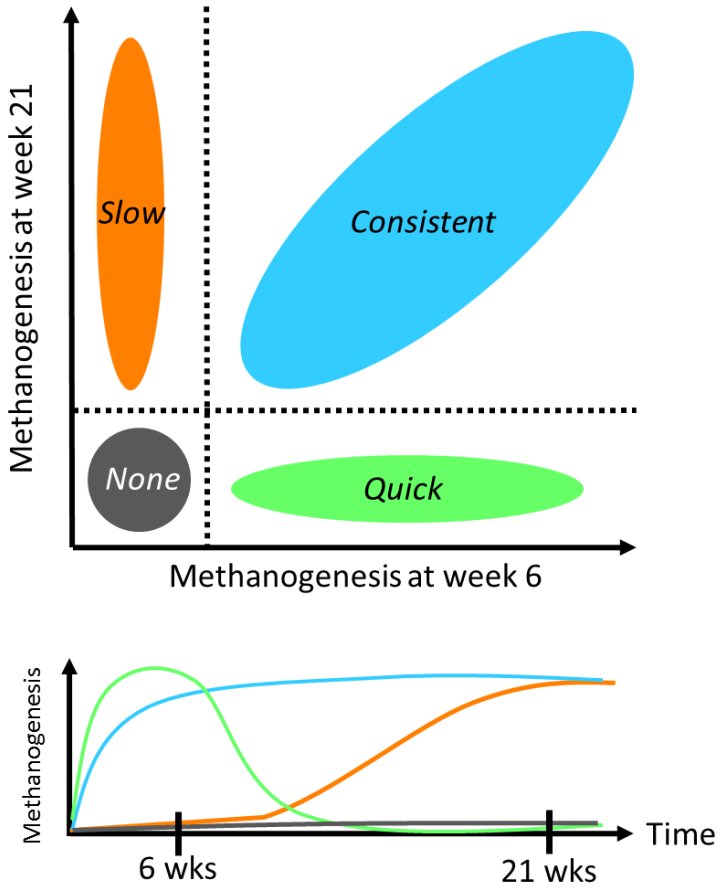


Figure 3.1: Conceptual diagram of groups based on methanogenic temporal dynamics. *Consistent* cores have detectable rates of methanogenesis at both 6 and 21 weeks, *Quick* cores have them only at week 6, *Slow* cores have them only at week 21, *None* cores do not have rates of methanogenesis above the detection limit at either 6 or 21 weeks.

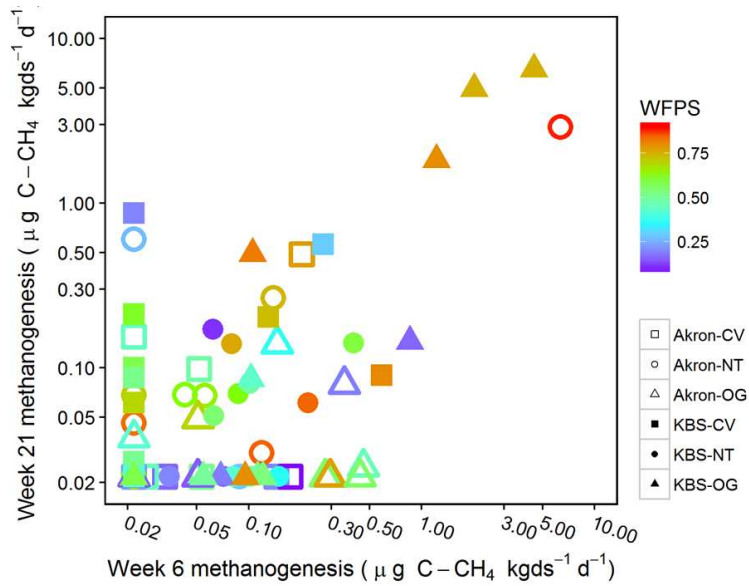


Figure 3.2: Methanogenesis at week 21 over methanogenesis at week 6, both log transformed. Matching the groups in fig. 3.1, cores falling on the x-axis are categorized as *Quick*, those falling on the y-axis are categorized as *Slow*, those falling on the origin (i.e.,  $x,y=0.021$ ) are categorized as *None*, all others are categorized as *Consistent*. Filled shapes are KBS, hollow shapes are Akron; squares are conventional, circles are no-till, triangles are organic. Abbreviations are, CV for Conventional, NT for No-till, OG for Organic. Shapes are colored by WFPS.

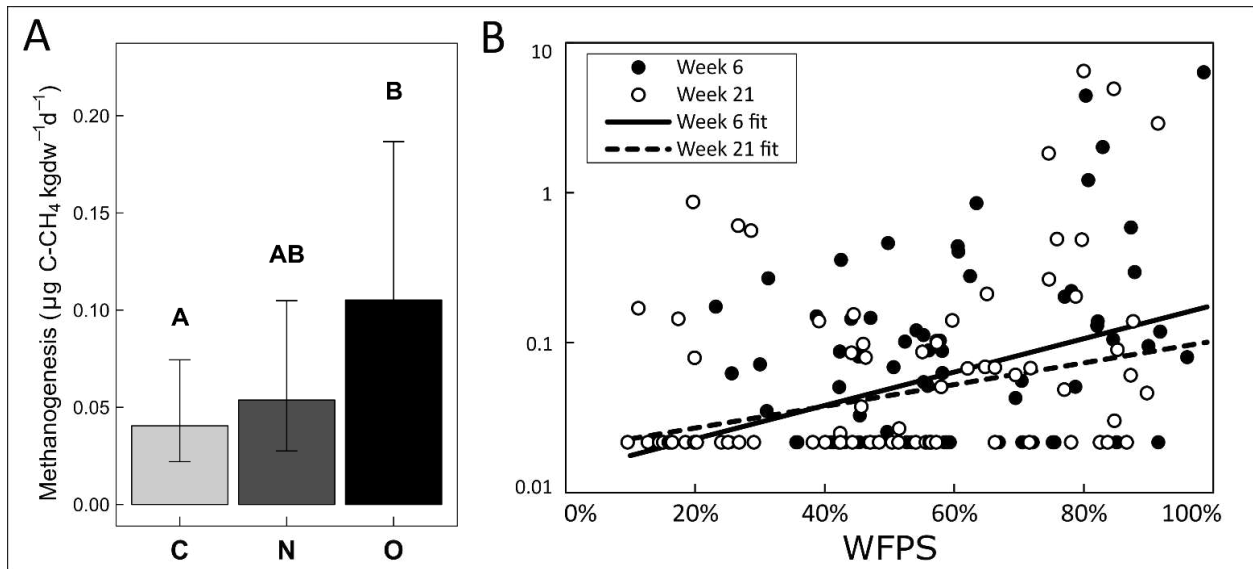


Figure 3.3: Week 6 methanogenesis fluxes over agricultural treatment, A, and,  $\log_{10}$ -transformed rates of methanogenesis during weeks 6 and 21 over WFPS, with regression fits from the ANCOVA WFPS coefficients, B. Tukey post-hoc letters come from the ANCOVA analysis, levels that share the same letter are not significantly different.

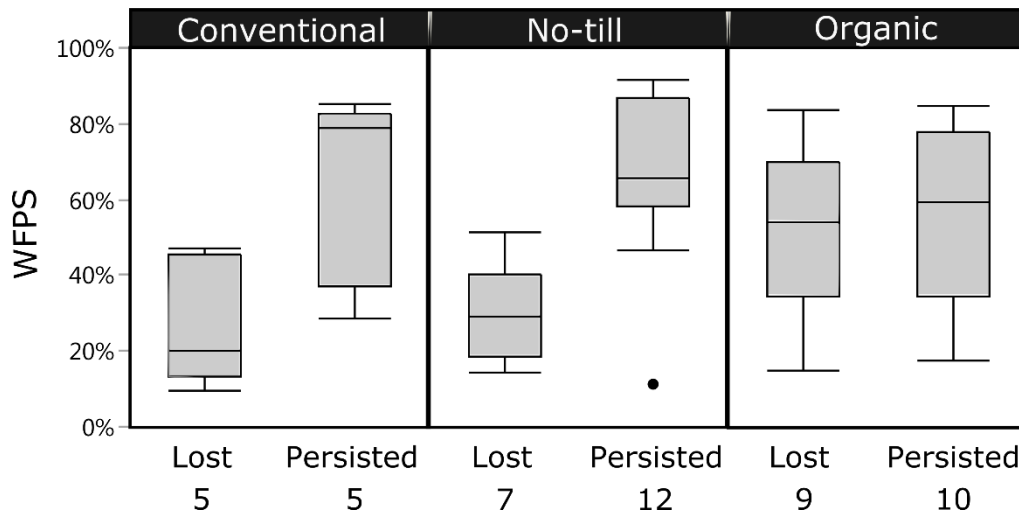


Figure 3.4: Percent WFPS over agricultural treatment and methanogenic persistence in standard box-and-whisker plots. Numbers below each column indicate the number of cores within that group.

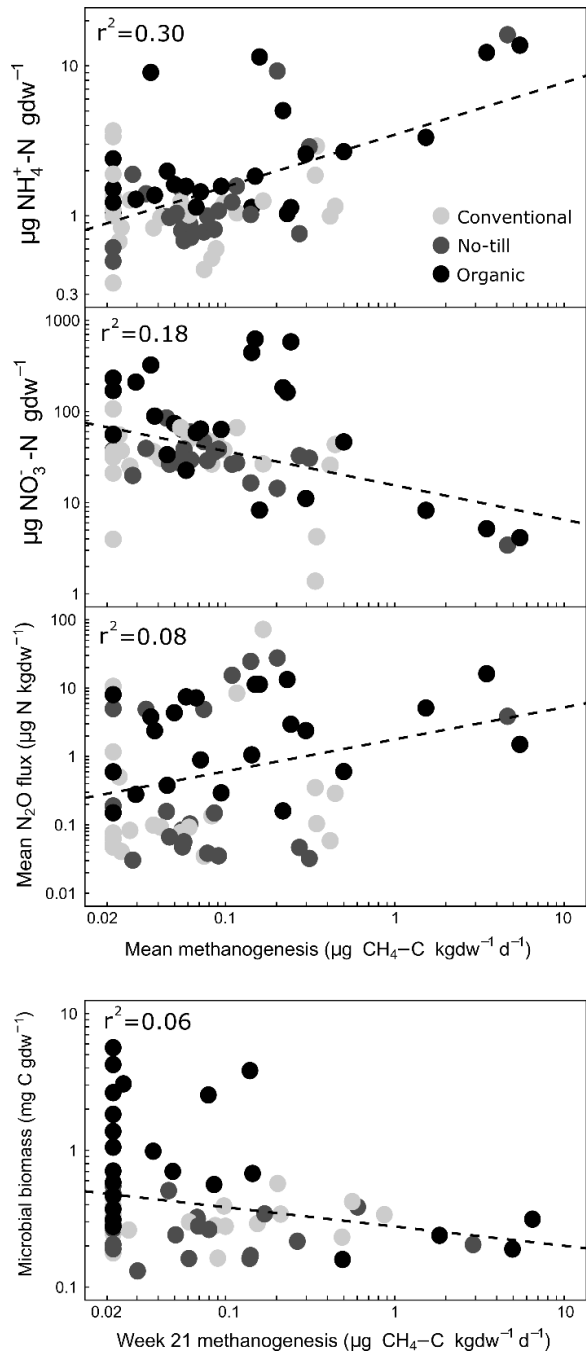


Figure 3.5: Inorganic N species and mean N<sub>2</sub>O emission regressed over the mean rate of methanogenesis. Rate of methanogenesis, NH<sub>4</sub><sup>+</sup>, NO<sub>3</sub><sup>-</sup>, and N<sub>2</sub>O emission are log transformed. Microbial biomass C regressed over week 21 methanogenesis.

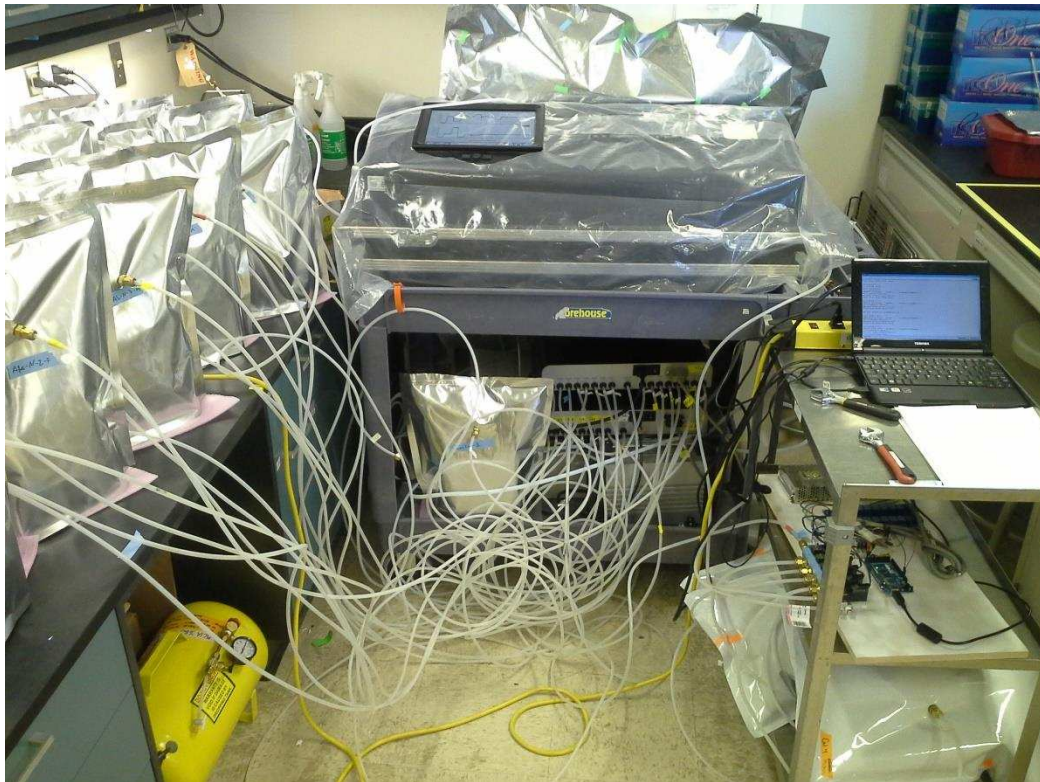


Figure 3.6: Instrumentation used in CH<sub>4</sub> isotope pool dilution method with dynamic headspace. Polyester foil bags with soil cores inside (left), MCI A (center, top of cart), manufacturer supplied gas manifolds (center, bottom of cart), high capacity check standard gas bag (center, behind MCI A), Arduino MEGA and lab-made gas manifold (right, middle of cart), Arduino control computer (right, top of cart), gas calibration standard bags (right, bottom of cart).

