

THE ECOLOGY OF NUTRITION: MANAGING SOIL ORGANIC MATTER TO
SUPPLY SOIL NUTRIENTS, INCREASE SOIL BIOTIC ACTIVITY AND
INCREASE CROP NUTRITIVE VALUE

by

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ABSTRACT

The increasing consumer interest in high quality foods – especially fruits and vegetables with high antioxidant phytochemicals – has led to interest in determining the effects of cropping system practices on phytochemicals over the last decade. Appropriate fertility management is critical to optimize agricultural production, both for yield and crop nutritive value, and minimize losses to the environment. In organic production systems, fertility management generally relies on soil microbial processes to decompose organic matter.

To better understand the dynamics of mulch decomposition and the resulting effects on soil fertility and crop yield, a three-year randomized strip-plot experiment was implemented on the Montana State University Horticulture Research Farm. Two mulch inputs with varying carbon to nitrogen ratio (C:N), decomposition rates and microbial responses were contrasted with two non-mulched treatments, urea N fertilizer and a no-treatment control. Spinach biomass, yield, total phenolics and antioxidant capacity were measured as plant response variables to changes in soil fertility and biology due to the different inputs over three years. Water-extractable organic matter (WEOM), available nitrogen (N), phosphorus (P) and potassium (K), carbon (C) respiration, N mineralization, soil enzyme activity, microbial biomass and mycorrhizal infectivity potential were measured to assess soil fertility and biology.

The hay mulch treatment increased nutrient availability and soil biological responses, and produced high spinach yields. The straw mulched treatment had a delayed effect on N availability and lower spinach yields initially, but in subsequent years both yield and biological parameters increased in the straw mulched treatments. Both mulch treatments produced cumulative spinach yields comparable to or exceeding the N-fertilizer plots. Only slight differences in total phenolic concentration and antioxidant capacity were measured among treatments indicating that other factors likely influence spinach phytochemicals more strongly than SOM. Measuring biological responses can be a sensitive measure of soil function and an important addition to farm management to better estimate how different management practices will affect soil processes, yields and the environment.

CHAPTER ONE

INTRODUCTION

This dissertation developed from my interest in understanding how decomposition of organic materials supplies plant available nutrients for sustainable vegetable production. I was also interested in how plants respond to the soil environments that develop from different fertility inputs and whether soil conditions affect vegetable crop nutritive quality for human consumption.

The idea of sustainable agriculture – maintaining the production of food and fiber and the ecosystem services provided by agroecosystems indefinitely into the future – has been in circulation since the 1970s (Gliessman 1998, Vandermeer 1995, Altieri et al. 1983). Sustainable agriculture includes management practices like intercropping (Vandermeer 1997), crop rotations (Gliessman 2001), maintaining soil organic matter (SOM) stores (Weil and Magdoff 2004) and integrated pest management (IPM; Altieri and Nicholls 2003), and integrates the social, economic and ecological frameworks of agriculture (Kibblewhite et al. 2008). The principles underlying sustainable agricultural management stem from ecological theory, including increasing diversity to maintain ecosystem stability (Shennan 2008, Swift et al. 2004), and attempting to close energy and nutrient cycles to mimic more natural systems (Vandermeer 1997). However, the practical application of sustainability remains elusive, in part because there is no single best-management practice to achieve sustainability.

One reason why there is not a single best way to achieve sustainability is because sustainability is a moving objective. Though humans only inhabit a fraction of a percent of the earth's landmass (Young and Ritz 2005), the effects of our actions are much farther reaching. Thus all landscapes fall on a continuum of degradation. Most agricultural lands tend to be highly degraded due to long-term intensive management that alters biotic community structure, energy flows, nutrient cycles, and soil architectural structure (Kibblewhite et al. 2008). In conventional, high synthetic input agricultural management, plant diversity is reduced to one or few species and the application of mineral fertilizers and pesticides subsume the ecosystem services provided by above- and below-ground biota (Altieri et al. 1983). The goal of conventional agricultural management is to minimize variability and thus control at least some of the many risks that farmers face. The result from conventional agricultural management is highly productive farmland producing relatively uniform quality food products. Additional results include loss of topsoil from erosion, increasing monetary costs of mineral fertilizers, and increasing pesticide resistance (Matson et al. 1997). Concerns regarding the degradative effects of conventional agriculture have led to the introduction of changes in agricultural practices over the last few decades. Changes to agricultural management include precision agriculture, the use of IPM, increased diversification in crop rotations, the use of shoulder-season crops like green manures to reduce fallow and further increase crop diversity, and no-till agriculture. In combination, these changes have lead to the reduction of erosion and decreased reliance on off-farm inputs (Magdoff 2007), shifting some agricultural landscapes away from the degraded end of the continuum.

From my work on this dissertation, I believe the practical definition of sustainability is to manage agricultural lands away from the degraded end of the continuum and towards more self-sustaining ecosystems, recognizing that sustainability is not a fixed point to achieve, but a moving target to work towards. In order to know whether their management is leading towards sustainability, farmers need a set of goals for their operation that includes social, economic and ecologic objectives. Farmers also need a robust understanding of the interconnections between soils, plants (both crop and non-crop), climate and management practices and a willingness to use adaptive management, incorporating on-farm experimentation, monitoring and subsequent adjustments to management practices to manage their land towards their goals. My desire with this dissertation was to investigate one small piece of sustainable agricultural management – how mulch additions contribute to soil fertility and crop productivity through time – and contribute to the knowledge base used by farmers to work towards sustainability.

Overview of Dissertation

To investigate mulch input effects on fertility and crop productivity, I implemented a small-plot experiment at the Montana State University Horticulture Farm. Our research plots were located adjacent to the Towne's Harvest Garden teaching and research vegetable farm (THG). The experimental design was a randomized strip-plot with four replicates of four soil treatments over 2000 m². The four soil treatments included barley-straw mulch, grass-legume mulch, urea N-fertilizer and a no-treatment

control. In these soil treatments we grew four crops: tomato (*Solanum lycopersicum* var. Juliet), broccoli (*Brassica oleracea* var. Arcadia), corn (*Zea mays* var. Vision) and spinach (*Spinacia oleracea* var. Space (F1)). For this dissertation, all plant sampling and analyses used only the spinach crop and all soil samples were also obtained from the spinach plots (Appendix B). I chose to focus research efforts on spinach plots for monetary and logistical constraints, preferring to investigate the relationship between fertility input, soil C and N cycling and crop productivity thoroughly with one crop. By choosing spinach, an early season crop, I were able to avoid most of the inclement weather events, including hail and early frost, that impact vegetable production in southwest Montana.

In Chapter 2, I measured the effects of different fertility treatments on spinach yield and soil fertility. Organically managed agroecosystems must rely on the decomposition of organic materials to supply plant available nutrients. But different types of organic inputs can have very different effects on microbial decomposition rates and subsequent nutrient cycling. The timing of nutrient release differed between the two mulched plots following their incorporation into the soil. The duration of the effects on available nutrients and yield also differed between the two mulches. Spinach yields were a sensitive response variable to the available nutrients in each treatment. Understanding the temporal dynamics of different organic inputs will help farmers make appropriate choices for their system and plan crop rotations to maximize the benefits from the inputs they use.

The increasing consumer interest in high quality foods – especially fruits and vegetables with high antioxidant phytochemicals – could have economic benefits to farmers. Accordingly, interest in determining the effects of cropping system practices on phytochemicals has increased in the last decade. Previous studies have found an increase in plant phytochemicals in organically managed systems compared with conventional systems (Lester et al. 2011, Mitchell et al. 2007). Understanding that a primary difference between organic and conventional management is the regular addition of SOM to organically managed soils (Doane et al. 2004), Chapter 3 describes the investigation of whether soil organic matter management affects spinach antioxidant production. Total phenolic concentration and oxygen-radical absorbance capacity (ORAC) were measured in spinach over three years. Understanding the drivers that affect plant biochemistry can be challenging as plants have many interacting pathways with feedbacks and complex regulating mechanisms, affected by both the above and below-ground ecosystem conditions. Antioxidants are temporally dynamic in plants and many factors, including SOM, may drive these dynamics (Figure 1).

Pre-seeding soil nitrate measures have been used in conventional agriculture to determine appropriate fertilizer application rates, following standard fertilizer recommendations (Jacobsen et al. 2005). In organic systems, where nutrients are added in the form of organic material, nutrient release is the result of organic matter decomposition. Chapter 4 investigates whether using the traditional NO_3 test is appropriate to estimate growing season available N in systems using organic fertility inputs. In comparison to the NO_3 test, water-extractable organic matter (WEOM), total

organic C, potentially mineralizable N and microbial C-respiration were measured from the soils from each treatment as alternative measures of N-availability in organic production. These measurements were compared with N-uptake from a greenhouse-grown bioassay species grown in soils from the different fertility treatments (Liu et al. 2011). Soil measures that capture biological processes can give producers a greater understanding of how their management practices affect the soil biological functions in their systems.