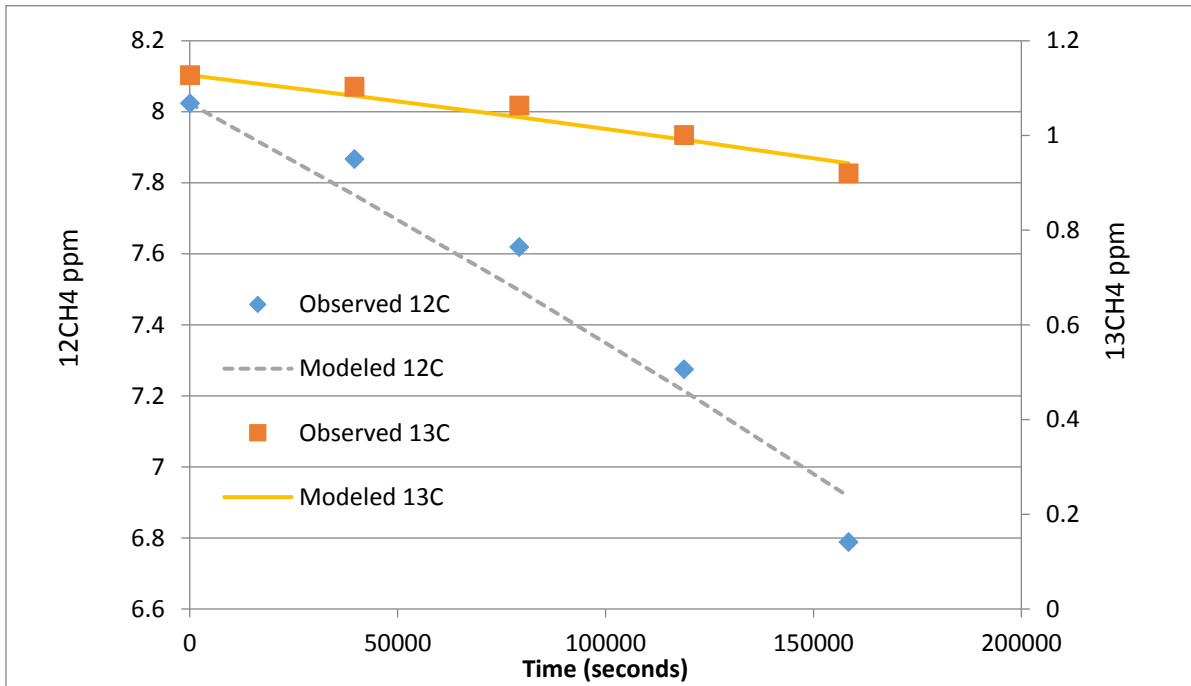


Figure 3.7: Observed and model predicted 12CH<sub>4</sub> and 13CH<sub>4</sub> concentrations.



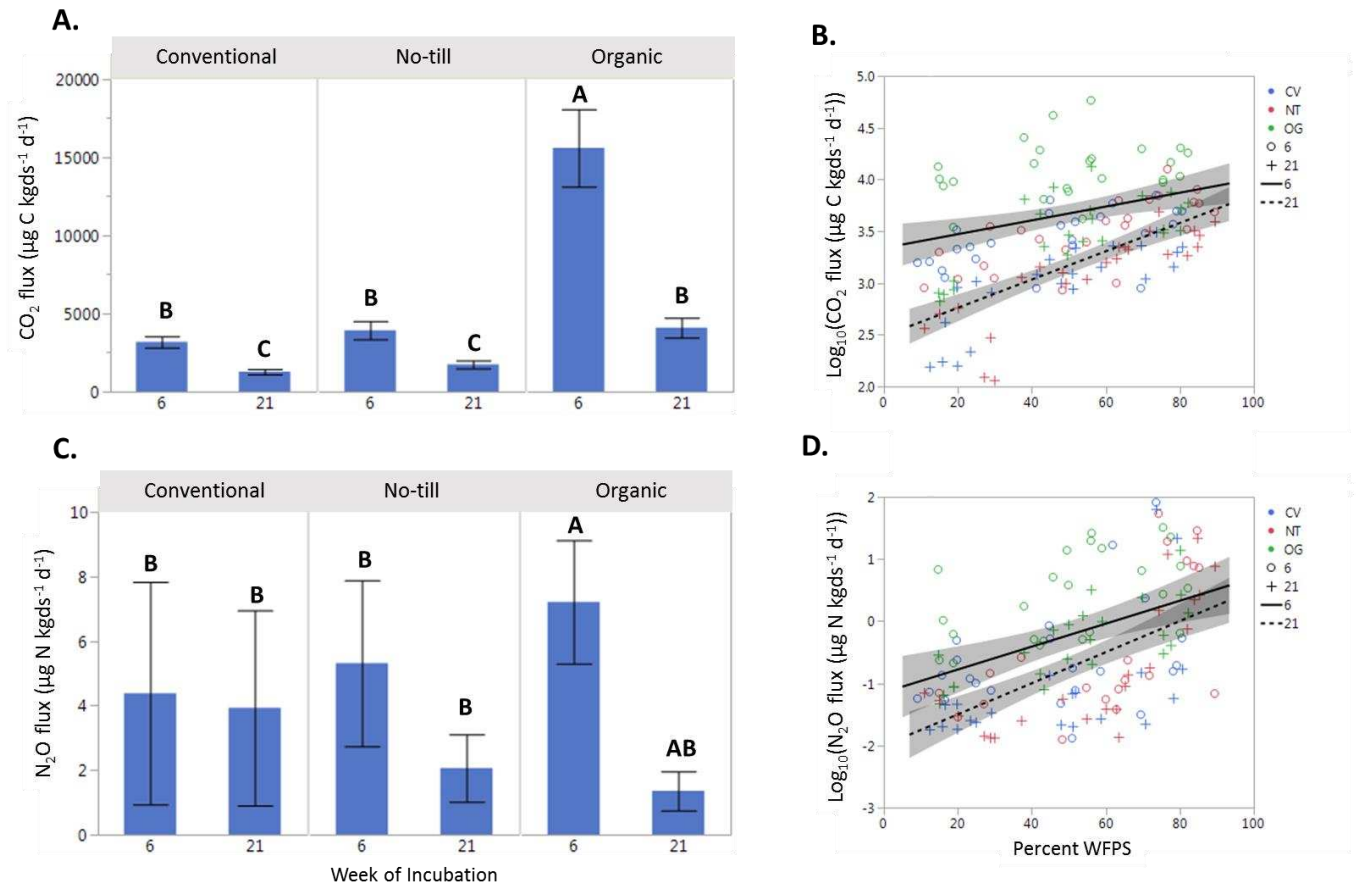


Figure 3.8: CO<sub>2</sub> flux and N<sub>2</sub>O flux over agricultural treatments and WFPS by sampling time. Panels A. and C. show untransformed fluxes with Tukey post-hoc connecting letters from ANOVAs performed on log-transformed fluxes (values that do not share the same letter are significantly different). Abbreviations are: CV for Conventional, NT for No-till, OG for Organic, 6 for week 6, 21 for week 21. Panel B.  $r^2$  are 0.14 and 0.49 for weeks 6 and 21 respectively; for panel D  $r^2$  are 0.19 and 0.42 for weeks 6 and 21 respectively.

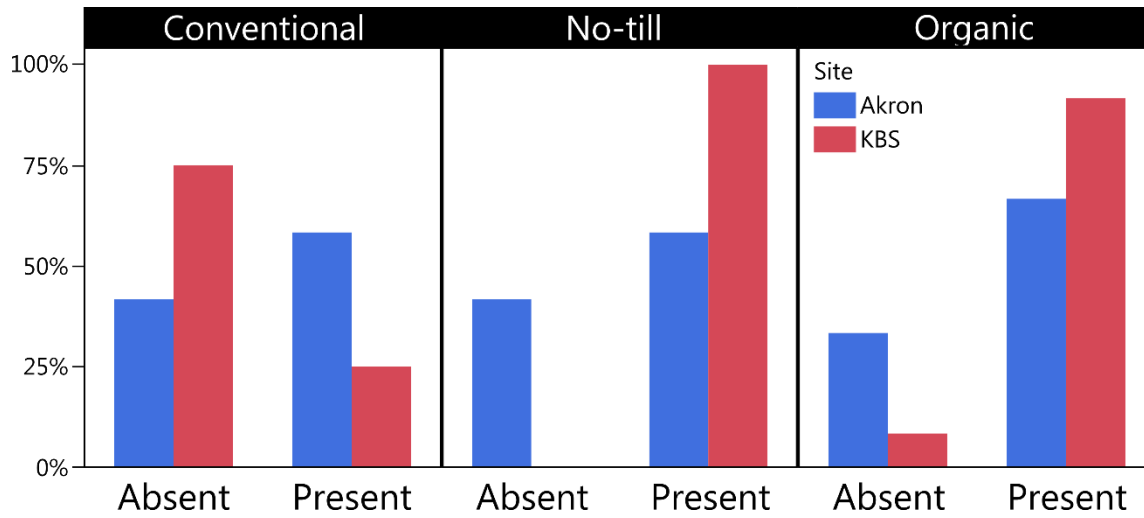


Figure 3.9: Percent of cores with methanogenesis present at week 6 by agronomic treatment and site. The percent is of the 12 cores in each site X agricultural treatment group (e.g. “KBS-No-till”). ANCOVA analysis found this relationship was significant ( $p=0.014$ ; table 2).

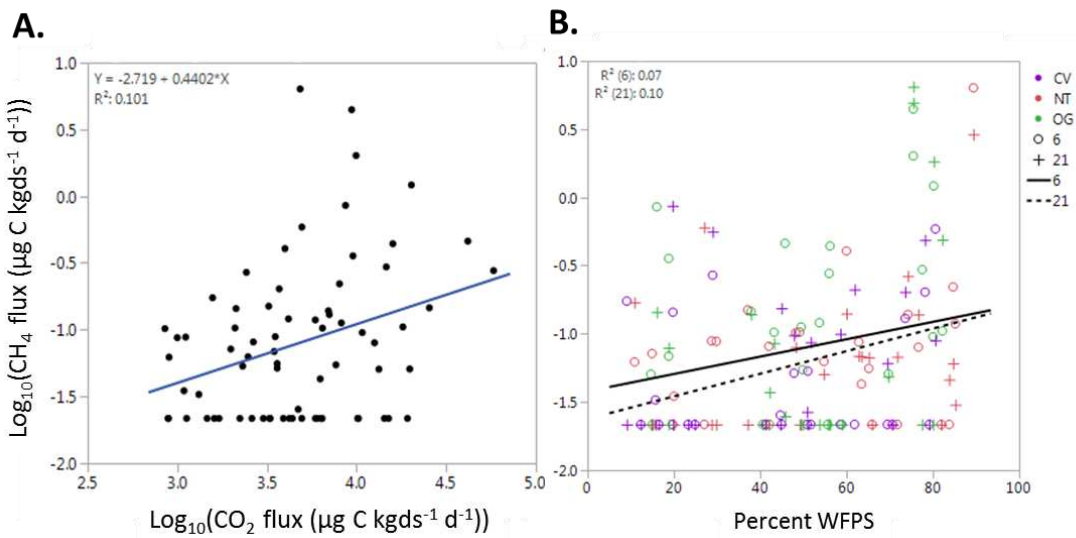


Figure 3.10: A, rates of methanogenesis from week 6 over CO<sub>2</sub> flux, and B, methanogenesis from weeks 6 and 21 over WFPS, with linear regressions. All gas fluxes are log<sub>10</sub> transformed. Abbreviations are: CV for Conventional, NT for No-till, OG for Organic, 6 for week 6, 21 for week 21.

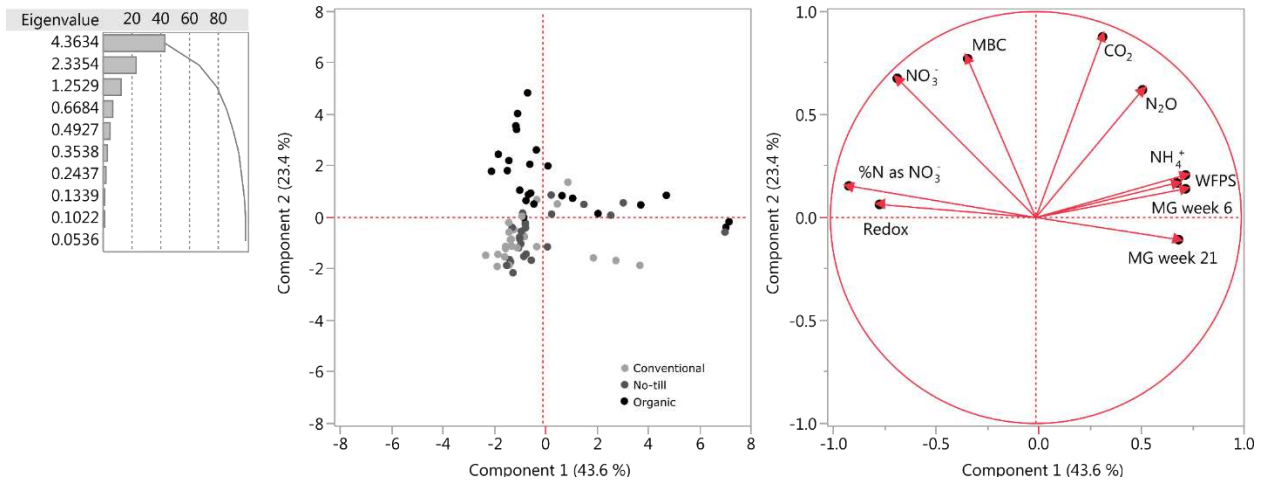


Figure 3.11: Principal components analysis of soil properties and gas fluxes. Prior to analysis MBC,  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , and all gas fluxes were log-transformed. Abbreviations are: CV for Conventional, NT for No-till, OG for Organic.

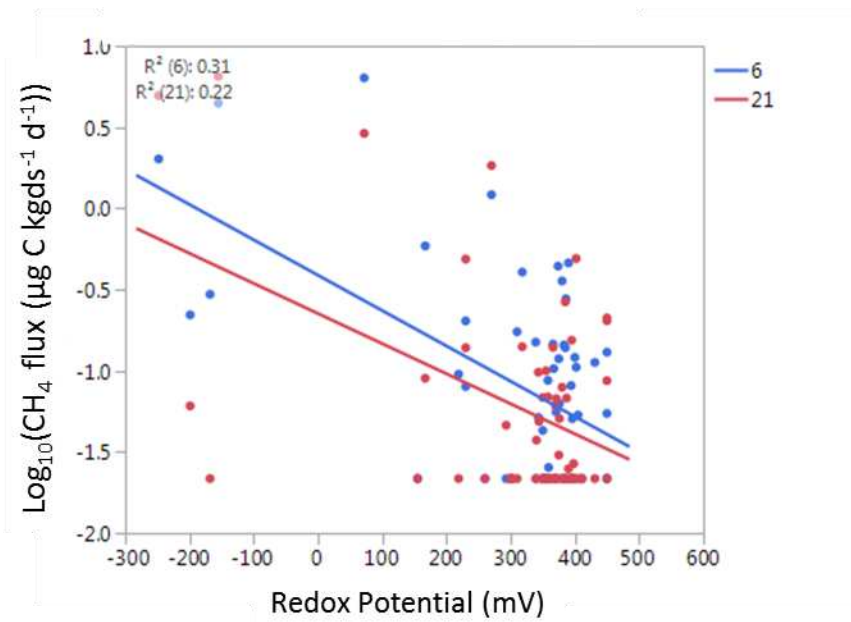


Figure 3.12: Week 6 and 21 methanogenic fluxes over the minimum redox potential of each core. Flux is  $\text{log}_{10}$  transformed, 6 and 21 indicate the week of the incubation.

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## 4 – Effects of spatial aggregation of plant litter on biogeochemical processes across a range of soil moisture

### **4.1 - Introduction**

Soil moisture and litter quality are widely recognized as major controls of decomposition (Jansson and Berg, 1985; Parton et al., 1987), however the limitations of current models (Parton et al., 2007; Currie et al., 2010) show that other soil and litter attributes may help drive decomposition, N cycling, and other soil processes. The spatial distribution of litter and other organic matter structures the soil habitat and can affect decomposition (Magid et al., 2006; Hewins et al., 2013), N<sub>2</sub>O production (Loecke and Robertson, 2009a), N availability (Ambus et al., 2001; Magid et al., 2006), and mesofaunal populations (Nielsen et al., 2010). There is evidence that the impact that spatial distribution of resources has on processes may depend on soil moisture and that it may have the strongest effects in the dry soils of arid and semi-arid environments (Loecke and Robertson, 2009a; Hewins and Throop, 2016). If so, the spatial aggregation of plant litter could have significant consequences for crop production in dry climates where crop residues are often left in the field and managed for moisture retention. However, we do not know what impacts litter aggregation has in drier soils, or even what the relative importance of it might be in affecting soil processes compared to soil moisture and litter quality.

The mechanisms of soil moisture and litter quality impacts on decomposition and N-cycling are well-established in ecosystems ranging from mesic to tropical. Greater moisture generally increases rates of decomposition and N-cycling until soil water begins to fill larger soil pores and restrict O<sub>2</sub> diffusion (del Grosso et al., 2005; Castellano et al., 2011), at which point decomposition and aerobic processes slow, N losses accelerate through leaching and denitrification, and methanogenesis can begin (Castellano et al. 2010; Castellano et al. 2011). Plant litters with higher C:N (i.e., lower N content)

decompose more slowly than litters that have C:N ratios at or below those of microbial growth requirements (C:N = 25:1) (Hobbie, 1992; Cotrufo et al., 1994; Chapin et al., 2011). This also leads to slower rates of N-cycling in ecosystems dominated by plants that produce high C:N litters (Hobbie, 1992). By building on these well-defined conceptual frameworks of moisture and litter quality effects on soil processes, researchers can extend studies to other controls on decomposition and N-cycling.

The spatial distribution of plant litter in soils affects soil habitat physically and chemically, and may ultimately lead to biological feedbacks. A given mass of litter can be aggregated (i.e., occur in fewer, larger patches) or dispersed (i.e., occur in many small patches) in soils. Aggregated litters can have comparatively less contact with mineral soil particles (Breland, 1994), create strong gradients of nutrients, O<sub>2</sub>, toxins (e.g. NO<sub>2</sub>) (van der Lee et al., 1999), retain moisture (Mulumba and Lal, 2008), and provide concentrated resource centers that can support larger and more complex trophic structures (Clarholm, 1985; Vestergaard et al., 2001). Less contact with soil minerals, greater moisture, faster biomass turnover should all increase rates of decomposition and N-cycling in litter patches (Loecke et al., 2009a). However, decreased diffusion of O<sub>2</sub> or nutrients from the soil to the center of a litter patch or concentration of toxins would slow decomposition and N-cycling and could increase N loss if litter areas become anoxic and host denitrification (Magid et al., 2006; Loecke and Robertson, 2009a; Keiluweit et al., 2016). If aggregation of litter creates a strong diffusion gradient, the effects of this could be more prominent in dry soils where rates of aqueous diffusion decrease due to reductions in porewater connectivity (Ebrahimi and Or, 2014).

Litter aggregation could be especially important for dryland crop production in semi-arid environments where soils are managed for moisture retention by growing crops under no-till practices and/or retaining crop residues to protect soils from drying (Halvorson et al., 1996; Nielsen et al., 2002). No-till agriculture decreases the physical cutting and distribution of belowground residues while aboveground residue management can leave large amounts of fresh litter on the soil surface or

incorporate the residue into the soil to improve moisture retention. In addition, organic agriculture practices typically involve the addition of significant amounts of organic matter in the form of plant litter, compost, or manure. These management practices determine the size and distribution of fresh organic matter in soils and could be altered to modify the distributions if there were benefits for soil C storage, N availability, water retention, or reductions in greenhouse gases.

To evaluate the relative importance of litter distributions as compared to soil moisture and litter type, we manipulated the aggregations of low and high C:N litters across a broad range of soil moistures (15%-85% water holding capacity). Our goals were to determine, 1) what is the relative importance of litter aggregation versus soil moisture and litter type in affecting soil processes, 2) are certain processes affected particularly strongly by litter aggregation, and, 3) how do effects of litter aggregation and litter type compare between very dry soils and wetter soils. To do this, we measured net CO<sub>2</sub> and CH<sub>4</sub> fluxes throughout a 100-day laboratory incubation as well as inorganic N, microbial biomass, and soil organic matter chemistry at the end of the incubation period.

## **4.2 - Methods**

### 4.2.1 - Soil and litter collection

Soil was collected in June 2011 from the Shortgrass Steppe LTER (Nunn, CO, USA). It was collected from three sites from high and low topologies and with varying textures to provide a functionally diverse microbial community. The soils were all Ustic Haplargids, two were fine sandy loams and the third was a coarse sandy loam; they were collected from the top 20cm of the horizon. Collected soils (approx. 60kg) were thoroughly mixed and placed in covered, new plastic buckets for storage. The mixed soil had a gravimetric moisture of 1.7%, a pH of 6.5, and was a sandy loam of approximately 70% sand. Mixed soil was stored at room temperature (20-25<sup>0</sup>C) and ambient humidity (typically <40% relative humidity) until mesocosms were constructed. Whole alfalfa plant and wheat

straw were field dried and collected from the Agricultural Research, Development and Education Center of Colorado State University (Fort Collins, CO, USA) over the summer of 2011. Since the litter hydration was low no further drying was necessary for processing. Litters were ground to 1mm in a Wiley mill and stored in sealed plastic bags. The alfalfa and wheat had C:N ratios of 14.5 and 37.0, respectively, as analyzed with a VG Isochrom continuous flow IRMS (Isoprime Inc., Manchester, UK), coupled to a Carlo Erba NA 1500 elemental analyzer.

#### 4.2.2 - Experimental design

Target soil moisture levels were determined by calculating the added water needed to reach 15%, 35%, 65%, or 85% water holding capacity (WHC). The source soils were dry enough that all levels required an addition of water. Litter patches were composed of either alfalfa or wheat straw. The litter patch size treatment was made up of four levels, one large patch, three medium patches, nine small patches, or a uniform but sparse, non-contiguous layer of litter – a treatment intended to form minimal sized litter patches (fig. 4.1). Control mesocosms were also constructed absent of litter but across the range of soil moisture levels. A full-factorial design was used, resulting in 36 treatments with five mesocosm replicates of each treatment (n=180). To enable the analysis of this large number of mesocosms, their construction and incubation was staggered in sets of 24 over the course of seven months with two to four weeks between set start dates. Treatments were evenly distributed across these sets and no significant set differences were found in any of our resulting data.

#### 4.2.3 - Mesocosm construction

Mesocosms were constructed in 500 ml Mason glass jars with 250 grams dry weight (gdw) of soil and 1 gdw of saturated alfalfa or wheat litter. Prior to construction 1ml, 3ml, and 10ml plastic syringes were cut to remove the nozzle end. Litter saturated with deionized water was loaded into

the syringes. The single patch treatment was made with all litter in a 10ml syringe, three patch treatments had litter split evenly into three 3ml syringes, and nine patch treatments had litter split into nine 1ml syringes. Syringes were placed in a consistent, repeated pattern for each mesocosm by attaching them to a plastic frame (see diagram in fig. 1).

First, 80 gdw layer of pre-moistened soil was placed in the bottom of the jar, then litter syringes were placed on top of that layer, in a vertical alignment. The second layer of 120 gdw soil was filled around the syringes, then the syringes were removed, leaving the litter patches in place. Finally, a third layer of 50 gdw soil was placed on top. If necessary, each layer was tamped down to maintain consistent mesocosm soil densities between soil moisture treatments. The jars were then covered with a thin low-density polyester film punctured with 20 small needle holes to allow gas diffusion but minimize water loss. The mesocosms were incubated in the dark at room temperature. Temperature was logged throughout the incubation and did not vary significantly between staggered incubation periods. Soil moisture was measured gravimetrically every two weeks and additions were made when soils dropped more than 1g below their target moisture.

#### 4.2.4 - Gas flux measurements

Net fluxes of CO<sub>2</sub> and CH<sub>4</sub> from mesocosm soils were measured on daily to bi-weekly intervals throughout the incubation period. Standard metal Mason jar lids were fitted with a black rubber septum (Geomicrobial Technologies, Ochelata, OK, USA). To begin a flux measurement, jars were removed from storage and lids with septa were fastened onto them with standard jar lid rings. Then the jar headspace was raised to 10 ppm CH<sub>4</sub> with a small injection of concentrated CH<sub>4</sub> and 30ml room air was added as makeup air for subsequent gas samples. After the headspace equilibrated with soil air over 30 minutes, the first gas sample was taken by mixing the headspace air three times then extracting 30ml of gas with a syringe. The gas sample was compressed into a sealed and evacuated

20ml glass. Following a 3-5 hour waiting period, the jars' headspace was sampled a final time. Mesocosm jars then had lids replaced with LDPE film and were returned to storage. Vials containing headspace gases were analyzed with a Los Gatos Research Fast Greenhouse Gas Analyzer (ABB - Los Gatos Research Inc., San Jose, CA, USA). Shimadzu *EZStart* software was used to calculate peak heights and areas from this instrument. All samples were calibrated against certified lab standard gases

#### 4.2.5 - Incubation end and soil assays

At the end of the incubation soils were sieved (2mm) and homogenized. Subsamples were frozen for subsequent pH measurement, maintained at 4<sup>0</sup>C for inorganic N and microbial biomass measures, or, in the case of soil organic carbon content, dried at 55<sup>0</sup>C for 24 hours. Gravimetric water content of soils was calculated as the difference in mass between wet soil and soil dried at 105<sup>0</sup>C for 24 hours. Inorganic nitrogen concentrations quantified colorimetrically (Alpkem Flow Solution IV, O.I. Analytical, College Station, TX, USA) were measured in 1M KCl solution after soil was extracted for 24 hours in 50ml *Whirlpak* bags (Nasco Inc., Fort Atkinson, WI, USA) in a 45ml KCl to 10 gdw mix. Measurements of pH were made with an Orion 3 Star pH meter (Thermo Scientific, MA, USA) in a 1:1 solution of soil and deionized water. Microbial biomass C and N were extracted using a chloroform slurry technique adapted from Fierer and Schimel (2003). Briefly, a soil sample was split into two 8-15g samples in ½ pint glass jars, then 40ml of 0.5M K<sub>2</sub>SO<sub>4</sub> was added to both jars and 0.5 ml chloroform was added to a jar designated as the cell lysis jar. Both jars were shaken at 200 RPM on a rotary shaker for four hours. The jars were removed, the sediment was allowed to settle for 40 minutes, then the contents were gravity filtered (Whatman 40 paper) for one hour. Finally, cell lysis extracts were bubbled with N<sub>2</sub> gas to remove any residual chloroform. All extracts were stored at -20C in 50ml plastic vials until analysis. Analysis of dissolved organic C and total N was performed on



a Shimadzu TOC-Vcsn carbon analyzer with a TNM-1 nitrogen module (Shimadzu Scientific Instruments Incorporated, Kyoto, Japan).

#### 4.2.6 - Statistics

Mean daily gas fluxes were calculated using flux measurement data, interpolating fluxes when measurements were not made daily. To determine the relative importance of experimental factors we performed full factorial ANOVAs for each measured gas flux and soil property. When error distributions failed assumptions of normality data sets were transformed appropriately. Both mean CO<sub>2</sub> flux and microbial biomass were log-transformed prior to ANOVA analysis. ANOVA predictors with  $0.05 < p < 0.10$  were given further attention and discussion given their likelihood of indicating an actual relationship. All statistics were performed R version 3.2.5 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria) and JMP Pro 12.2 (SAS Institute Inc., Cary, NC, USA).