Soil biota are receiving considerable attention in agricultural soils currently as recognition of their role in regulating soil processes increases. Chapter 5 looks more closely at the biological processes that drive nutrient availability in organic and sustainably managed agricultural systems and the global implications of managing SOM to meet fertility needs. Measuring soil enzyme activity, microbial biomass, arbuscular mycorrhizal infectivity, N mineralization and C-respiration allowed assessment of the biological responses to the different soil inputs. Patterns of resource use were measured that would not have been apparent looking at nutrient availability alone. Relying on organic inputs to provide plant available nutrients requires an understanding of soil ecosystem processes and the effects that management practices have on soil community function and nutrient availability.

CHAPTER TWO

INPUT C:N EFFECTS ON SOIL FERTILITY AND SPINACH YIELDS OVER THREE YEARS

Contribution of Authors and Co-Authors

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Contributions: Obtained partial funding, assisted with study design, discussed the results and methods for data analysis and edited the manuscript at all stages.

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CHAPTER TWO

INPUT C:N EFFECTS ON SOIL FERTILITY AND SPINACH

YIELDS OVER THREE YEARS

Abstract

Maintaining soil fertility is a critical concern for all farmers; for organic farmers, soil fertility is often achieved by adding fresh or composted plant material to their soils. Straw and hay mulches are two such inputs used by organic farmers. A challenge of using organic fertility inputs is relying on microbial decomposition of materials with variable C:N, given N is often limiting. We investigated how mulch inputs with contrasting C:N affect spinach grown in a mixed-vegetable production system in southwest Montana. Using a grass-legume hay mulch, a straw mulch, urea N-fertilizer and a no-treatment control, we measured spring available nutrient levels, SOM quantity and quality, and spinach yield and biomass over three years. The timing of nutrient release differed between the two mulches and the effects from straw mulch were longer-lived than that of the hay. Three years is a short period to study agroecosystem dynamics and longer experiments are warranted to discover the long-term effects of mulch additions and how to best integrate them into mixed-vegetable crop rotations.

Introduction

One of the fundamental challenges of agriculture is to offset the annual removal of nutrients. To replace nutrients removed by the crop, agricultural systems require regular resupply of nutrients to feed soil communities or risk depleting soil stores (Denison and Kiers 2005). Microbial recycling of stored energy and nutrients from soil organic matter (SOM) plays a large role in supplying plant-available nutrients, even in agricultural systems (Tu et al. 2006, Seiter and Horwath 2004, FlieBbach and Mader 2000). In conventional agriculture where SOM levels can be low, mineral fertilizers are added to replace the removed nutrients and microbial communities can become C-starved (Schimel and Weintraub 2003, Scow 1997), slowing nutrient cycling. Organic agricultural systems use organic materials, like green manures, mulches and compost to resupply C-bound nutrients. These systems rely on microbial processes to release nutrients bound in SOM (Magdoff 2007, Francis et al. 2003). SOM quality and quantity have a large impact on microbial communities and their subsequent capacity for nutrient cycling (Blagodetskaya et al. 2009, Tu et al. 2006). We are interested in measuring how organic matter with different C:N affects nutrient cycling, SOM stores and crop yields over time.

Because all OM decomposes through the same set of biochemical pathways, a strong correlation exists between litter C:N and the rate of litter mass-loss, thus C:N is often used as a measure of SOM quality (Ågren et al. 2013, Fierer et al. 2009). Organic management practices generally increase soil organic C and N, despite frequent and intensive tillage across a variety of soil types (Marriott and Wander 2006). Plant-available

N is tightly linked to C availability in soils as microbes require energy from labile C for N mineralization (Culman et al. 2013, Haney et al. 2008, FlieBbach and Mader 2000), and though the amount and timing of available nutrients often differ between conventional and organic vegetable production systems, crop yields often remain similar (Agehara and Warncke 2005, Doane et al. 2003, Kramer et al. 2002, Poudel et al. 2002).

Yield is the most commonly measured indicator of agroecosystem function, as the crop integrates soil conditions and the soil integrates management practices (FlieBbach and Mader 2000). Though we have successfully produced high yields relying solely on fertilizers, the ecosystem services provided by functional soils, including decreased surface runoff and erosion, C-sequestration and decreased nutrient leaching are recognized as incentives for maintaining soil resources (Kibblewhite et al. 2008). Management practices that maintain or increase SOM have a high propensity to supply adequate plant nutrients (Seiter and Horwath 2004), and agroecosystem management that builds both short and longer term C and N pools tend to be more productive and ecologically stable (Sanchez et al. 2004). The physical benefits of soil organic matter are known, including increased water holding capacity and cation exchange capacity and reduced bulk density, especially in intensively tilled systems like vegetable production (Kibblewhite et al. 2008, Seiter and Horwath 2004, Loveland and Webb 2003, Hudson 1994). What is less well understood is the relationship between SOM quality, plant available nutrients and crop yields through time.

The objective of this research was to investigate how inputs with different C:N affect soil nutrient availability and spinach yields over three growing seasons. We

hypothesized that: 1) Low C:N inputs will deliver more nutrients more quickly than high C:N inputs, and result in higher crop yields; 2) High C:N inputs will have a delayed benefit to crops; and 3) High C:N inputs and their corresponding effects on available nutrients will persist longer, over multiple growing seasons, than low C:N inputs, due to a longer residence time in the soil. To achieve our objective, we implemented a small plot experiment contrasting two mulched treatments with different C:N against two non-mulched treatments. The treatments included: barley straw mulch (high C:N input), grass-legume hay mulch (low C:N input), urea fertilizer, and a no-treatment control. We measured N, phosphorus (P), and potassium (K) each spring prior to seeding spinach to determine plant-available nutrients under each fertility input. We also measured the quantity of soil organic C (SOC) and the SOM quality, using SOM C:N, water-extractable OM C:N (WEOM) and dissolved organic N (DON) at harvest each year. WEOM is the labile portion of SOM and is maintained in soil solution, in equilibrium with solid-state SOM (Toosi et al. 2012, Chantigny 2003), and is often more immediately responsive to changes in soil fertility management (Haney et al. 2012, Weil et al. 2003). Similarly, DON represents the quantity of soil N available and accessible for microbial mineralization (Haney et al. 2012, Cabrera et al. 2005). From these data, we determined the relationships between available nutrients, SOM and spinach yield over three years.

Methods

Field Study

We conducted a complete randomized block experiment of four replicates of four soil treatments in a 2000 m² field, growing spinach (*Spinacia oleracea* var. Space (F1)) over three growing seasons, from 2011-2013. The research took place within the Towne's Harvest Garden (THG) research vegetable farm, located at the Montana State University (MSU) Horticulture Farm in Bozeman, MT (45.66° N, 111.07° W). Our research plots were under conventional cereal-fallow management for 20 years, prior to mixed vegetable production. The soil is a Turner loam with 400 g kg⁻¹ sand, 370 g kg⁻¹ silt and 230 g kg⁻¹ clay, pH of 7.3 and an initial organic carbon (C) content of 23 g kg⁻¹.

Soil treatments consisted of two mulch additions, barley straw and legume-grass hay, a N-fertilizer treatment and an untreated control. Straw mulch was applied at a rate of 5.5 kg m⁻² and hay mulch at 9 kg m⁻², at the recommendation of local vegetable farmers. Both mulches were applied only once over the course of the experiment, in October 2010. The mulches overwintered on the surface and were incorporated into the soil via tilling to 30 cm in June 2011. Mulch additions differed in C:N (t-test; $t = 2.5$, $p = 0.049$, $n = 4$), and averaged 26.5 g g⁻¹ for the hay mulch and 41.6 g g⁻¹ for the straw treatment. The N fertilizer was added in the form of urea (46-0-0). Existing NO₃-N was determined by sampling the soil prior to planting (Section 2.3). Urea was side-dressed each year following MSU recommendations for Montana vegetable production (0.0159 kg N m⁻²), approximately 10 days after spinach emergence (Dinkins et al. 2010). The untreated control had no fertility additions over the course of the experiment.

Plant Analysis

Spinach was harvested in July and August each year. We measured spinach biomass of a randomly chosen subsample of three plants in each plot. Sampled spinach plants were appropriate for sale – not excessively small, large or damaged. The duration of the growing period varied from year to year. Spinach was harvested according to size and market demand, not a set growing period, consequently spinach grew for 59 d in 2011, for 53 d in 2012 and for 36 d in 2013. Aboveground biomass was clipped at the soil surface, dried at 75 °C for 48 h and weighed to determine biomass (g). Dried tissue was ground with a mortar and pestle and combustion-analyzed for C and N content, using a LECO analyzer (LECO Corporation, USA). Remaining plants in each plot were harvested and weighed to determine yield (kg m^{-2}). Average plant biomass (g plant^{-1}) was also calculated using yield data and the number of plants per plot.

Soil Analysis

Soils were collected each spring for nutrient analysis prior to planting spinach in the field (Agvise Laboratory, ND). Ten 1-cm dia x 15-cm deep cores were collected and composited from each plot.

At spinach harvest, soils were sampled from the root zones of sampled plants. A soil core (diameter equivalent to the diameter of the outer rosette leaves and 40 cm deep) containing the root mass of the spinach plant was extracted from the soil. Soil adjacent to the roots were collected from this core and all visible roots were removed. Soils were air dried overnight, sieved to 2 mm and held at 4 °C until analyzed. Soils were analyzed for $\text{NO}_3\text{-N}$, Olsen phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and CEC

(Agvise Laboratory, ND). Post-harvest soils were also used for WEOM analysis and SOM C and N concentration. WEOM analysis was performed following Toosi et al. (2012) and extracts were analyzed with a TOC-V analyzer (Shimadzu Corporation, Japan) to determine WEOM C:N and dissolved organic N (DON). In 2011 and 2013, dried soils were milled using a ball mill and analyzed for organic C and N using a combustion-analyzer (LECO Corporation, USA) to calculate SOC and SOM C:N.

Statistical Analysis

To determine differences in yield between treatments and differences in fertility and SOM between treatments across the three years of the experiment, 2-way Analysis of Variance (ANOVA) was performed and the least significant differences (LSD) test was used for multiple means comparison using R (version 2.15.2, R Core Team 2012, Mendiburu 2014). Variables were tested for non-normality using the Shapiro-Wilk test and for unequal variance using the Fligner-Killeen test for homogeneity of variances. For variables with non-normality and unequal variance, we used the Kruskal-Wallis (K-W) test for differences between groups and determined differences based on ranks comparison. We used Pearson's correlation analysis to determine the relationship between yield and field conditions. All graphics were made using ggplot2 (Wickham 2009).

Results

Input Effects on Soil Nutrients and OM

Pre-seeding soil analyses allow farmers to determine the baseline nutrient availability for that season's crop growth. Spring NO_3 differed among treatments, among

years and among treatments from year to year (Table 1). NO_3 at planting in 2011, nine months after mulch application, was nearly ten times higher in the hay treatment than in the straw treatment and about twice as high as the non-mulched treatments (Figure 1a). Pre-seeding NO_3 in 2012 was twice as high in the hay treatment than in the other three treatments, but by 2013 spring NO_3 was similar across all experimental treatments. Pre-seeding soil samples were used to inform the fertilization rate for the N-fertilizer treatment each year, resulting in little difference in $\text{NO}_3\text{-N}$ between the N-fertilizer and no-treatment control soils each spring. Hay mulched plots had greater P in 2011 than the N-fertilizer plots. In 2012 and 2013, P was similar among treatments (Figure 1b). Pre-seeding K was consistently higher in the mulched treatments over the course of the experiment (Figure 1c). While K differed between treatments and years, it was consistent within treatments from year to year. Neither CEC, Ca nor Mg varied between treatments or years, or within treatments from year to year (Appendix B).

Rhizosphere-sampled SOC content differed among treatments and between years and within treatments from year to year ($F_{3,83} = 20.9$, $p < 0.001$ and $F_{1,83} = 11.1$, $p = 0.001$, $F_{3,83} = 4.87$, $p = 0.003$, respectively; Figure 2a). OC was higher in both mulched treatments than in the N-fertilizer or no-treatment control in 2011 and the control treatment was higher than the N-fertilizer treatment. In 2013, SOC in the straw mulched treatment was higher than in the other treatments and the hay mulched treatment was higher than N-fertilizer treatment. Rhizosphere SOM C:N did not differ significantly between treatments in any year, but it did increase slightly overall by the end of the

experiment (ANOVA $F_{3,82} = 0.11$ $p = 0.95$, $F_{1,82} = 23.91$ $p < 0.001$, respectively; Figure 2b).

WEOM C:N from rhizosphere samples was very responsive to the soil treatments. Straw mulched plots had a WEOM C:N four times higher than that of the other three treatments in 2011 (K-W: $H_3 = 12.32$ $p = 0.006$, Figure 2c), and it changed very little over the duration of the experiment. Meanwhile, rhizosphere WEOM C:N was initially very low in both the hay mulch and N-fertilizer treatments, but increased in both treatments to a high point in 2012 (K-W: $H_3 = 7.26$ $p = 0.06$). By 2013 WEOM C:N in all treatments except for the straw mulch were similar (K-W: $H_3 = 9.81$ $p = 0.02$). DON was also very responsive to the soil inputs and the amount of DON in rhizosphere soils of each treatment changed over the course of the experiment (Figure 2d). Hay mulched treatments had nearly twice as much DON as the other treatments in 2011, following mulch incorporation (K-W: $H_3 = 14.38$ $p = 0.002$), but by 2012 straw mulched plots had the greatest DON (K-W: $H_3 = 13.04$ $p = 0.005$). By the end of the experiment all treatments had similar amounts of DON (K-W: $H_3 = 2.22$ $p = 0.53$).

Input Effects on Spinach Average Biomass and Yield

Crop yields reflect the combined effects of nutrient management practices and growing season conditions (Table 2). Yields differed between treatments, years, and between treatments from year to year (ANOVA $F_{3,38} = 3.96$ $p = 0.02$, $F_{3,38} = 20.14$ $p < 0.001$, and $F_{3,38} = 5.61$ $p = 0.0003$, respectively). Yields were highest in the hay mulched treatment and lowest in the straw mulched treatment in 2011. In 2012, both mulched treatments had approximately four- to five-fold higher yields than the non-mulched

treatments. There were no yield data to report for 2013 because severe weather in June reduced germination rates.

Inter-annual variation in plant biomass was expected because we grew the plants for different lengths of time each year – harvest was dictated by market demand and plant size, not absolute growing period, as we were trying to mimic farmer decision making. Spinach average biomass differed between treatments only in 2011 ($F_{3,12} = 7.8$ $p = 0.004$, Table 1) with greater biomass in the hay mulched treatments than in the N-fertilizer and straw mulched treatments. Spinach average biomass differed between years only in 2013 ($F_{2,35} = 28.1$ $p < 0.001$).

Environmental Conditions Effects on Yield

Plant biomass and yield not only reflect aboveground growing conditions, but are the aboveground indicators of below-ground conditions, as plants integrate the soil conditions in the rhizosphere. Spinach can be sensitive to heat stress (Seaman 2013) and the temperatures experienced by spinach plants varied over the growing period from year to year. In 2011 spinach experienced 49 days above 27 °C and 18 nights above 10 °C. In an 840 growing degree days (GDD) period in 2011, 80% of the days were above 27 °C and 30% of nights were above 10 °C. In 2012, there were only 26 days above 27 °C and 5 nights above 10 °C and 546 GDD over the growing period, with only 50% of the days above 27 °C and only 10% of nights above 10 °C. In 2013 there were 24 days above 27 °C or 70% of the days in the growing period, 13 nights, or 40%, with temperatures above 10 °C and 499 GDD. Average spinach biomass was negatively correlated with both the

proportion of days over 27 °C and the proportion of nights above 10 °C. Total yield was only negatively correlated with GDD (Table 3).

Spring NO₃ and average spinach biomass were positively correlated ($r = 0.62$, $p < 0.001$ Table 3) in 2011 and 2012. Spring NO₃ and total yield were also positively correlated in 2011 and 2012 ($r = 0.46$, $p = 0.005$). The other predominant relationships were between spinach biomass and rhizosphere SOM C:N, WEOM C:N and DON (Table 5). Spinach yield was also correlated with SOC, SOM C:N, WEOM C:N and DON.

Discussion

Nutrient Availability From Inputs With Different C:N

If the goal of sustainably managed agriculture is to maintain agroecosystem goods, services and functions indefinitely, the efficacy of different fertility management practices must be assessed through time. By comparing the effects of a low C:N mulch input to a high C:N input and contrasting both mulches against a N-fertilizer treatment and a no-treatment control, we hypothesized that low C:N inputs would deliver nutrients more quickly and result in higher spinach yields. Adding inputs with different C:N affected pre-seeding nutrient availability in our soil. The largest mulch effect was an initial increase in spring available N in the hay mulched treatments, an effect that lasted through the second growing season post-mulch incorporation. Available N was very low in straw mulched treatments in the first year, but was similar to the non-mulched treatments by the second growing season as N that was immobilized after incorporation became mineralized.

Available N was moderate in both non-mulched treatments through the course of the experiment. The N-fertilizer treatment was similar with the no treatment control because soil sampling occurred prior to N-fertilizer side-dress application each year. By the final growing season in 2013, there were no differences in available N between mulched and non-mulched treatments. NO_3 may have been affected by the temporal dynamics of NO_3 in soils, including temperature and microbial activity at the time of sampling resulting in no measurable differences among treatments. Also, THG had high fertility to begin with and often management effects are often not seen in short-term agricultural research due to existing high soil fertility (Denison and Kiers 2005, Doane et al. 2003).