### 4.3 - Results

#### 4.3.1 - Inorganic nitrogen

The dominant controls of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> were soil moisture and litter type, litter aggregation was also a significant predictor of NH<sub>4</sub><sup>+</sup>. The concentrations of NH<sub>4</sub><sup>+</sup> among moisture X litter type groups reveal multiple relationships (fig.2). Concentrations of NH<sub>4</sub><sup>+</sup> were nearly four-fold higher in the 15% WHC alfalfa soils than other groups. Concentrations in other groups were similar to the control soils, however 15% and 85% WHC wheat soils had less NH<sub>4</sub><sup>+</sup> than the controls. NH<sub>4</sub><sup>+</sup> concentrations decreased consistently as litter was more distributed, with single patches having 60% more NH<sub>4</sub><sup>+</sup> than uniformly distributed litter (fig. 4.5). This relationship was particularly strong in the 15%WHC alfalfa and wheat soils (fig. 4.6).

Soil  $\text{NO}_3^-$  across soil moisture had an inverse pattern compared to  $\text{NH}_4^+$ , with the highest  $\text{NO}_3^-$  concentrations in the moderate moisture soils (fig. 4.3). Alfalfa had twice the  $\text{NO}_3^-$  as wheat soils, and, unlike  $\text{NH}_4^+$ , wheat soils had similar levels of  $\text{NO}_3^-$  as control soils.

#### 4.3.2 - $\text{CO}_2$ fluxes

Cumulative  $\text{CO}_2$  rose steadily with soil moisture, quadrupling from 15% WHC to 85% WHC, and alfalfa soils produced 25% more  $\text{CO}_2$  than wheat soils. However, these effects were primarily driven by the difference in wheat and alfalfa  $\text{CO}_2$  from the 15% WHC soils, at this level alfalfa soils produced twice the  $\text{CO}_2$  that wheat soils did (fig. 4.3). Soils with single patches of alfalfa litter produced 20% more  $\text{CO}_2$  than the uniformly distributed litter, but litter aggregation did not affect cumulative  $\text{CO}_2$  in wheat soils (fig. 4.6).

#### 4.3.3 - $\text{CH}_4$ fluxes

In contrast to the non-wet soils (15%, 35%, 65% WHC) that exhibited moderate rates of net  $\text{CH}_4$  consumption and similar variances, many of the wet soils (85% WHC) produced large amounts of  $\text{CH}_4$  and had radically higher variance of net  $\text{CH}_4$  flux. To maintain valid inference in light of this heteroscedasticity we analyzed the  $\text{CH}_4$  fluxes of non-wet and wet soils separately. The wet soil  $\text{CH}_4$  fluxes were not significantly predicted by litter type or aggregation (fig. 4.7), however the non-wet soils were affected by these factors in a number of ways. For  $\text{CH}_4$  flux from non-wet soils, soil moisture and litter type had high levels of explanatory power with litter aggregation less important, though a little more important than it was for  $\text{CO}_2$  flux (fig. 4.2).

The treatment groups of these drier soils typically had net  $\text{CH}_4$  consumption, though some had nominal positive  $\text{CH}_4$  fluxes. The driest soils had nominal rates of  $\text{CH}_4$  uptake (and some passing instances of  $\text{CH}_4$  production), but most of the 35% and 65% WHC soils had uptake rates falling

between 0.6 and 1.0 ng C gdw<sup>-1</sup> d<sup>-1</sup>, which is comparable to other grassland soils (Kammann et al., 2009). Interestingly, litter quality had nearly as large an effect on CH<sub>4</sub> uptake as moisture (fig. 4.3). Comparing the samples to unamended control soils it is clear that mesocosms with alfalfa had much lower rates of CH<sub>4</sub> uptake than the controls, but that wheat caused no measurable difference in uptake – this relationship is consistent in moderate soil moistures of 35% and 65% WHC (fig. 4.3).

The significant interaction of litter aggregation and soil moisture appears to show that less aggregation lead to greater CH<sub>4</sub> uptake in moist soils, but it lead to less uptake in the driest soil, since all three moisture levels had indistinguishable uptake rates when litter was aggregated into a single patch but as the litter was more dispersed the change in fluxes diverged.

More than half of the wet soil CH<sub>4</sub> fluxes were positive (n=22/40), indicating methanogenesis was present, and mean CH<sub>4</sub> flux was 47.28 ng C gdw<sup>-1</sup> d<sup>-1</sup>. ANOVA analyses of wet soil CH<sub>4</sub> flux did not indicate that litter C:N or aggregation were significant predictors (fig. 4.7), but this appears to be largely due to a non-normal distribution of errors that cannot be log transformed because some flux values are negative (i.e., CH<sub>4</sub> uptake). Alfalfa soils produced 75.99 ng C gdw<sup>-1</sup> d<sup>-1</sup>, nearly four-fold the 18.69 ng C gdw<sup>-1</sup> d<sup>-1</sup> that soils with wheat produced and much more than the 3.05 ng C gdw<sup>-1</sup> d<sup>-1</sup> produced by unamended control soils. The uniform distributions of litter had the highest CH<sub>4</sub> fluxes for both alfalfa and wheat, producing approximately double what the other litter distributions averaged (fig. 4.7). Fluxes of CH<sub>4</sub> in wet soils had fairly strong correlations with inorganic N, increasing CH<sub>4</sub> production with greater NH<sub>4</sub><sup>+</sup> (r<sup>2</sup>=0.63) and decreasing with greater NO<sub>3</sub><sup>-</sup> (r<sup>2</sup>= 0.42; fig. 4.8).

#### 4.3.4 - Microbial biomass

Microbial biomass was greatest in the driest soils (1.3 mg C gdw<sup>-1</sup> d<sup>-1</sup>) and decreased as soil moisture increased (fig. 4.4). This was true of soils with litter and the control soils with no litter added. Microbial biomass decreased with increasing mean CO<sub>2</sub> flux (p=0.0001, r<sup>2</sup>= 0.25, fig. 4.9).

#### 4.3.5 - Review of 15% WHC relationships

Respiration and  $\text{NH}_4^+$  concentrations increased consistently with greater aggregation of alfalfa litter in 15% WHC soils, both were 65-70% greater in single patches as opposed to uniform distributions. Wheat litter had 50% greater  $\text{NH}_4^+$  in single patches than uniform distributions at 15% WHC. These effects were not present at the other moisture levels, except a similar increase in  $\text{NH}_4^+$  in 85% WHC soil. At 15% WHC there was no difference in  $\text{NO}_3^-$  due to litter type, while in the other moisture levels alfalfa had much greater  $\text{NO}_3^-$  than wheat. Concentrations of  $\text{NO}_3^-$  in 15% WHC alfalfa soils mirrored the increase with litter aggregation in  $\text{NH}_4^+$ , but this 70% increase from uniform to a single patch was not significant in this ANOVA.

#### 4.3.6 - Relative importance

We illustrate the relative importance of predictors using an ANOVA framework where we compare the Sum of Square variance explained by each factor. We found that soil moisture was the most important predictor of variation in all gas fluxes and soil properties (fig. 4.2). The only exception was the non-wet (i.e., 15%, 35%, 65% WHC) soils'  $\text{CH}_4$  flux for which litter type was most important. However, that set of  $\text{CH}_4$  flux data excluded the wet (85% WHC) soils which had much higher fluxes than the other moisture levels. For gas fluxes, the second and third most important factors were litter type and the interaction of moisture and litter type (fig. 4.2 and 4.3). Many soil properties had litter type or an interaction with litter type as the second most important factor.

For responses including  $\text{CO}_2$  flux,  $\text{NH}_4^+$ , and late and average non-wet  $\text{CH}_4$  fluxes, either litter aggregation or an interaction that included aggregation was a significant predictor for (Table 4.1). For  $\text{CO}_2$  and  $\text{NH}_4^+$  the impact of litter aggregation were driven by large differences in the driest soils (i.e., 15% WHC), but for  $\text{CH}_4$  fluxes the effects appeared to occur in multiple moisture levels.

## 4.4 - Discussion

This and other studies have found that the distribution of organic matter at millimeter scales can affect soil processes, although compared to impacts of soil moisture and organic matter quality the effects of organic matter aggregation are often small. Here we show that litter aggregation can have major impacts on decomposition and N availability, but that this occurs primarily in dry soils. We found when litter was aggregated into a single patch rather than being dispersed throughout, soil concentrations of  $\text{NH}_4^+$  increased 50-70% and, for alfalfa-amended soils, C-mineralization rates increased 65%. Litter aggregation had smaller effects on  $\text{CH}_4$  flux. While there appear to be occasional effects of litter aggregations in moist and wet soils, the strongest impacts were in the driest soils (i.e., 15% WHC).

### 4.4.1 - Inorganic nitrogen

Variations in  $\text{NH}_4^+$  and  $\text{NO}_3^-$  concentrations were consistent with expectations based on litter N content, diffusion limitations, and biomass turnover. While litter C:N is most likely responsible for the greater  $\text{NH}_4^+$  in alfalfa versus wheat amended soils, diffusion limitation probably played the largest role in creating the observed patterns of  $\text{NH}_4^+$  across soil moistures and litter aggregation. The low diffusivity of  $\text{NH}_4^+$  in water causes it to accumulate in areas of N mineralization (Reddy and DeLaune, 2008) and the diffusivity is even lower in drier soils with limited water film connectivity (Ebrahimi and Or, 2014). However, it is also possible the larger litter patches created resource centers hosting large food webs with enhanced N remineralization. Increasing litter particle size five-fold can double the nematodes present after 70 days (Vestergaard et al., 2001), so our increase in aggregation by over 100-fold (single particle size versus largest litter patch size) could have greater impacts on populations of bacterial and fungal consumers. Soil mesofauna increase bacterial and fungal biomass turnover and rates of N-mineralization (Beare et al., 1992), and at the site where our soils were sampled they can

contribute a third of the available N (Hunt et al., 1987). Moderate moisture levels appear to provide the best conditions for  $\text{NO}_3^-$  accumulation which may have been caused by a combination of decomposition and nitrification with minimal denitrification. The lower  $\text{NO}_3^-$  in the wettest soils does not appear to be caused by inadequate decomposition so it is more likely due to  $\text{NO}_3^-$  loss through denitrification, rates of which typically increase exponentially between 60 and 80% water-filled porespace (Linn and Doran 1984; Loecke and Robertson, 2009a).

Although litter aggregation altered  $\text{NH}_4^+$  this effect did not extend to  $\text{NO}_3^-$  availability. Unlike  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  is highly mobile in soil porewater so we expect any effect that litter aggregation had on  $\text{NO}_3^-$  would likely have been transient. Magid et al. (2006) showed that highly aggregated, high C:N litter can cause the depletion of soil N to be slowed over long periods (i.e., 50-200 days), but Vestergaard et al. (2001) observed no significant effect of litter particle size on  $\text{NO}_3^-$  over time. However, both studies observed dynamics of  $\text{NO}_3^-$  over 50-200 days, and other studies have shown that denitrification peaks in the first 20 days (Ambus et al., 2001; Loecke and Robertson, 2009a) indicating that our study may have simply missed the effects of litter aggregation on  $\text{NO}_3^-$  due to timing. Ultimately, our observations indicate that litter aggregation may extend or enhance  $\text{NH}_4^+$  availability, but the potential for temporal  $\text{NH}_4^+$  dynamics should be investigated to determine how this might affect seasonal nutrient availability.

We expected that decomposition of the high C:N wheat litter would have greater N demand and decrease soil inorganic N. Soil  $\text{NH}_4^+$  was lower in wheat soils but not soil  $\text{NO}_3^-$  so it appears this may have only affected  $\text{NH}_4^+$ . This could have been caused by trophic remineralization dynamics if that was a more important control of  $\text{NO}_3^-$  concentrations than decomposition demand at day 100 of the incubation. Increased prominence of remineralization altered the soil N content prior to the 100<sup>th</sup> incubation day in a similar study (Magid et al., 2006)

#### 4.4.2 - Carbon mineralization

Despite its low N content, wheat amended soil only had lower C mineralization than alfalfa amended soil at 15% WHC. This difference was likely caused by N limitation of wheat decomposition. Decomposition of high C:N litters require N to diffuse to the litter from surrounding soil (Vestergaard et al., 2001, Magid et al., 2006), but diffusion would be retarded in these dry soils, slowing decomposition of the wheat litter while the alfalfa litter contains ample N, not requiring inorganic N to diffuse from the surrounding soil.

The increase in alfalfa-amended soil respiration with greater litter aggregation could be caused by larger food webs, rates of predation, and C and N remineralization around large litter patches (Hunt et al., 1987; Breland, 1997). The  $\text{NH}_4^+$  patterns we observed also support this cause. Alternatively, soil moisture may have been better retained in larger litter patches that have lower surface area:volume ratios and, thus, slower diffusion of  $\text{H}_2\text{O}$  out of them into the soil (Mulumba and Lal, 2008). This increase in litter moisture would have the most noticeable impact on microbial activity in the driest soils where this litter aggregation relationship was strongest.

A similar increase in  $\text{CO}_2$  flux with greater particle size was observed with rye litter (C:N=9; Angers and Recous, 1997). Other studies have found the opposite trend for litter that is instead composed of high C:N material (Vestergaard et al., 2001; Magid et al., 2006) and we observed this trend in the driest wheat amended soils, but it was not significant. When aggregation decreases  $\text{CO}_2$  flux from high C:N litters, the relationship is attributed to N requirements of the litter decomposition and multiple studies have established this mechanism causing lower  $\text{CO}_2$  flux from more aggregated high C:N litter (Vestergaard et al., 2001, Magid et al., 2006).

#### 4.4.3 - CH<sub>4</sub> flux relationships

The lower rates of CH<sub>4</sub> uptake in alfalfa soils compared to wheat soils could be caused by NH<sub>4</sub><sup>+</sup> inhibition of methanotrophy or stimulation of methanogenesis. A study of CH<sub>4</sub> inhibition found that 30 μg N gdw<sup>-1</sup> NH<sub>4</sub><sup>+</sup> slowed growth of methanotrophs and uptake of CH<sub>4</sub> (Mohanty et al., 2006) and it may occur at lower concentrations. Although homogenized soil NH<sub>4</sub><sup>+</sup> was between 2 and 20 μg N gdw<sup>-1</sup> in our alfalfa soils it is reasonable to assume the NH<sub>4</sub><sup>+</sup> was much higher closer to litter patches, so the concentration experienced by microbes in parts of the mesocosm would have been higher than the homogenized soil NH<sub>4</sub><sup>+</sup> concentration. Alternatively, alfalfa patches could have contained methanogenic microsites, which would increase gross CH<sub>4</sub> production and cause the net CH<sub>4</sub> flux to become less negative (Brewer, chapter 2). These microsites could have formed if O<sub>2</sub> consumption in litter patches was so great that the center of the patch became anoxic for extended periods (Keiluweit et al., 2016). Both of these effects are possible but it is more likely that NH<sub>4</sub><sup>+</sup> inhibition played a more prominent role in decreasing CH<sub>4</sub> uptake in alfalfa soils because none of these soils exhibited consistent net CH<sub>4</sub> production, which likely would have happened with some if methanogenic microsites were present.