The mulch additions initially boosted already high available P levels in our research plots, but treatments equilibrated by the second growing season. K remained the highest in the mulched treatments over the course of the experiment, as a result from the large quantities of added organic material (Clark et al. 1998). Surprisingly, there was no difference in CEC between treatments over all three years of our experiment. SOM is touted for increasing soil CEC due to the increase in reactive sites and surface area (Kaiser et al. 2008). No change in CEC may be a residual effect of initial high CEC.

SOM Effects from Inputs with Different C:N

Adding mulches affected both OM quantity and quality in our soil. SOC increased in both mulched treatments compared with the non-mulched treatments. The straw mulch remained high through the three years of the experiment. The hay mulch treatment

remained higher than the N-fertilizer treatment but did not differ from the control treatment by 2013, indicating a shorter residence time compared with the straw mulch.

WEOM C:N in rhizosphere soils differed among treatments over the course of the experiment. WEOM C:N represents the quality of the most labile portion of SOM as the ratio between soluble organic C and dissolved organic N. At the beginning of the experiment, rhizosphere WEOM C:N was four times higher in straw mulched treatments than in the hay mulch and N-fertilizer treatments. Over the following two years, the WEOM C:N in the rhizosphere soils of the hay mulch, N-fertilizer and no-treatment control plots all increased while the straw mulch showed little change. To explain this we looked at the patterns of DON. In the second growing season, DON in straw mulched plots increased compared with DON in the first growing season. The mineralization of initially immobilized N from a flush of microbial population growth may be part of the reason for the delayed increase in DON (Kaiser et al. 2014). These data support our second hypothesis that straw mulch treatments would have a delayed benefit to crops. In the hay mulch treatments, the DON pattern was opposite, with initially high DON dropping by about half in the second growing season. As a legume-grass mixture, the hay mulch treatment resulted in soils with a larger proportion of labile N-compounds in the initial growing season than the soils of the less nutrient-rich straw mulch treatment. No differences were seen in SOM C:N among treatments.

The most consistent differences in OM quantity and WEOM C:N over the duration of the experiment were between the straw mulch treatment and the N-fertilizer treatment. These two soil inputs represent the ends of the fertility-addition spectrum, one

with high C and little N (straw mulch), the other with high N and no C (N-fertilizer). WEOM C:N represents the dissolved OM, the OM fraction most accessible to microbial decomposition and also the pool that receives the majority of microbial decay as populations turn over in the soil (Kaiser et al. 2014). It is possible that the straw mulched treatment maintained higher levels of microbial biomass and microbial biomass turnover, thus the lower WEOM C:N through time, compared with the other treatments. Also, initial spinach yields and biomass were higher in the N-fertilizer treatment and likely those soils received an OM addition from crop roots and residues that was greater the first year than in the straw mulch treatment. However, by the second growing season biomass and yields in spinach were similar in both the N-fertilizer and straw mulch treatments. Over a longer experimental period, the differences in available C and N for both microbial and plant uptake between the N-fertilizer treatment and straw mulch treatment would likely increase as microbes consumed the available C from existing SOM in the N-fertilizer treatment.

Crop Response to Inputs With Different C:N

Our third hypothesis was that high C:N inputs and their corresponding effects on available nutrients will persist longer – over multiple growing seasons – than low C:N inputs, due to a longer residence time in the soil. Though available N was similar in all treatments by 2013, there were longer residual effects from the straw mulch treatment in WEOM C:N and total SOC. The biggest differences seen in yield and biomass were between spinach from the hay mulch and straw mulch treatments in the first year after the

mulch additions. Yield and biomass were initially very high in the hay mulch treatment and remained high through the second growing season, while yield and biomass were very low in the straw treatment. Low available N in the straw treatment in the first growing season likely contributed to the low yields and small plants, but increased surface roughness from the straw mulch may have also negatively affected germination rate and seed contact with soil. Surface moisture and temperature were also likely different in both mulched treatments compared with the non-mulched treatments, though these were not measured in this experiment. Manipulating soil surface conditions could be a potential benefit of using mulches in rotations if certain crops benefit from the additional moisture and protection that they may provide. The differences in spinach growth between the two mulch treatments disappeared in the second growing season when both mulched treatments produced high yields compared with the non-mulched treatments. These data support literature findings of similar vegetable yields between crops grown in organic systems compared with crops grown in synthetic fertilized systems (Doane et al. 2003, Kramer et al. 2002).

Conclusions

Inputs with different C:N have the potential to affect soil nutrients and spinach yields through time supporting all three of our hypotheses. Data from this experiment show that in conditions similar to those experienced in southwestern Montana, a high C:N mulch will initially have a negative effect on N-demanding crops like spinach, but that N availability increases through time. A low C:N mulch provides high N availability

immediately after incorporation. Overall, the effects from the hay mulch were transient while the effects from the straw mulch were more persistent. Over the course of the experiment, both mulches produced equal or greater amounts of spinach than the N-fertilizer treatment.

A long view is necessary in sustainable production. OM decomposition rates can vary with location and inputs, resulting in varied nutrient availability. Thus, individual farmers must consider temperature, moisture and existing soil C:N of their system when making fertility management decisions (Agehara and Warncke 2005). Other environmental factors can also influence plant growth and yields, in addition to nutrient availability. In this research, spinach yield was well correlated with temperatures and the length of the growing period. Though we did not explicitly measure this, we observed that adding mulches can also change surface conditions, affecting seed bed preparation, moisture retention and wind speed. Mulches could have benefits or detriments from these non-nutrient related effects that should be considered.

Though the straw mulch negatively affected spinach yields in the first growing season, in a full mixed-vegetable crop rotation, a less N-hungry crop may thrive immediately following straw mulch additions. Planning crop rotations that take advantage of the annual differences in nutrient availability and surface conditions of straw mulch may be a way to optimize yields but obtain the longer-term benefits we saw in the straw mulch treatments. Straw mulch appears to be an affordable organic fertility input, though it is also necessary to consider where the nutrients in the straw originated and the long term sustainability of utilizing off farm inputs to maintain soil fertility.

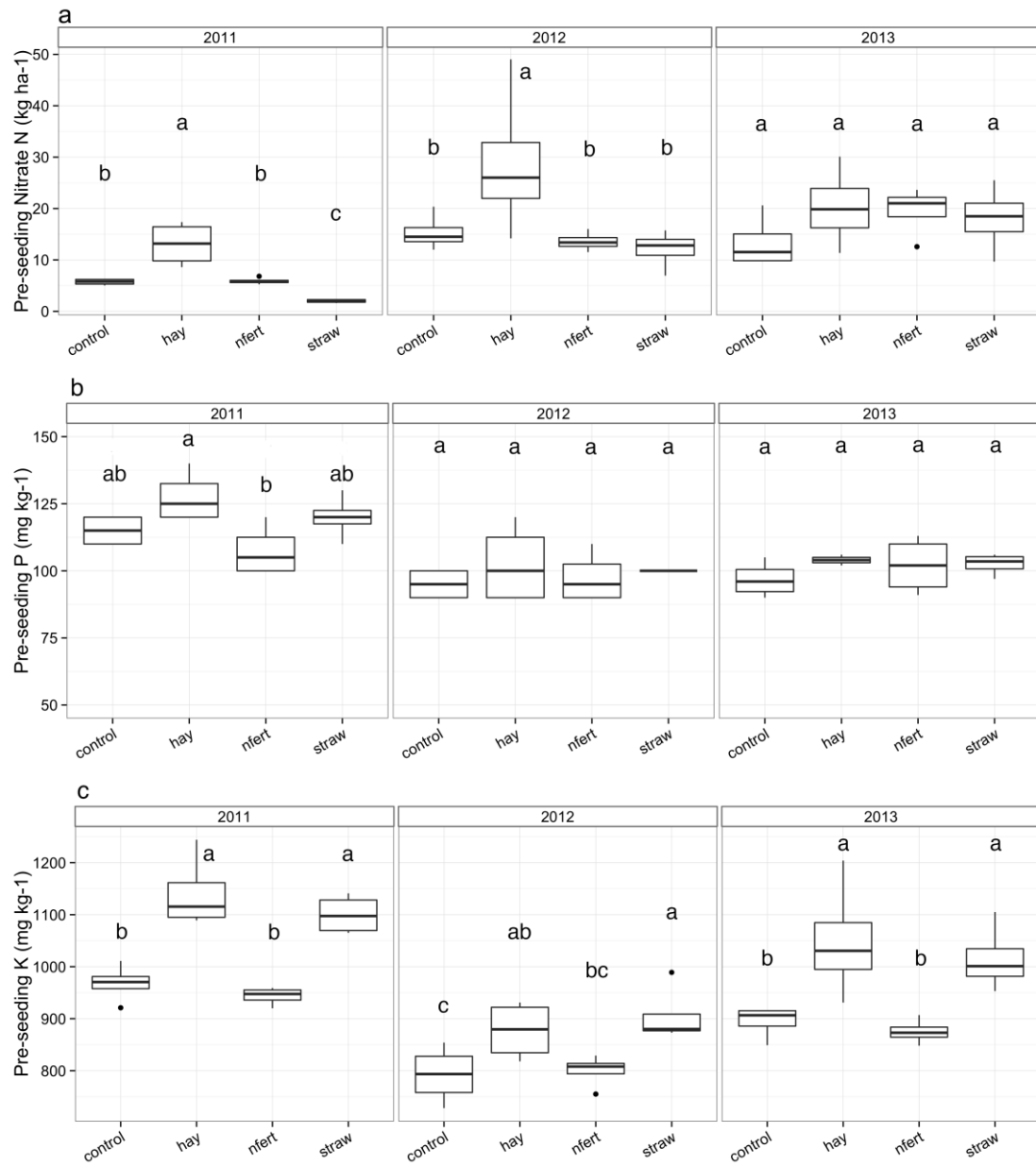


Figure 1. Pre-seeding nutrients from four soil fertility inputs over three growing seasons, Nitrate-N (a), P (b), and K (c). Letters represent differences between treatments at $p < 0.05$. Soils sampled to 15 cm prior to crop seeding.

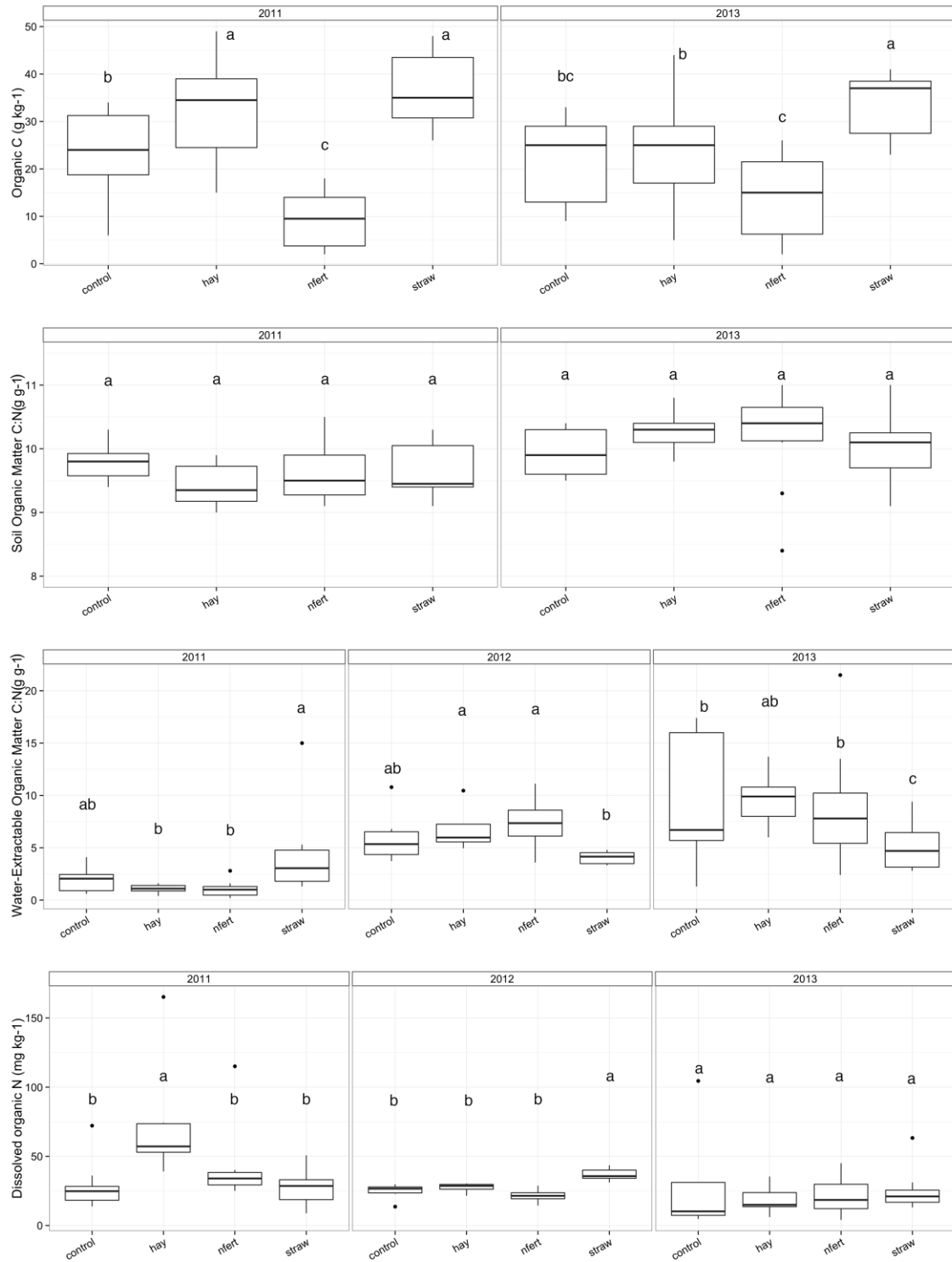


Figure 2. SOC quantity (a) and quality represented by SOM C:N (b), WEOM C:N (c) and DON (d). Letters represent differences between treatments at $p < 0.05$

Table 1. Results of two-way ANOVA for pre-seeding NO₃, P and K.

	df *	Pre-seeding NO ₃			Pre-seeding P			Pre-seeding K		
		MS	F	p	MS	F	p	MS	F	p
Treatment	3	220.8	7.40	0.0003	204.8	0.99	0.41	55085	20.51	< 0.001
Year	3	521.9	17.49	< 0.001	1913.2	9.20	< 0.001	169414	63.07	< 0.001
Treatment x Year	6	53.6	1.80	0.12	323.4	1.56	0.18	2921	1.09	0.12

*The numerator degrees of freedom for all factors was 50.

Table 2. Spinach yield and average plant biomass, mean (standard error). No yields in 2013 due to severe weather conditions post-planting.

	2010		2011		2012		2013	
	yield kg m ⁻²	average biomass g plant ⁻¹	yield kg m ⁻²	average biomass g plant ⁻¹	yield kg m ⁻²	average biomass g plant ⁻¹	yield kg m ⁻²	average biomass g plant ⁻¹
no treatment	0.92 (0.27)	--	0.66 b (0.04)	0.23 a (0.005)	0.13 (0.02) b	0.3 a (0.05)	--	0.005 a (0.0003)
N-fertilizer	--	--	0.48 c (0.03)	0.32 a (0.04)	0.22 (0.36) b	0.29 a (0.04)	--	0.009 a (0.001)
hay	--	--	0.86 a (0.03)	0.31 a (0.008)	0.78 (0.18) a	0.42 a (0.09)	--	0.008 a (0.0003)
straw	--	--	0.09 d (0.01)	0.09 b (0.005)	0.82 (0.06) a	0.48 a (0.03)	--	0.014 a (0.002)

Table 3. Correlations between average plant biomass, spinach yield and field conditions.

***p < 0.001; **p < 0.01; * p < 0.1

	GDD	Proportion of days above 27 °C	Proportion of nights above 10 °C	pH	pre-seed NO ₃ kg ha ⁻¹	pre-seed P mg kg ⁻¹	pre-seed K mg kg ⁻¹	OC g kg ⁻¹	SOM C:N g g ⁻¹	WE OM C:N g g ⁻¹	DON mg kg ⁻¹	avg plant biomass g plant ⁻¹
average plant biomass g plant ⁻¹	0.19	-0.29 *	-0.65 ***	-0.33 *	0.17	0.2	-0.2	-0.26	-0.45 *	-0.32 *	0.35 *	1
yield kg m ⁻²	0.40 **	--	-0.21	-0.11	0.13	0.28 *	0.15	0.30 *	-0.35 *	-0.42 **	0.51 ***	0.81 ***

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CHAPTER THREE

ORGANIC MATTER EFFECTS ON SPINACH
ANTIOXIDANT PRODUCTION

Contribution of Authors and Co-Authors

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Contributions: Assisted with study design and discussed the results and edited the manuscript.

Co-Author: Bruce D. Maxwell

Contributions: Obtained partial funding, assisted with study design and methods of data analysis, discussed the implications of the results and edited the manuscript.

Co-Author: Catherine A. Zabinski

Contributions: Obtained partial funding, assisted with study design, discussed the results and methods for data analysis and edited the manuscript at all stages.