The small interactive effect of litter aggregation and moisture appears to be caused by greater CH<sub>4</sub> uptake in soils with a single wheat patch. These mesocosms consumed CH<sub>4</sub> while the 15%WHC controls did not, so the aggregated wheat litter may have retained soil moisture or otherwise provided good methanotrophy habitat.

Fluxes of CH<sub>4</sub> in the wet soils (85% WHC) were dominated by net production. Though these fluxes did not significantly relate to experimental factors they were strongly correlated with inorganic N. A common cause of decreased surface CH<sub>4</sub> uptake is inhibition of methanotrophy by NH<sub>4</sub><sup>+</sup> (Aronson and Heliker, 2010), but it appears that the relationship here may have been related to methanogenesis, not methanotrophy. In this case we may have observed inhibition of methanogenesis



by  $\text{NO}_3^-$ , which has been observed before but primarily in mineral  $\text{NO}_3^-$  additions (Nazaries et al., 2013). This effect was not related to litter aggregation but N content was much greater in alfalfa litters at this moisture.

#### 4.4.4 - Microbial biomass

There are multiple reasons we may have found relatively high amounts of microbial biomass in very dry soils. When microbes are stressed by low moisture, they accumulate osmolytes which can increase their biomass by 25-35% (Schimel et al., 1989; Schimel et al., 2007). This is consistent with the ~50% increase in biomass we observed going from 35% to 15% WHC levels and the negative relationship of MBC and  $\text{CO}_2$  flux. Biomass could decrease as moisture increases from 15% WHC to 65% WHC because greater moisture increases connectivity of porespace allowing for more mobility and predation by microviborous protozoans, nematodes, and other soil fauna (Hunt et al., 1987). However, from 65% WHC to 85% WHC one does not expect pore connectivity to increase dramatically. Here the decrease in biomass may also be caused by greater anoxic microsite volume in the soils, shifting activity from aerobes to anaerobes which grow slower (Brune et al., 2000).

#### 4.4.5 - Litter aggregation in dry soils

This study was novel in comparing litter aggregation and type across a soil moisture gradient that included very dry soils typical of arid and semi-arid climates. Half of the significant impacts of litter aggregation of litter type were only present or were strongest in the driest soil group, indicating that these litter attributes may be particularly important for ecosystems in drier climates. And high N content litter has the greatest potential to affect C and N cycling in dry soils since the impacts of litter aggregation we observed were generally stronger in soils amended with alfalfa than wheat straw.

Dry soils may exhibit strong impacts from litter aggregation because their limited water film connectivity causes spatial arrangements of organic matter to determine the available microbial resources. In contrast, the greater diffusion and mobility of compounds and organisms in a wetter soil would mute effects of spatial resource distributions. Interactions of dissolved organic C with mineral particles should also be decreased in soils with larger litter patches since the C compounds will be slow to diffuse to the mineral soil, so the causes of this dry soil litter aggregation effect may not be entirely biological.

However, the specific mechanisms causing higher rates of C mineralization and  $\text{NH}_4^+$  concentrations in soil with aggregated litter remain unclear. Future studies could focus on food web structures, diffusional constraints, physical protection of C, and temporal dynamics of these factors to uncover the responsible drivers.

#### 4.4.6 - Implications

Since decomposition and availability of soil inorganic N are important for plant growth the large changes in C and N cycling in soils with aggregated litters could alter productivity in agricultural and unmanaged settings. Even when the effects of litter dispersal are small, they could be meaningful for farmers who cannot control soil moisture or organic amendment quality, but can alter how organic matter is incorporated into cropland and rangeland soils. In particular, organic matter aggregation may have larger effects on processes with high spatial heterogeneity, like rhizosphere nutrient availability, than soil  $\text{CO}_2$  and  $\text{CH}_4$  flux. Aggregated litter amendments were found to increase corn biomass by 14% compared to dispersed litter in a mesic climate (Loecke and Robertson, 2009b), so the heightened importance of aggregation for dry soil processes portends strong potential for aggregated organic matter to increase productivity in semi-arid and arid climates. These and other effects of litter aggregation may be long-lived, persisting until decomposition is complete.

Table 4.1: Results of full factorial ANOVAs of each gas flux and soil property measured. MBC is microbial biomass. CH<sub>4</sub> data exclude fluxes from the 85% WHC soils in this analysis. CO<sub>2</sub> and MBC were both log-transformed prior to analysis.

		<b>CO<sub>2</sub></b>	<b>CH<sub>4</sub></b>	<b>NH<sub>4</sub><sup>+</sup></b>	<b>NO<sub>3</sub><sup>-</sup></b>	<b>MBC</b>
	Model R <sup>2</sup>	0.93	0.79	0.82	0.70	0.35
<b>Predictor</b>	df	Predictor p-value				
Soil H <sub>2</sub> O	3	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>
Litter type	1	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	0.66
Litter aggregation	3	0.18	0.68	<b>0.010</b>	0.42	0.38
H <sub>2</sub> O X Type	3	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	0.90
H <sub>2</sub> O X Agg.	9	0.97	0.056	0.105	0.54	0.82
Type X Agg.	3	<b>0.035</b>	0.32	0.072	0.55	0.76
H <sub>2</sub> O X Type X Agg.	9	0.091	0.090	0.23	0.94	0.94

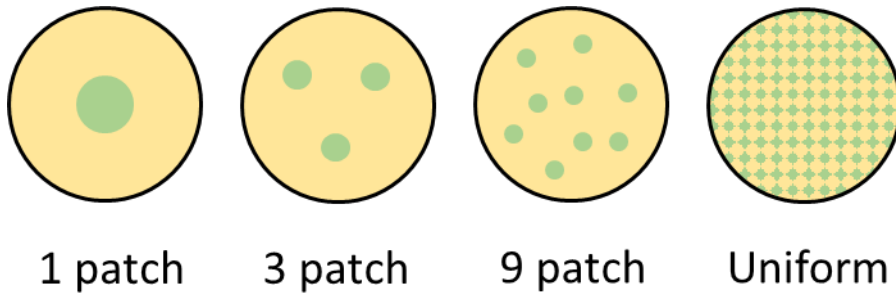


Figure 4.1: View from above of arrangement of litter patches in different litter aggregation treatments.

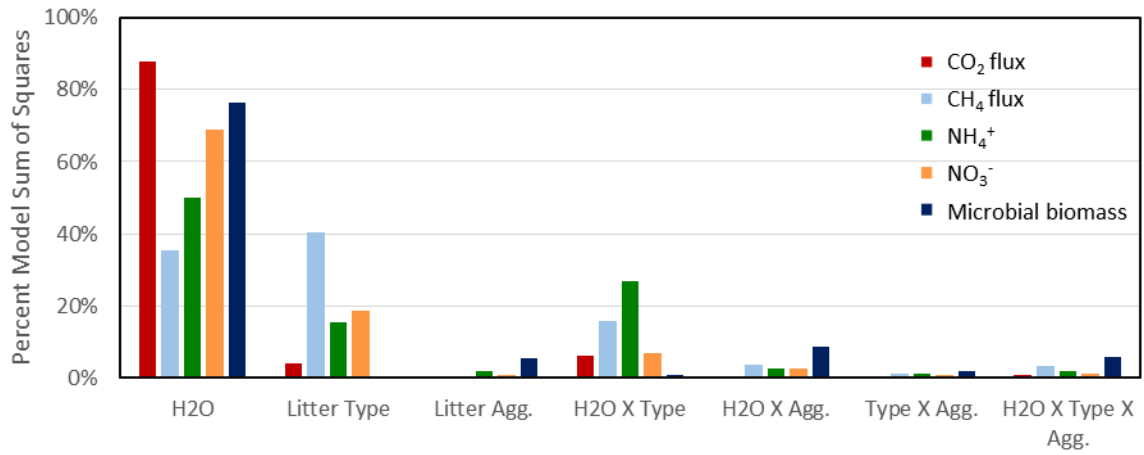


Figure 4.2: The percent of each response variable's model sum of squares attributed to each main effect and interaction from ANOVAs constructed with full factorial predictors of experimental factors.

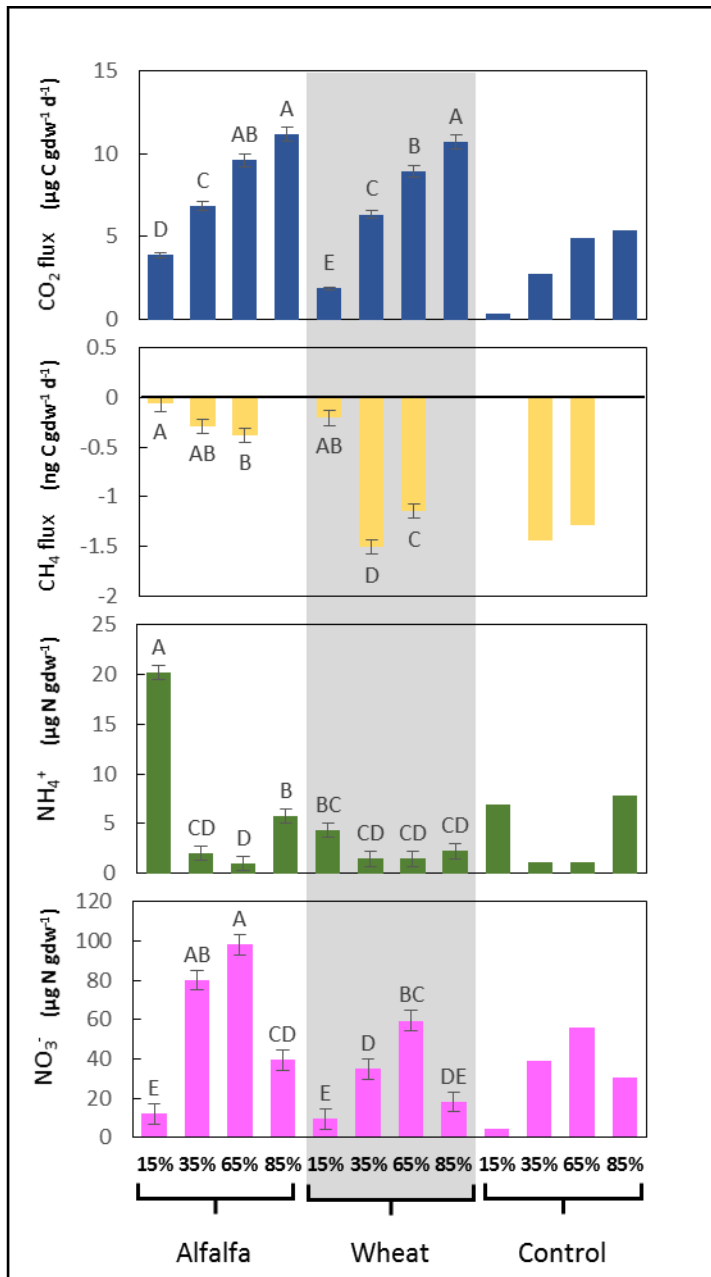


Figure 4.3: Gas fluxes and soil properties over litter type and soil moisture levels. This interaction was significant for each of these factors. The data is grouped by litter type and then sorted by soil moisture level. Error bars are standard errors, values that do not have shared letters are significantly different. Neither error bars nor letters are shown on the controls because they have much higher variance than the other treatments and were not statistically compared.

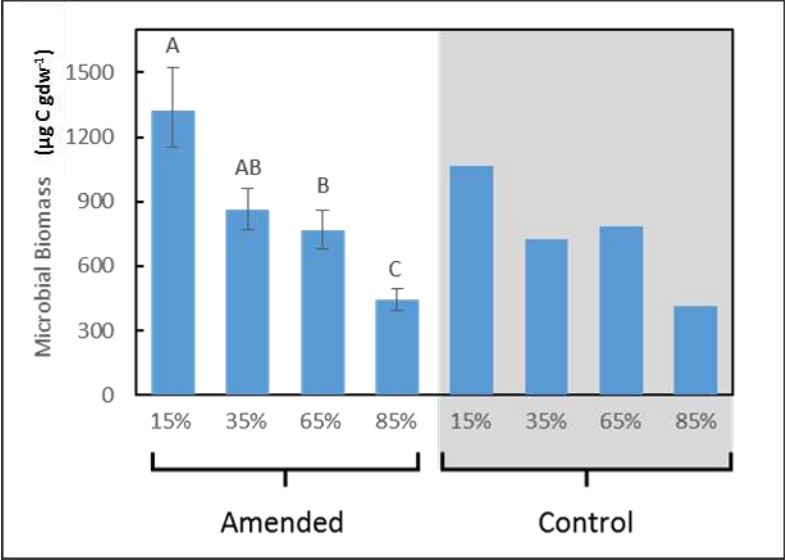


Figure 4.4: Microbial biomass over soil moisture. Error bars are standard errors, values that do not have shared letters are significantly different. Neither error bars nor letters are shown on the controls because they have much higher variance than the other treatments and were not statistically compared.

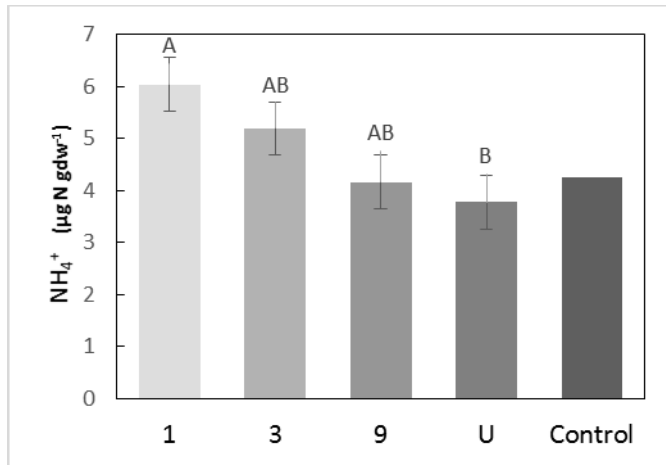


Figure 4.5:  $\text{NH}_4^+$  concentration over litter aggregation. Error bars are standard errors, values that do not have shared letters are significantly different. Neither error bars nor letters are shown on the control because it has much higher variance than the other treatments and was not statistically compared.