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CHAPTER THREE

ORGANIC MATTER EFFECTS ON SPINACH
ANTIOXIDANT PRODUCTIONAbstract

BACKGROUND: Vegetable consumption makes available an array of vitamins and phytonutrients reported to have antioxidant properties. Increasingly consumers are demanding nutrient-rich produce. The demand for high quality vegetables has a potential economic benefit for farmers. However much of the research on what factors influence increased phytochemical production in crops are inconclusive. Few studies have investigated what system characteristics may contribute to the differences in antioxidant content between cropping systems. We investigated factors affecting spinach phenolic concentration and antioxidant capacity, including soil organic matter (SOM) quantity and quality (C:N), nutrient availability and growing season temperatures, using a combination of small-plot research and on-farm sampling in southwestern Montana.

RESULTS: We found that while SOM quality has some influence on spinach phenolics and antioxidant capacity, it is not the only factor, or even a primary factor, contributing to dietary antioxidant concentrations. High temperatures and high available N negatively affected spinach phenolics and antioxidant capacity, indicating that stress likely contributes more than soil conditions to spinach phenolic biosynthesis.

CONCLUSION: Sustainable agricultural production relies on site-specific management decisions to accommodate differences in site history, edaphic features and farm

objectives. Experimenting with management practices that strategically increase crop stress to promote dietary antioxidant concentrations and choosing crop varieties that are well suited to a specific farm's objectives and conditions may be the optimal way of maximizing the nutritive quality of vegetables.

Introduction

Many of the nutrients important to human health that we derive from vegetables are vitamins and phytochemicals that have antioxidant, anti-inflammatory, anti-mutagenic and anti-cancerous properties (1,2)). As consumer interest in and demand for high quality food increases, producing vegetables with higher quantities of human-health benefiting phytochemicals could have economic benefits to farmers. Accordingly, interest in the effect of cropping system practices on phytochemicals that may benefit human health has increased in the last decade. Despite the research done to date, consensus has not been reached on whether agronomic factors can influence vegetable phytochemical production or the human health benefit of consuming dietary antioxidants in fruits and vegetables (3–6). From a soil perspective, one difference between organic and conventional cropping systems is the quantity of soil organic matter (SOM) in organic systems due to differences in fertility management practices (7,8). SOM quantity can affect plant available nutrients over the growing season through decomposition and release of organic nutrients (9,10). The goal of this research was to explore factors influencing total phenolic concentration and antioxidant capacity in spinach crops grown in southwestern Montana by testing three hypotheses: H_0) There are no differences in total phenolic

concentration or antioxidant capacity in spinach sampled across different soil conditions and management practices in southwestern Montana; H_A) High nutrient availability as a result of high quantities of SOM will result in higher total phenolic concentration and antioxidant capacity in spinach; H_B) Suboptimal growing conditions, including temperature and nutrient stress, will result in higher total phenolic concentration and antioxidant capacity in spinach crops.

Cropping System Management and Plant Phytochemical Biosynthesis

The historical objective of sustainable agriculture was to grow food, fiber and fuel indefinitely (8,11). To achieve this long-term goal, agricultural management practices must look beyond resource use efficiency for annual production (12) and consider how year-to-year practices can promote soil quality, including soil fertility, structural integrity and biodiversity, for the long-term (11–15). Broadly, soil quality refers to a soil's capacity to maintain biological and ecosystem functions like nutrient cycling, water and air quality, and plant productivity (13,16). But soil quality can be difficult to practically define and manage due to the high inherent heterogeneity of soils and the diverse functions soil provides for human enterprise (13). In spite of the ambiguity in defining soil quality, it is widely acknowledged that soil organic matter (SOM) is a key component of productive agricultural soils due to the physical and biological benefits it provides (7,13,17).

Numerous studies have attempted to determine whether organically or sustainably grown fruits and vegetables are higher in dietary antioxidant phytochemicals

than conventionally grown fruits and vegetables (4–6,18–26). In addition to investigating whether cropping system management affects vegetable phytochemical content, other studies have looked at latitude, season, and varietal effects on phytochemical production (2,27–29). From meta-analyses, organically grown fruits and vegetables have been found to have higher dietary antioxidant phytochemical and micronutrient content in a higher number of reports than conventional fruits and vegetables (22) and the differences seen between organic and conventional systems were often related to differences in fertility management (20). However when abiotic factors like soil type and harvest timing were considered in the analyses, fewer significant differences between cropping system type were found (19,22).

In studies comparing spinach varieties grown under different nutrient conditions, cropping systems and growing seasons, organically grown spinach had higher dietary antioxidant phytochemical levels than conventionally grown spinach and phytochemical content was affected by genotype, growing season, pest pressures and N-supply all affected spinach phytochemical content (2,28,30). From the existing body of research, it is clear that biotic, abiotic and edaphic factors can all influence fruit and vegetable phytochemical production (22,27,31,32).

Plant Phenolics

Plant phytochemicals come in many forms, from many different plant metabolic pathways. Phenolics are one large family of plant phytochemicals, ranging from simple monomers to large polymers, common in agricultural species, and they represent a precursor to dietary antioxidants (33,34). The phenolics family include diverse

compounds, such as lignin and tannins, aromatic volatiles like caffeic acid and vanillic acid, flavonoids, and anthocyanidins. Phenolics are synthesized by plants as a result of environmental stressors, including injury, infection, nutrient deficiencies, and UV radiation (18,34,35), and can be both constitutive and induced defenses (36,37). But phenolics are not only defensive chemicals – they are also produced for normal plant function including the formation of structural compounds (lignin) and in reproductive and symbiont signaling (33,35,38,39). The quantity and types of phenolic compounds can be influenced by genetics (34) and epigenetics (2,27,33,40–42).

All phenolics are synthesized via the shikimate pathway. The vast diversity of shikimate-derived compounds contributes to the complexity in determining what external factors can be manipulated to control phenolic expression (38,43). Part of this complexity stems from plants having several copies of PAL genes, a critical enzyme in phenolic biosynthesis (44). Spinach, the focus of this study, is a diploid with four copies of PAL (45). The expression of each gene copy is developmentally and spatially unique, meaning that the triggers for phenolic biosynthesis may vary depending on the plant and even the tissue under scrutiny, resulting in unique chemical phenotypes (38,39). Due to the variety of phenolic compounds in a plant, it can be impractical to quantify all of the individual compounds contained in a sample (1,2). Quantifying the antioxidant capacity of a sample can be an efficient alternative. There are many assays available to determine antioxidant capacity, including Trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), 2,2-Azinobis 3-ethylbenzthiazoline-6-sulphonic acid radical scavenging (ABTS⁺) and oxygen radical absorbance capacity (ORAC). For this study we

measured total phenolics and ORAC. The Folin-Ciocalteu analysis for estimating total phenolics colorimetrically measures the total reducing power of a sample, while ORAC measures the suppression of an oxidizing agent like peroxy radicals (33,46–49).

The Folin-Ciocalteu analysis can overestimate total phenolics as compounds like ascorbic acid (Vitamin C) also have reductive capability, but it remains a useful and commonly used tool in broadly assessing the antioxidant quality of a sample (33,50).

ORAC is generally considered a biologically relevant method of measuring antioxidant capacity because it demonstrates chain-breaking antioxidant activity via hydrogen transfer (1,46,48,51,52). However, many antioxidant capacity assays, including ORAC, have been questioned due to the lack of method standardization across the field and subsequent low reproducibility of results between studies (47,48) and because of limitations in the radical sources used as oxidizing agent in each assay and their relevance *in vivo* (53). There is also concern that antioxidant capacity assays lack biological relevance, especially post-consumption of vegetables (33). However, antioxidant capacity assays are widely used, despite flaws because they can be useful for comparing treatments within an experiment, though caution must be applied when attempting to compare results among experiments (2,33,52,54,55). We chose to use ORAC as a complement to measuring total phenolic concentration because the kinetic nature of the assay allows for assessment of slow- and fast-acting antioxidant compounds in a sample (48,51) and because of reported correlation between total phenolics and ORAC, as phenolics are a large and common family of dietary antioxidants in plant tissue (2,50).

Factors Influencing Crop
Phytochemical Biosynthesis

In order to look more closely at how field conditions may contribute to the differences seen between phytochemical biosynthesis in vegetables grown in organic and conventional cropping systems, we investigated the role of SOM quantity and quality (C:N) in spinach total phenolic biosynthesis and ORAC in southwestern Montana soils with different fertility inputs. SOM C:N is negatively correlated with SOM decomposition rates and nutrient release, thus C:N is often used as a measure of SOM quality, with low C:N indicating a high quality SOM substrate (56,57). SOM also increases soil surface area and CEC, increasing nutrient retention in soils (58). Crop resource allocation has been linked to management practices (42,59), and a continuous adequate supply of nutrients throughout the growing season can result in allocation to defense, resulting in higher phytochemical production (24,31). The combined benefits of SOM to soil nutrient availability may result in greater nutrient supply to plants and therefore higher plant phytochemical production. Conversely, increases in plant phytochemical production have also been correlated with increased plant stress, including nutrient deficiency and suboptimal growing temperatures (10,30,34,44,60,61). We combined a small-plot experiment with on-farm sampling to test whether spinach total phenolics and antioxidant capacity differ among farms with varied growing conditions and management practices and to assess the effects that SOM quantity, nutrient availability and temperature stress have on total phenolics and antioxidant capacity in spinach.

Methods

Field Study

To measure the relationship between SOM quantity and quality and spinach total phenolics and antioxidant capacity, we implemented a small-plot field trial at Towne's Harvest Garden (THG) research vegetable farm. THG is located within the Montana State University (MSU) Horticulture Farm, on the MSU campus in Bozeman, MT (45.66, -111.07; Figure 1). Our research plots were under conventional barley-fallow management for 20 years prior to the start of our experiment. The soil is a Turner loam with 400 g kg⁻¹ sand, 370 g kg⁻¹ silt, 230 g kg⁻¹ clay, 20.3 g kg⁻¹ organic carbon, a cation exchange capacity (CEC) of 22 cmol kg⁻¹ and a pH of 7.6. The legacy of this farm includes intensive tillage, and high phosphorus (P) and potassium (K) inputs from the cereal-fallow management, following standard cereal fertilization recommendations (62) for 20 years prior to this experiment.

We conducted a complete randomized block experiment with four replicates of four soil treatments across 16 plots in a 2000 m² field, growing spinach (*Spinacia oleracea* var. Space (F1)) over three growing seasons, from 2011-2013 (Appendix A). Soil treatments consisted of two mulch additions—barley straw and sanfoin (legume)-grass hay—a N-fertilizer treatment, and an untreated control. Straw mulch was applied at a rate of 5.5 kg m⁻² and hay mulch at 9 kg m⁻², at the recommendation of local vegetable farmers. Mulch additions differed in C:N (t-test; $t = 2.5$, $p = 0.049$, $n = 4$; Appendix A), and averaged 26.5 for the hay mulch and 41.6 for the straw treatment. Mulches were surface applied in the fall of 2010 and incorporated into soils in the spring of 2011 via

rototiller. The N fertilizer was added in the form of urea (46-0-0). N fertilizer rate followed a standard recommendation based on sampling the soil for nutrient content 10 d prior to planting each year. Urea was side-dressed following recommendations for Montana vegetable production (159 kg N ha^{-1}), (62), approximately 10 days after spinach emergence. The untreated control had no fertility additions over the course of the experiment.

Gallatin Valley Farms Study

In June, July and October 2013, we sampled spinach tissue and soil from five mixed-vegetable production farms around Gallatin Valley, MT (Figure 1) in order to assess phytochemical production in spinach from a wider range of soil types, locations and management practices (Table 1; Appendix A). Amaltheia Farm (AMA) has been producing vegetables since 2012. Between 2012 – 2009 it was in a mixed forage rotation and in alfalfa hay production prior to 2009. In 2012 and 2013 it transitioned to mixed-vegetable production. Fertility was managed in 2012 with a winter pea, vetch, winter wheat cover crop mixture following fall-applied pig compost at 45 t ha^{-1} . Root crops were grown in 2012 prior to spinach in 2013. AMA is on the windward side of the Bridger Mountain range, resulting in a high frequency of summer storms. Gallatin Valley Botanical (GVB) has been in production since 2008. Prior to vegetable production, the land was used for pasture and hay since the 1980's. Fertility is managed by manure application in alternate years, last applied in the spring of 2012. The preceding crop in 2012 was lettuce and salad greens. The location of GVB results in late, cool spring conditions. Gallatin Grown (GG) has been in vegetable production since 2012. Prior to

that the land was used for conventional potato production in a 7-year rotation with wheat and alfalfa. Fertility is managed with winter cattle grazing. GG is farthest west from Bozeman and has hotter summers compared with GVB. 3-Fiddles Farm (FID) has been producing vegetables in Bridger Canyon since 2008. Prior to vegetable production the land was used as grazing forage since the 1990's. Fertility is managed using a fall-seeded legume green manure. The preceding crop is unknown. FID has the highest elevation (1.6 km) and a very short growing season (approximately 60 days). Three Hearts Farm (HEA) has been in production since 2008. Prior to vegetable production it was perennial pasture since the 1970's. Fertility is managed with fall-applied manure and legume cover crop. The preceding crop was lettuce and salad greens. HEA is most similar to THG in location and environmental conditions.

Plant Analysis

Spinach was harvested in July and August each year (Table 2). Three spinach plants were randomly chosen for analysis each year and all were at the 9-12 leaf stage and appropriate for sale. Seedling spinach, very large plants and plants with tissue damage were avoided. Three 1-g samples of leaf tissue were cut from each selected plant and frozen in the field in liquid N. Frozen tissue was held at -80 °C until extraction, within 60 days of sampling. No fresh tissue was analyzed as phytochemicals are highly dynamic and tissue concentrations can change rapidly post-harvest ((21,26). Aboveground biomass of each sampled plant was clipped at the soil surface, dried at 75 °C for 48 hours and weighed.

Samples were extracted following Prior et al. (63; Appendix A). Frozen spinach tissue (1 g fw) was homogenized with acidified acetonitrile (5:1 v:w) using a Polytron homogenizer (Brinkmann, Switzerland). After incubation at 20 °C and centrifugation, a 2 mL aliquot of the supernatant was reserved and stored at -20 °C until antioxidant analysis.

Total phenolic concentration was quantified using the Folin-Ciocalteu colorimetric assay, using gallic acid as a standard, following Singleton et al. (64; Appendix A) Absorbance at 765 nm was determined using a U-2000 UV/Vis Spectrophotometer (Hitachi High-Technologies Corporation, Japan).

ORAC concentration was quantified using fluorescein as the probe, 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) as a source of peroxy, and Trolox to develop the standard curve, following Held (65) and Gillespie et al. (66; Appendix A) Fluorescence decay was measured every minute for one hour using a KC4 microplate reader (BioTek, USA) with excitation/emission wavelengths of 485/20 and 528/20, respectively.

Dried spinach tissue was ground with a mortar and pestle and analyzed for N concentration using a TruSpec CN combustion analyzer (LECO Corporation, USA).

Soil Analysis

Soils were collected each spring at THG for NO₃-N analysis prior to planting spinach in the field (Table 2). Ten randomly located 1-cm dia x 15-cm deep cores were collected and composited from each plot to measure bulk soil pre-seeding available N (62). Spring soil samples were not collected at Gallatin Valley farms.

At spinach harvest in the experimental plots and on Gallatin Valley farms, 1000 g of soil were sampled from the root zones of the same three spinach plants selected for tissue analysis in order to measure the soil conditions experienced by each sampled plant (Table 2, 3). After tissue sampling, a soil core (diameter equivalent to the diameter of the outer rosette leaves and 40 cm deep) containing the root mass of the spinach plant was extracted from the soil. Soil adjacent to the roots were collected from this core and all visible roots were removed. Soils were air dried overnight, sieved to 2 mm and held at 4 °C until analyzed, within 20 days post-sampling. Soils were analyzed for NO₃-N, OM (LOI), Olsen-P, K and CEC (Agvise Laboratory, ND). Post-harvest soils were also used for WEOM analysis and SOM C and N composition. Water extractable organic matter analysis was performed following Toosi et al. (67; Appendix A) and extracts were analyzed with a TOC-V analyzer (Shimadzu Corporation, Japan). Dried soils were milled using a ball-mill and analyzed for organic C and N using a TruSpec CN combustion analyzer (LECO Corporation, USA).

Statistical Analysis

To determine differences in crop responses among treatments, 1-way Analysis of Variance (ANOVAs) were performed and protected-LSD tests were used for multiple means comparison ($\alpha < 0.1$) using R statistical software, version 2.15.2. Variables were tested for non-normality using the Shapiro-Wilk test and for unequal variance using the Fligner-Killeen test for homogeneity of variances. For variables with non-normal distributions and unequal variance, we used Kruskal-Wallis (K-W) to test for differences between treatments and *post hoc* determination of differences between treatments based

on the mean rank of the groups. (68). We used Pearson's correlation analysis on normally-distributed data to assess the relationship between the two antioxidant measures and Spearman's correlation analysis for variables with non-normal distributions and unequal variance to quantify the degree of relationships between plant phytochemical concentrations.