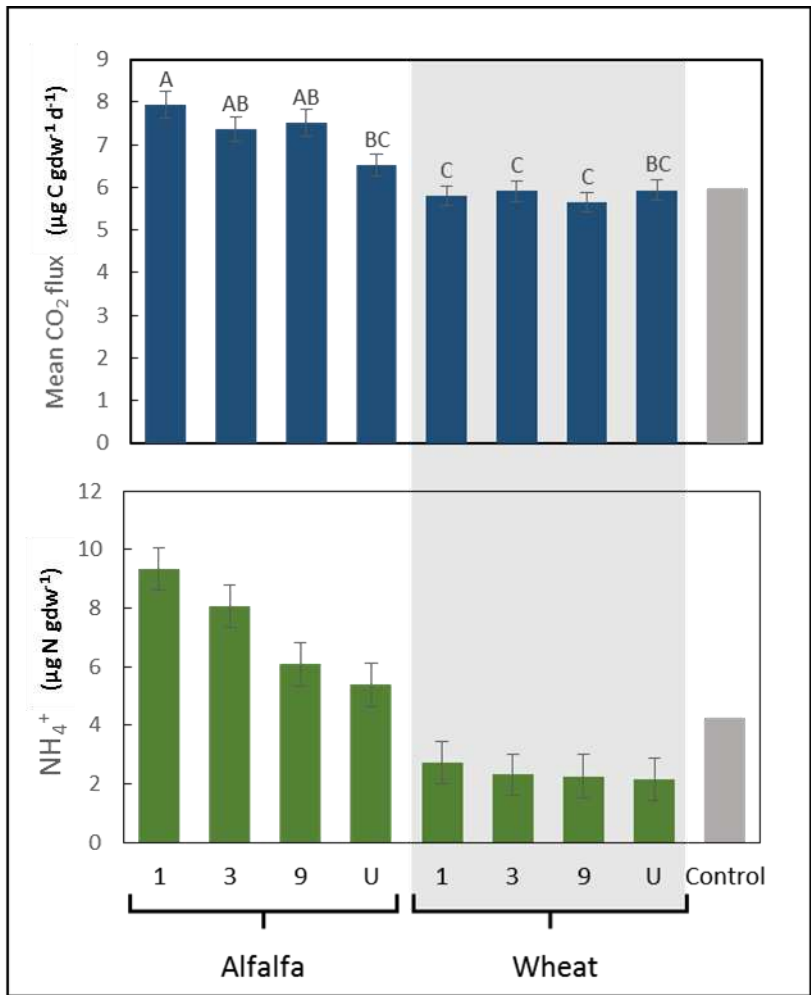


Figure 4.6: CO<sub>2</sub> and NH<sub>4</sub><sup>+</sup> over litter type and aggregation level. This interaction was not significant in the ANOVA for NH<sub>4</sub><sup>+</sup>, but it helps illustrate the importance of litter types. . Error bars are standard errors, values that do not have shared letters are significantly different. Neither error bars nor letters are shown on the controls because they have much higher variance than the other treatments and were not statistically compared.

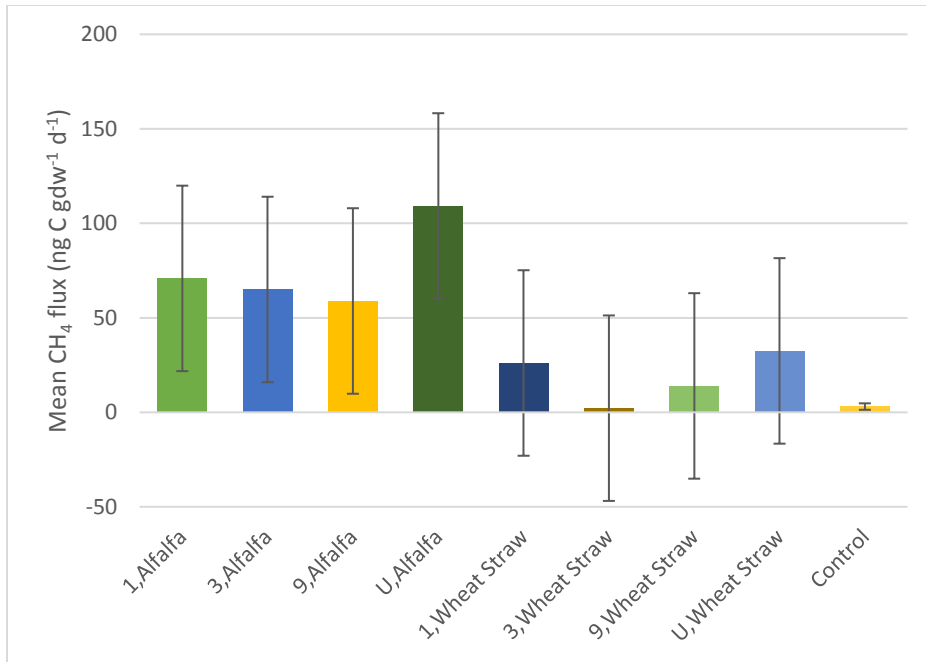


Figure 4.7: CH<sub>4</sub> flux of 85% WHC soils over litter type and aggregation level. Neither was a significant predictor of CH<sub>4</sub> for this moisture level.



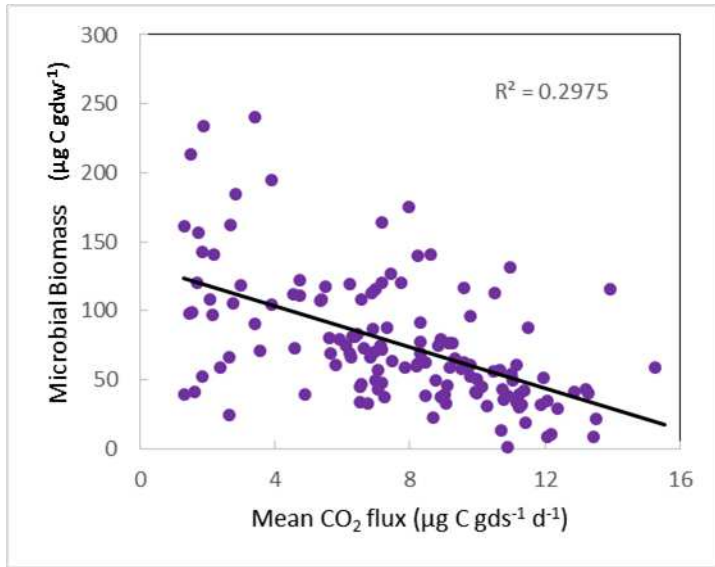


Figure 4.9: Microbial biomass C regressed over mean CO<sub>2</sub> flux.

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## 5 – Conclusion

### **5.1 - Summary**

In my dissertation research I sought to characterize relationships between soil heterogeneity and processes critical for ecosystem function. Specifically, I addressed gaps in our knowledge of how heterogeneity of organic matter, oxygen, and anaerobic activities alter carbon cycling, nitrogen availability, greenhouse gas fluxes, microbial biomass, and other biogeochemical processes.

Methanogenesis is more than a flux of CH<sub>4</sub>, it is also an indicator of deeply reduced conditions and anaerobic microbial activity (Conrad, 1996). Because of this I view it as an indicator of anoxia and greater potential for other anaerobic processes. This perspective allowed me make inferences about the likelihood of anaerobic processes occurring in study soils without destroying soil structure to make direct O<sub>2</sub> or reduction-oxidation potential measurements. I used this approach in my first two chapters.

In the first chapter I measured gross CH<sub>4</sub> production and consumption in established tillage treatments over two growing seasons during which the semi-arid fields were under wheat then fallow rotations. I observed much higher rates of methanogenesis in soils under no-till wheat production than tilled or fallow soils. Because this elevated methanogenesis was present during the season of wheat production but not in the same plots during a fallow year I concluded there is a wheat related cause of methanogenesis which is likely either methanogenic microsites forming around dead roots or macrofaunal gut methanogenesis. High rates of methanogenesis were complemented by similarly high rates of methanotrophy in no-till wheat plots that prevented CH<sub>4</sub> produced in the soils from reaching the atmosphere. No-till soils can become stronger CH<sub>4</sub> sinks than tilled soils after being established for >10 years (appendix 1) but until now it has been unclear if this was due to greater methanotrophy or increased gas diffusivity. While no-till plots did have

greater gas diffusivity than tilled plots, multiple regression analysis indicated that it was the elevated rates of methanotrophy in these 46 year old no-till plots that caused them to be stronger sinks than the adjacent tilled soils. The coincidence of high rates of methanotrophy and methanogenesis match findings of others (Kammann et al., 2009; Yang and Silver et al., 2016) and, together, these studies indicate that upland methanogenesis may enable growth of methanotrophs and higher net CH<sub>4</sub> uptake in grassland and cropland soils. We conclude that common agricultural practices have large impacts on microbial activity that may be best understood by using conceptual models that explicitly consider management-soil environment-microbe relationships.

In my second chapter I examined the impacts that soil moisture and agronomic practices (conventional, no-till, and organic) have on soil properties and the presence and persistence of anaerobic activities in an intact core laboratory experiment. I made measurements of methanogenesis at week 6 and 21 of the experiment and found that early rates of methanogenesis were correlated with agricultural practice (organic soils producing more CH<sub>4</sub> than conventional soils) and soil respiration while later rates were better predicted by water-filled porespace. The persistence of methanogenesis between measurements was also related to higher water-filled porespace, and this relationship was especially strong in no-till soils. Because methanogenesis was associated with high CO<sub>2</sub> flux and water-filled porespace at different times in the incubation it appears that anoxic microsite dynamics can result from changes in the underlying biological and physical mechanisms driving anoxia (i.e., O<sub>2</sub> consumption and diffusion). Higher rates of methanogenesis were associated with higher soil NH<sub>4</sub><sup>+</sup>, less NO<sub>3</sub><sup>-</sup>, more N<sub>2</sub>O emissions, and less microbial biomass. Moreover, anoxic microsites and their impacts may form and persist more often in wet soils or high SOC soils, such as those under organic agricultural production.

My third chapter summarizes an experiment of spatially dispersed organic matter across multiple manipulated factors in full factorial. I constructed soil mesocosms across four levels of litter

aggregation (a single litter patch, three patches, nine patches, or uniformly distributed litter), two litter types (alfalfa or wheat straw), and four soil moistures ranging from 15% water holding capacity to 85%. Net CH<sub>4</sub> production was observed in the wettest treatments and though it was not affected by litter aggregation it was correlated with higher NH<sub>4</sub><sup>+</sup> and lower NO<sub>3</sub><sup>-</sup>. When compared to the full range of effects of soil moisture and litter type, litter aggregation was not generally important for CO<sub>2</sub> or CH<sub>4</sub> gas fluxes or inorganic N and soil organic matter properties. However, in the driest soils litter aggregation created a number of significant differences in soil processes, these soils exhibited 50%-70% greater NH<sub>4</sub><sup>+</sup> in single litter patches compared to uniformly distributed litter. In addition, the alfalfa amended soils had 65% greater respiration between those same litter aggregation treatments. Dry soils may have exhibited stronger impacts of litter aggregation because limited water film connectivity caused spatial arrangements of organic matter to determine the available microbial resources, where greater diffusion in wetter soils would mute effects of spatial resource distributions (Moyano et al., 2013; Ebrahimi and Or, 2015). The impacts of litter aggregation on carbon and nitrogen cycling could affect dryland agriculture in semi-arid climates where tillage and crop residue management alter distributions of buried litter.

## 5.2 - Synthesis

Multiple studies have now found that high rates of methanogenesis and methanotrophy frequently co-occur in upland soils (chp. 2; Kammann et al., 2009; Yang and Silver, 2016), and it is intuitive that local CH<sub>4</sub> production would stimulate growth of methanotrophs and their functional capacity (Hatamoto et al., 2010). However, my experiment of intact cores with manipulated moistures (chp. 3) did not find that methanotrophy was stimulated by methanogenesis. This could be because growth of methanotrophs at the low concentrations of CH<sub>4</sub> typical of soils requires many months or even years. Stimulation of methanotrophy by methanogenesis could be responsible for

the 5-10 year recovery of CH<sub>4</sub> net consumptive capacity observed after cropland soils are converted to no-till, a time frame that agrees with slow methanotrophic growth (Maxfield et al., 2006). If that is the case, this is a fascinating relationship: small-scale organic matter and O<sub>2</sub> heterogeneity causes methanogens to flourish and produce CH<sub>4</sub>; methanotroph populations grow, stimulated by an increase in substrate and eventually are able to consume all the CH<sub>4</sub> produced; the greater methanotrophic capacity of the no-till soils now also affects an ecosystem process, increasing the soil's net surface CH<sub>4</sub> uptake beyond its original capacity. More generally, soil management alters soil O<sub>2</sub> heterogeneity and sub-surface CH<sub>4</sub> cycling to result in an enhanced ecosystem function.

CH<sub>4</sub> production was also reliably associated with elevated levels of NH<sub>4</sub><sup>+</sup> and N<sub>2</sub>O, but decreased levels of NO<sub>3</sub><sup>-</sup> and microbial biomass (chp. 3 & 4). These relationships are consistent with greater rates of methanogenesis indicating larger portions of soil under anoxic conditions since that would slow nitrification, increase denitrification, and shift metabolic dominance from aerobes to slower growing anaerobes (Conrad, 1996). And, together, these findings suggest that greater frequency of anoxic microsites and anaerobic activity in upland soils may slow nutrient release or increase gaseous N loss. Here, methanogenesis is an indicator of changes in nitrogen cycling that are likely related to soil O<sub>2</sub> availability. Thus, gross CH<sub>4</sub> has potential for use as an indicator in field or lab settings for potential changes in nutrient availability.

Spatial effects of heterogeneous litter distributions had strong effects on nitrogen availability and carbon mineralization in very dry soils. While the methanogenesis-related effects of heterogeneity appear to be dominated by biological factors, these effects of space interacting with moisture may be primarily physical, caused by the limited diffusion and strong diffusional gradients present when pore connectivity is lost. This shows that soil physical properties can control effects of chemical or biological heterogeneity, particularly under certain conditions, and that soil pore

structure may be especially important in mediating effects of soil heterogeneity in arid and semi-arid ecosystems (Moyano et al., 2013; Lee et al., 2014; Hewins and Throop, 2016).

### **5.3 - Future directions and limitations**

Measuring gross CH<sub>4</sub> production is a promising non-destructive approach to studying anoxic microsites and anaerobic activity in soils, however it is not without caveats. Dynamics of methanogenesis and other anaerobic processes are likely to be asynchronous at times due to different controls dominating them (Pandey et al., 2012), I did not observe the strong correlations between methanogenesis and N<sub>2</sub>O flux I would expect if methanogenesis and denitrification were tightly linked. Methanogenesis also demonstrates a much lower predictability than CO<sub>2</sub> and N<sub>2</sub>O fluxes based on soil properties (e.g., moisture, organic matter) which could either pose challenges to its use or could be a benefit if this occurs because the soils where methanogenesis is present are biogeochemically distinct in other important ways.

My set of studies provides a variety of findings that encourage deeper investigations and new questions. What are the causes of upland methanogenesis, how do they vary over space and time, do they affect other processes? How does methanogenesis relate to denitrification, sulfate and iron reduction? Is methanogenesis an indicator of altered carbon cycling and turnover, does it affect iron-related carbon cycling? Do nutrient effects of litter aggregation or methanogenesis translate to effects on plant productivity in field settings? And, perhaps most importantly, how can ecologists, farmers, conservationists and other land managers use knowledge of soil heterogeneity to improve our use of soils and their sustainability as a critical resource?

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## Appendix 1 - Effect of No-till Agriculture on Surface CH<sub>4</sub> Fluxes

No-till agriculture (NT) can affect surface CH<sub>4</sub> fluxes in comparison to conventionally tilled crop production (CT). An early review of this topic found that NT is a stronger sink of CH<sub>4</sub> than CT soils (Six et al., 2004), but some studies since then have found NT soils to be a weaker CH<sub>4</sub> sink (Plaza-Bonilla et al., 2014) or even a stronger source (Alluvione et al., 2009) compared to CT soils. A more recent review illustrates this uncertainty of the NT effect and concludes that wetter climates may cause NT soils to be weak sinks or sources (Abdalla et al., 2013).

To determine if an analysis of multiple factors with updated set of studies could elucidate a consistent and predictable effect of NT on CH<sub>4</sub> flux I performed a simple meta-analysis of existing studies that compare NT and CT soils in field settings. In this vote-counting analysis I recorded each studies' finding of a difference and its sign (i.e., negative if NT is a stronger sink or weaker source than CT, positive if the opposite) or, if there was no significant difference, considered it a finding of zero response. I then plotted these categories of response (negative, zero, positive) over the time since conversion to NT. To obtain my set of candidate studies I searched in scholar.google.com with search terms including “tillage”, “no-till”, “CH<sub>4</sub>”, “methane”, and “crops”. After finding an initial set of studies that had the necessary measurements and comparisons I reviewed all publications those studies cited as well as the later studies that cited the initial set studies. I found 14 studies that fit the needed measurements for this analysis (table 1).

Individual studies of upland, cropped soils that compare CT to NT soils show a variety of effects, higher CH<sub>4</sub> flux, lower flux, or no effect, but taken together the body of literature indicates a clear, but delayed, effect of conversion to NT on CH<sub>4</sub> flux in soils. Soils that have had longer durations of NT are more likely to be stronger sinks (or weaker sources) than CT soils (fig. 1). Within the first 7 years following conversion to NT, NT generally has greater flux than CT, but it

appears that 8-10 years after conversion soils have consistently lower CH<sub>4</sub> flux. This summary indicates that ultimately NT decreases the CH<sub>4</sub> flux of the cropped field, though there is a multi-year recovery of a soil's capacity for CH<sub>4</sub> uptake after conversion to NT. Some researchers previously noticed poor CH<sub>4</sub> uptake immediately following conversion to NT and attributed this to slow methanotrophic recovery (Hutsch, 1998) but past reviews of tillage effects on CH<sub>4</sub> flux have not noted the delayed conversion effect our summary shows (Six et al., 2004; Abdalla et al, 2013). In dry climates there is a similar delayed effect on N<sub>2</sub>O fluxes following conversion to NT: NT N<sub>2</sub>O efflux is greater than CT for the first 10 years following conversion, but after that NT N<sub>2</sub>O efflux is smaller than CT (Six et al., 2004; van Kessel et al., 2013). The authors hypothesized that older NT soils have greater SOM content (Conant et al., 2007) and improved soil structure that decreases the number of anoxic microsites hosting denitrification (van Kessel et al., 2013). Many fields in the United States are cycled from NT to CT in periods <10 years, so managers may be missing some of the best climate benefits of NT.

While this analysis provides compelling evidence of a time-since-conversion effect on CH<sub>4</sub> fluxes of NT soils, I will continue to improve it with standardized search criteria and deeper analyses of site factor relationships to CH<sub>4</sub> fluxes. I will also explore the possibility of employing meta-analytical tools beyond the vote-counting approach.

Table A.1: Studies and experiments used in meta-analysis. The far right column indicates the finding of the paper in regards to NT CH<sub>4</sub> flux vs CT.

Authors	Year	Journal	Study Location	Average # years since conversion to NT	NT vs CT CH <sub>4</sub> flux (pos, neg, or zero)
Plaza-Bonilla et al	2014	Plant and Soil	Spain	16	-1
Plaza-Bonilla et al	2014	Plant and Soil	Spain	5	1
Nyakatawa et al	2011	Applied and Environmental Soil Sc	Alabama	11	0
Jin et al	2014	Bioenergy research	9 sites across U.S. corn belt		1
Alluvione et al.	2009	J of Environmental Quality	Fort Collins, CO (ARDEC)	6.5	1
Robertson, et al	2000	Science	Michigan (KBS)	7	0
Gregorich et al	2006	SBB	Ottawa, Ontario: Agriculture and Agri-Food C	7	0
Kessavalou et al	1998	Atmospheric Pollutants and Trace	Sidney, NE; High Plains Agricultural Resear	24	-1
Mosier et al	1991	Nature	Colorado SGS, CPER (3 sites total)	9	0
Hutsch	1998	Biological Fertility of Soils	Bruchköbel near Hanau (Germany)	16	-1
Tian et al	2013	PLOS One	North China Plain	6	1
Tian et al	2012	PLOS One	North China Plain	4	1
Sainju et al	2012	J of Environmental Quality	Western North Dakota	28.5	-1
Cochran et al	1997	Can. J of Soil Science	Alaska	11	-1
Liu et al	2006	Plant and Soil	Colorado (ARDEC)	5	0
Liu et al	2006	Plant and Soil	Colorado (ARDEC)	5	1
Omonode er al	2007	Soil and Tillage Research	Indiana	29.5	0

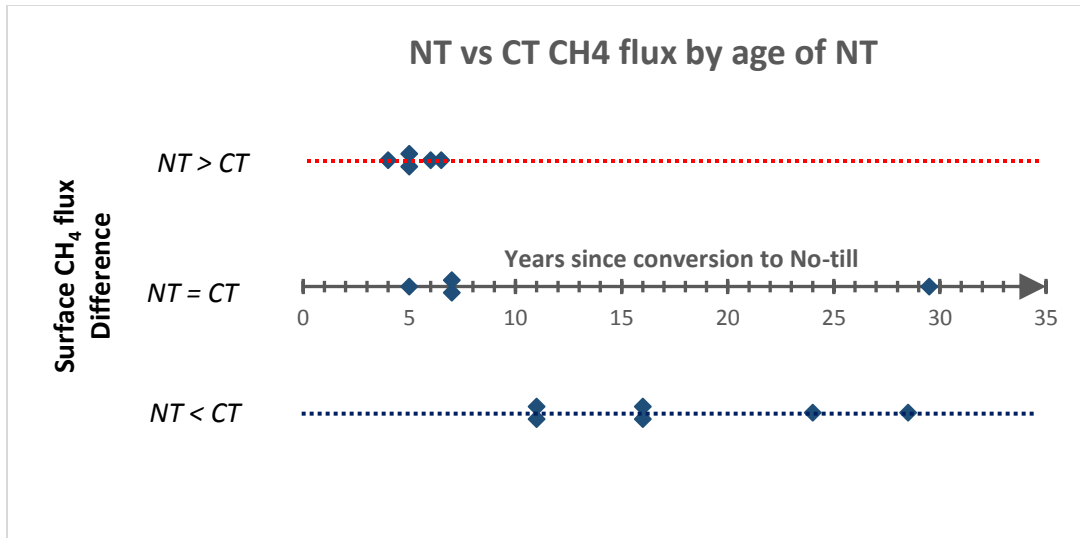


Figure A.1: Difference between fluxes of conventionally tilled (CT) and no-till (NT) soils in previous studies. *NT > CT* (red line) indicates NT soils had more positive fluxes than CT (i.e., less uptake), *NT = CT* indicates that soils did not have significantly different fluxes, *NT < CT* (blue line) indicates that NT soils had greater surface uptake (or lower emission) than CT soils. Data were taken from 14 studies (15 total experiments; see table 1). Some points are vertically offset to make duplicate results of same age clear.

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## Appendix 2 - R code used to fit and optimize the MASMC model

This abridged R code covers the important steps and aspects of the model implementation and usage, but does not contain all the script since my input files will vary from those of other researchers. You can contact me if I have overlooked a necessary detail at paul.brewer@gmail.com

```
#####  
## steady_state_gas_model.R  
##  
## A script to fit the MASMC model to data that includes surface  
## chamber flux and soil air gas measurements.  
##  
##  
  
library("optimx") # for optimization  
library("pracma") # for error function erf  
library("data.table") #for rbindlist  
library("GenSA") # for optimization  
library("pso") # for optimization  
  
options(digits=12) # use more sig digits so that deviances, etc are calculated very accurately  
  
## Define site that current data analysis is for:  
site<-"Akron" # Either "KBS" or "Akron"  
  
## Define gas that is currently being evaluated:  
gas<-"CH4"  
  
## Declare whether gas height or area are to be used:  
gas_metric<-"Area" ## either "Ht" or "Area", typically Area for CH4 and Ht for N2O  
  
max_iter<-5000 # Maximum # of iterations allowed in the model  
  
## Define gas of interest and related constants.  
## Gas constants and model limits (from Butter's table)  
Gas_nameV<-c("CH4","CO2","N2O")  
DairV<-c(13.74,9.72,10.08)  
KhV<-c(29.3, 1.2, 1.6)  
DfreewaterV<-c(0.001104,0.001056,0.001104)  
D_IV<-c(1e-14,1e-14,1e-14)
```



```

D_uV<-c(7,5,6)
mu_IV<-c(1e-7,1e-7,1e-7)
mu_uV<-c(3,10,100)
gamma_IV<-c(1e-7,1e-7,1e-7)
gamma_uV<-c(10,50000,5000)

gases_df<-data.frame(Gas_name=Gas_nameV,Dair=DairV,Kh=KhV,Dfreewater=DfreewaterV,
  D_lower_limit=D_IV,D_upper_limit=D_uV,mu_lower_limit=mu_IV,
  mu_upper_limit=mu_uV,gamma_lower_limit=gamma_IV,
  gamma_upper_limit=gamma_uV,
  stringsAsFactors=FALSE, check.names=T)
gas_info<-gases_df[gases_df$Gas_name=="gas,] # current gas info list

# The constants I originally used for CH4
Dair<-gas_info$Dair# cm2/min
Kh<-gas_info$Kh # Henry's constant for CH4, dimensionless
Dfreewater <-gas_info$Dfreewater #cm2/min

## Define model limits, in order of D, mu, gamma
upper_limits<- c(gas_info$D_upper_lim,gas_info$mu_upper_lim,gas_info$gamma_upper_lim)
lower_limits<-c(gas_info$D_lower_lim,gas_info$mu_lower_lim,gas_info$gamma_lower_lim) ##
Original values: c(0,0.00001,0.00001)

#####
# Define Steady-state gas model (ie, the MASMC model)
## Statement to use only for model testing: D<-D0; mu<-mu0; gamma<-gamma0

SS.model.fit<-function(D,mu,gamma){
  t<-chamber_timepts
  # Recalc variables
  beta<-sqrt(mu*a/D)
  new_lamda<-mu/R
  new_r<-(H/(a*R))*beta
  C_mod<-C_0_0-gamma/mu
  sigma<-c(-1/(2*new_r)*(1+(4*new_r^2+1)^(1/2)),-1/(2*new_r)*(1-(4*new_r^2+1)^(1/2))) #
sigma variables 1 and 2
  lamda<-c(-1,1,-sigma[1],-sigma[2]) # lamda variables 1 through 4
  LAMDA<-c(1/(1-sigma[2])-1/(1-sigma[1]),1/(1+sigma[2])-1/(1+sigma[1]),
    1/(1+sigma[1])-1/(-1+sigma[1]),1/(-1+sigma[2])-1/(1+sigma[2])) # Capital lamdas, 1
through 4
  pi_term<-(1/(pi*new_lamda*t))^(1/2)

```

```

S_matrix <- matrix(, nrow = length(t), ncol = 4) # define the S matrix with t rows and j columns
for(j in 1:4){ # Ie, cycle through j lamdas
  Sj_t_vector<-vector('numeric')
  for(i in 1:length(t)){ #cycle through all t timepoints
    S_j_t<-pi_term[i]-lamda[j]*exp(new_lamda*chamber_timepts[i]*(lamda[j])^2)*
      (1+erf(-lamda[j]*(new_lamda*chamber_timepts[i])^(1/2))) ## Effectively, I use the
complimentary error function
    Sj_t_vector<-rbind(Sj_t_vector,S_j_t)
  }
  S_matrix[j,]<-Sj_t_vector
}
summation_t_vector<-
LAMDA[1]*S_matrix[,1]+LAMDA[2]*S_matrix[,2]+LAMDA[3]*S_matrix[,3]+LAMDA[4]*S_matri
x[,4]
exponential_t_vector<-exp(-new_lamda*t)*(-1/(2*new_r*(sigma[2]-sigma[1])))
C_t_model<-gamma/mu+C_mod*(1+exponential_t_vector*summation_t_vector)
C_t_relative_deviance_sq<-(C_t_model-
C_t_chamber_df$C_t_data)^2/(StdDev_C_t_data/C_0_0)
C_t_SSQ<-sum(C_t_relative_deviance_sq)
C_x_model<- C_mod*exp(-beta*C_x_soil_df$Depth)+gamma/mu
C_x_relative_deviance_sq<-(C_x_model-C_x_soil_df$C_x_data)^2/(StdDev_C_x_data/C_0_0)
C_x_SSQ<-sum(C_x_relative_deviance_sq)
C_total_SSQ<-C_t_SSQ+C_x_SSQ

## Calculate some other stats
Max_percent_C_x_deviance<-max(C_x_relative_deviance_sq)/C_x_SSQ
Max_percent_C_t_deviance<-max(C_t_relative_deviance_sq)/C_t_SSQ
ave_C_x_data<-mean(C_x_soil_df$C_x_data[])
soil_air_C_diff<-C_x_soil_df$C_x_data[2]-C_x_soil_df$C_x_data[1] ## Difference between C_x
at 10cm and C_x at 0cm (ie, the C_t_1 value)

if(log_model_data==1){
## Copy model data to a logging df:
model_data<-list(C_x_max=max(C_x_soil_df$C_x_data[]),
  C_x_ave=ave_C_x_data,
  Soil_air_difference=soil_air_C_diff,
  C_t_0=C_t_data[1],
  StDev_C_x=StdDev_C_x_data,
  StDev_C_t=StdDev_C_t_data,
  Max_C_x_dev_percent_of_x_SSQ=Max_percent_C_x_deviance,
  Max_C_t_dev_percent_of_t_SSQ=Max_percent_C_t_deviance,
  SSQ_C_x=C_x_SSQ,
  SSQ_C_t=C_t_SSQ,
  SSQ_total=C_total_SSQ)
}

```

```

Model_data_df<-rbindlist(list(Model_data_df,model_data))}

return(C_total_SSQ)
} ### End SS model fit function

log_model_data<-0 ## set the model data logging off for the start

# ## declarations for testing:
# current_cham_samp_ID<-38
# current_plot<-"C1"
# current_chamber<-1

## Initialize a dfs for model output and various metadata
#
Sample_meta_df<-data.frame(Chamber_sampling_ID=integer(),
                           Probe_sampling_ID=integer(),
                           Sampling_Date=character(),
                           Sampling_Time=character(),
                           Plot_name=character(),
                           Treatment=character(),
                           Chamber_rep=integer(),
                           Effective_HS_height=double(),
                           Chamber_area=double(),
                           Porosity=double(),
                           Soil_VWC=double(),
                           Air_filled_space=double(),
                           Air_filled_pore_space=double(),
                           Water_filled_pore_space=double(),
                           D_of_soil_estimate=double(),
                           stringsAsFactors=FALSE)

Model_meta_df<-data.frame(Max_iterations=double(),
                           D_init=double(),
                           Mu_init=double(),
                           Gamma_init=double(),
                           stringsAsFactors=FALSE)

Model_data_df<-data.frame(C_x_max=double(),
                           C_x_ave=double(),
                           Soil_air_difference=double(),
                           C_t_0=double(),
                           StDev_C_x=double(),
                           StDev_C_t=double(),

```

```

Max_C_x_dev_percent_of_x_SSQ=double(),
Max_C_t_dev_percent_of_t_SSQ=double(),
SSQ_C_x=double(),
SSQ_C_t=double(),
SSQ_total=double(),
stringsAsFactors=FALSE)
Model_fit_param_df<-data.frame(D_est=double(),
  Mu_est=double(),
  Gamma_est=double(),
  Traditional_flux=double(),
  SS_flux=double(),
  Rsq_flux=double(),
  P_slope=double(),
  Counts=integer(),
  stringsAsFactors=FALSE)

## Subsample sampling info df to control which data is processed in the loops
Sampling_info_df<-Sampling_info_full_df[(Sampling_info_full_df$Site==site),]# &
  #(Sampling_info_full_df$Year==2014),]

## Set which data sets are selected
selected_soil_air_df<-(get(paste(site,"_soil_air_df",sep="")))
selected_chamber_df<-(get(paste(site,"_chamber_df",sep="")))

for(current_cham_samp_ID in(Sampling_info_df$Chamber_samp_ID)){
  ## Chamber and soil air have different sampling IDs on the same sampling date, so I define both
  current_samp_info<-
(Sampling_info_df[Sampling_info_df$Chamber_samp_ID==current_cham_samp_ID,])# All info
on current samp
  current_sair_samp_ID<-current_samp_info$Soil_air_samp_ID
  samp_year<-current_samp_info$Year
  if(samp_year=="2013"){rotation<-"Wheat"}else{rotation<-"Fallow"}

  for(current_plot
in(unique(selected_soil_air_df[(selected_soil_air_df$Gas_sampling_ID==current_sair_samp_ID),"
Plot"]))) {
    treat=substr(current_plot,1,1)
    ## Build soil air df
    soil_air_data<-
selected_soil_air_df[(selected_soil_air_df$Gas_sampling_ID==current_sair_samp_ID) &
  (selected_soil_air_df$Plot ==current_plot),]
    soil_air_depths<-soil_air_data[, "Depth_or_timepoint"] # the depths of soil air probes in cm.
    n_soil_depths<-length(soil_air_depths)

```

```

# Make a df to hold current soil air data
# For data where C_x=0 row is missing, add one. (ie, 2013 data or where Surface air
measurement was bad)
if(soil_air_depths[1]!=0){ probe_i<-1}else{probe_i<-0}
C_x_soil_df<-
data.frame("Probe"=((1+probe_i):(n_soil_depths+probe_i)), "Depth"=soil_air_depths,
stringsAsFactors=F)
C_x_data<-soil_air_data[,paste(gas_metric,gas,"ppm",sep="_")]
C_x_soil_df<-cbind(C_x_soil_df,C_x_data)
if(samp_year==2013){
  surface_data_row<-list(1,0,0)
  C_x_soil_df<-rbindlist(list(surface_data_row,C_x_soil_df))
}

for(current_chamber
in(unique(selected_chamber_df[(selected_chamber_df$Gas_sampling_ID==current_cham_samp_I
D)&
(selected_chamber_df$Plot==current_plot),"Chamber_rep"]))) {
  ## Build chamber data df
  chamber_data<-
selected_chamber_df[(selected_chamber_df$Gas_sampling_ID==current_cham_samp_ID) &
(selected_chamber_df$Plot ==current_plot)&
(selected_chamber_df$Chamber_rep==current_chamber),]
  ## Get the true sampling data from the chamber date
  samp_date<-chamber_data[1,"True_Sampling_Date"]
  samp_time<-chamber_data[1,"Sampling_start_time"]
  ## Define chamber timepoints
  chamber_timepts<-chamber_data[, "Depth_or_timepoint"]
  chamber_timepts<-(chamber_timepts-1)*current_samp_info$Timept_minutes ## Traslate
into minutes elapsed
  if(chamber_timepts[1]==0){chamber_timepts[1]<-chamber_timepts[1]+0.001} ## Make first
time non-zero for model fit
  n_chamber_timepts<-length(chamber_timepts)
  # Make a df to hold current chamber data
  C_t_chamber_df<-
data.frame("Timepoint"=(1:n_chamber_timepts), "Timepoint_min"=chamber_timepts,
stringsAsFactors=F)
  if(site=="Akron"){gas_string<-
paste(gas_metric,gas,"ppm",sep="_")}else{(if(site=="KBS"){gas_string<-("ppm")})}
  C_t_data<-chamber_data[,gas_string]
  C_t_chamber_df<-cbind(C_t_chamber_df,C_t_data)
  StdDev_C_t_data<-sd(C_t_chamber_df$C_t_data)

```

```

C_x_soil_df$C_x_data[1]<-C_t_chamber_df$C_t_data[1] # Make soil air C_0cm same as
chamber C_t=0, as is done in excel
StdDev_C_x_data<-sd(C_x_soil_df$C_x_data)

##### Calculate constants for model fit of this combination of soil air and chamber data
anchor_area<-current_samp_info$Anchor_area #cm2, varies by site
if(site=="Akron"){chamber_vol<-
current_samp_info$Chamber_vol}else{if(site=="KBS"){chamber_vol=11000}} #cm3, varies by
site.
anchor_ht<- chamber_data[1,"Ave Chamber Height:"] #cm, varies by plot
H<-(chamber_vol+anchor_ht*anchor_area)/anchor_area # Effective headspace height above
surface

### These reflect plot level bulk density (2014) and daily VWC
por<-
Soil_BD_df2[[(Soil_BD_df2$Site==site)&(Soil_BD_df2$Plot_name==current_plot)],"por_est"]
# porosity, estimated as 1-bulkdensity/particledensity(ie, 2.65)
if(is.na(por)){por<-0.38}

#VWC_col_name<-paste(treat,"_0to30",sep="")
# Find current VWC value
if(samp_date %in% Soil_VWC_model_df$Gas_sampling_date){ ## test if date exists in VWC
data
VWC_perc<-
Soil_VWC_model_df[(Soil_VWC_model_df$Gas_sampling_date==samp_date)&(Soil_VWC_mod
el_df$Rotation==rotation)&
(Soil_VWC_model_df$Tillage==treat),"Final_VWC_0_to_30cm"]

wc<-1/100*VWC_perc
#Soil_VWC_model_df[Soil_VWC_model_df$Close_samp_date==samp_date,VWC_col_name]
} else {print("No VWC found."); wc<-numeric()}

a<-por-wc # air-filled porosity, % of total soil volume that is air-filled (NOT air filled
porespace)
tort<-a^(10/3)/por^2 # tortuosity
Dsoil<-Dair*tort #cm2/min, a soil model estimate of what diffusion of gas in the soil would be
Dsoilwater<-Dfreewater*(wc^(10/3))/(por^2) # diffusion of compound through water filled
porespace (ie, in solution)
contribution<-Dsoilwater*wc/Kh # contribution of Dsoilwater to total diffusion of compound
Dgasandwater<-Dsoil+contribution # shows that relative addition of diffusion of compound in
solution is very low
# unless the compound's solubility is high and wc is also very high (e.g. wc=.45, por=0.47)
R<-1+(wc/Kh)/a # a SS model component

```

```

#####
## Initialize model
#

# Initial values
D0<-Dsoil; mu0<-0.002; gamma0<-0.00001 # These are fairly typical values for CH4 - I
could randomize to see if there's an effect on the fits

C_0_0<- C_t_chamber_df$C_t_data[1] # Concentration at t=0, x=0 (ie, surface)
params<-c(D_mod=D0,mu_mod=mu0,gamma_mod=gamma0) # Define initial model
parameters

# Optimization with GenSA:
SS_fit<-GenSA(par=params,function(params) SS.model.fit(params[1],params[2],params[3]),
lower=lower_limits,
upper=upper_limits, control=list(maxit=max_iter))
# Some optimized model output:
D_fit<-SS_fit$par[1]; mu_fit<-SS_fit$par[2]; gamma_fit<-SS_fit$par[3]; min_SSQ<-
SS_fit$value; iters<-SS_fit$counts

# Log the final model internal data (I have to rerun the model fit with the final params to do
this)
log_model_data<-1
SS.model.fit(D_fit,mu_fit,gamma_fit)
log_model_data<-0

# Calculate Steady state and traditional soil surface fluxes
SS_flux<-(-(C_0_0-gamma_fit/mu_fit)*((D_fit*a*mu_fit)^.5)) ## SS flux in ppm*cm/min.
These are odd units so I convert.
C_t_linear_reg<-lm(C_t_chamber_df$C_t_data~C_t_chamber_df$Timepoint_min)
Trad_flux<-C_t_linear_reg$coefficients[2]*H # Traditional flux in ppm*cm/min
## Get r-sq and P-value of trad flux fit's slope
summary_flux<-summary(C_t_linear_reg)
P_of_slope<-summary_flux$coefficients[2,4]
rsq_trad_flux<-summary_flux$r.squared

sample_metadata<-list(Chamber_sampling_ID=current_cham_samp_ID,
Probe_sampling_ID=current_sair_samp_ID,
Sampling_Date=samp_date,
Samplnig_Time=samp_time,
Plot_name=current_plot,
Treatment=treat,
Chamber_rep=current_chamber,
Effective_HS_height=H,

```

```

    Chamber_area=anchor_area,
    Porosity=por,
    Soil_VWC=wc,
    Air_filled_space=a,
        Air_filled_pore_space=a/por,
        Water_filled_pore_space=wc/por,
    D_of_soil_estimate=Dsoil)
Sample_meta_df<-rbindlist(list(Sample_meta_df,sample_metadata))

model_metadata<-data.frame(Max_iterations=max_iter,
    D_init=D0,
    Mu_init=mu0,
    Gamma_init=gamma0)
Model_meta_df<-rbindlist(list(Model_meta_df,model_metadata))

Model_fit_param<-data.frame(D_est=D_fit,
    Mu_est=mu_fit,
    Gamma_est=gamma_fit,
    Traditional_flux=Trad_flux,
    SS_flux=SS_flux,
    Rsq_flux=rsq_trad_flux,
    P_slope=P_of_slope,
    Counts=SS_fit$counts)
Model_fit_param_df<-rbindlist(list(Model_fit_param_df,Model_fit_param))

### A test output:
print(paste(samp_date,current_plot,current_chamber,D_fit,mu_fit,
    gamma_fit,min_SSQ,"VWC=",wc,sep=" "))
print("5 second sleep...")
Sys.sleep(5)
print("...next data set starting now.")

} ## End Chamber sampling loop

} ## End Plot sampling loop

} ## End Date sampling loop

### Combine all the output dfs and output a file:
full_output_df<-cbind(Model_fit_param_df,Sample_meta_df,Model_data_df,Model_meta_df)

current_time_date<-format(Sys.time(), "%m_%d_%y at %H_%M_%S")

```



```
write.table(full_output_df,file=paste(current_time_date,gas,"SS_model_output.csv",sep="_"),row.names=FALSE, col.names=TRUE, sep=",")
```

```
## End of code for running the model ##
```

```
#####  
#####
```