To identify the functional relationship between total phenolics and soil and environmental parameters derived from the quality and quantity of SOM, we ran linear multiple regression analysis using a generalized linear model (GLM) on the log-transformed levels of both total phenolic concentration and ORAC concentration. Regression models used combinations of the explanatory variables: available N (NO_3), SOC, SOM C:N, WEOM C:N, the number of days over 27 °C, P, CEC and a mulch indicator dummy variable. Available N and P are critical nutrients for plant growth and CEC is a measure of potential soil nutrient storage and often related to SOM quantity. We did not include K in regression analysis because though K is an important plant nutrient, it was not judged to be limiting at any of our sampling locations based on standard fertilizer recommendations from soil tests. SOC and the mulch indicator dummy variable both represent SOM quantity indicators. SOM C:N and WEOM C:N represent SOM quality indicators, with WEOM C:N representing the quality of the labile, accessible SOM pool and SOM C:N indicating the quality of the larger, more stable solid-state SOM pool. Spinach is a cool season crop and excessive heat will cause bolting, negatively affecting crop yields (27, 71) so the number of days in the growing season greater than 27 °C were calculated as a climatic stressor that may influence phytochemical production.

As additional environmental variables, we also calculated the frequency of nights above 10 °C (heat stress) and the frequency of temperatures below 0 °C (cold stress) over the growing period (Appendix B). The frequency of nights above 10 °C was autocorrelated with days above 27 °C and not included in regression. As there were fewer than two subzero temperatures during the growing period in any year, the frequency of below 0 °C temperatures was also not included in the regression. To rank multiple candidate models, Akaike's Information Criterion (AIC_C) was used, corrected for small sample size (69,70).

Results

Plant Response to Soil Treatments

In 2011 there were no differences among soil fertility treatments in mean total phenolic concentration or ORAC (ANOVA $F_{3,41} = 0.60$, $p = 0.62$, $F_{3,39} = 0.77$, $p = 0.52$, respectively; Figure 2a and 2c). In 2012, total phenolic concentration differed among treatments, but ORAC did not differ between treatments (ANOVA $F_{3,43} = 2.48$, $p = 0.07$, $F_{3,43} = 0.004$, $p = 1$, respectively). Spinach grown in the hay mulch treatment in 2012 had lower total phenolic concentration than spinach grown in the N-fertilizer treatment and the straw mulch treatment. In 2013, there were no differences in total phenolic concentration or ORAC among treatments (ANOVA $F_{3,35} = 1.60$, $p = 0.21$, $F_{3,35} = 1.39$, $p = 0.26$, respectively).

Spinach biomass was four times greater in the hay mulch treatment than in the straw mulch treatment in 2011, but in 2012 spinach biomass was similar across all treatments (K-W $H_3 = 13.08$, $p = 0.004$, $H_3 = 2.87$, $p = 0.4$, respectively; Figure 2e).

Spinach biomass did not differ among treatments in 2013 (K-W $H_3 = 3.09$, $p = 0.38$). Spinach N concentration was almost 1% higher in straw mulched plants than in the no-treatment plants in 2011, but comparable across treatments by 2013 (ANOVA $F_{3,28} = 2.722$, $p = 0.06$, $F_{3,35} = 0.67$, $p = 0.58$, respectively; Figure 2g).

In 2011, the correlation between total phenolic concentration and ORAC in the experimental plots was $r = 0.69$ ($p < 0.001$), by 2012 the correlation was $r = 0.42$ ($p = 0.004$), and by 2013 there was no correlation between ORAC and total phenolics ($r = 0.21$, $p = 0.20$).

Across Gallatin Valley farms in 2013, total phenolic concentration and ORAC were greatest in spinach grown at GVB compared with that from the other sampled farms (ANOVA $F_{5,18} = 6.81$, $p = 0.001$, $F_{5,18} = 3.81$, $p = 0.02$, respectively, Figure 2b and 2d). Spinach biomass was five times greater at FID than at AMA but all other locations had comparable biomass (K-W, $H_5 = 11.73$, $p = 0.04$, Figure 2f). Spinach N concentration was comparable at all farms except GVB, which had about half as much tissue N (ANOVA $F_{4,16} = 10.21$, $p = 0.0003$, Figure 2h).

Correlation between ORAC and total phenolic concentrations was $r = 0.72$ ($p < 0.001$) for plants collected on Gallatin Valley farms. Differences seen in total phenolic concentration and ORAC between farms were not due to differences in spinach variety (Table 1; ANOVA $F_{2,21} = 1.44$, $p = 0.26$ and $F_{2,21} = 0.48$, $p = 0.63$, respectively).

Soil Conditions with Different Fertility Inputs

SOC was highest initially in both mulched treatments compared with the non-mulched treatments and SOC remained the highest in the straw mulched treatments for the duration of the experiment (Table 3, Chapter 2). SOM C:N did not differ across treatments for the duration of the experiment and WEOM C:N was initially highest in straw mulch treatment plots, but by the end of the project in 2013, straw had the lowest WEOM C:N, while the other soil treatments were comparable with each other and values were twice as high as in straw mulched soils (Table 3, also Chapter 2). Immediately following the mulch additions, available N was two times higher in hay mulch plots than in the N fertilizer and no treatment plots, and 10 times higher than in straw mulch plots. By spring 2013, all treatments had similar NO_3^- (Table 3, Chapter 2). P and K were also initially highest in the hay mulch treatment, and K remained higher in the mulched treatments over the course of the experiment. By 2012 P was similar across all treatments and CEC did not ever differ among treatments (Table 3).

SOC ranged from 1% to 6% across Gallatin Valley farms in 2013. WEOM C:N also ranged widely, between 10 to 50, but SOM C:N was comparable around 11 at all farms except GG which had a SOM C:N of 22 (Table 4). P varied by a factor of 10 across Gallatin Valley farms, ranging from 11 to 120 mg kg^{-1} and was potentially limiting to plant growth at GVB. K varied by a factor of 5 across Valley farms, but was in adequate supply for spinach production at all farms (71). CEC ranged between 20 – 30 cmol kg^{-1} (Table 4).

In 2012 in the field study, spring NO_3^- was negatively correlated with total phenolic concentration (Pearson's $r = -0.54$, $p = 0.04$) and spring K was negatively

correlated with total phenolic concentration in 2013 ($r = -0.29$, $p = 0.08$). No other correlations were found in any year between phenolic concentration and soil nutrients. WEOM C:N was positively correlated with total phenolic concentration in the experimental plots across all years ($r = 0.29$, $p = 0.07$). No other correlations existed between SOM quality or quantity parameters and total phenolics or ORAC in our experimental plots. Total phenolic concentration was negatively correlated with the number of days above 27 °C during the growing period ($r = -0.57$, $p < 0.001$) and negatively correlated with the number of nights above 10 °C ($r = -0.20$, $p = 0.01$). ORAC was negatively with days above 27 °C ($r = -0.20$, $p = 0.02$), but not correlated with nights above 10 °C ($r = -0.02$, $p = 0.81$).

On Gallatin Valley farms in 2013, total phenolic concentration was positively correlated with WEOM C:N ($r = 0.49$, $p = 0.01$) and negatively correlated with K ($r = -0.37$, $p = 0.08$), while ORAC was negatively correlated with K ($r = -0.41$, $p = 0.05$) and P ($r = -0.42$, $p = 0.04$). There was a negative correlation between spinach N concentration and total phenolic concentration across all farms ($r = -0.54$, $p = 0.01$), but no correlation between spinach N and ORAC ($r = -0.31$, $p = 0.14$).

Phytochemical Production Under Different Growing Conditions

We used regression analysis to examine which edaphic and abiotic factors are important to phytochemical production in spinach. For total phenolic concentration, the model with the most support given our data included available N, SOM C:N, WEOM C:N and the number of days above 27 °C ($\Delta AICc$ 5.58 over the second model; Table 5).

The top model explained 54% of the variance in our data. For ORAC, the model with the most support given our data included available N, SOM C:N, and WEOM C:N (ΔAIC_c 2.89 over the second model; Table 6), but explained only 15% of the variance in our data.

Discussion

SOM Quality and N Availability_ Effects on Phytochemical Production

Adding significant quantities of mulch altered below-ground conditions over the course of this experiment. SOC was high in both mulched treatment plots for the first two years and the straw mulched treatment remained high for the duration of the project. Mulch effects on nutrient availability were variable, with an initial increase in N and P in both mulched treatments and sustained increase in K in mulched plots compared with non-mulched plots. WEOM C:N was also variable, especially in the straw mulched treatment, which initially had the highest WEOM C:N and became the lowest by 2012. By 2013, available N was similar among treatments, but WEOM C:N was the lowest in the straw mulched treatment by 2012 and stayed that way through 2013. The straw mulch was composed of only barley straw while the hay mulch was a mixture of grass and sanfoin (legume). As a single substrate, straw mulch had a more constant rate of decay than the combination of grass and legume in the hay mulch and resulted in different ratios of available nutrients in the labile OM fraction of the hay mulched treatment (72–74).

The changes in the soil nutrient availability from the different inputs affected spinach biomass in the first two years. However phenolic concentration only differed among treatments in one year. In 2011, soil treatments resulted in large differences in

spinach biomass between the two mulch treatments, with high biomass in the hay mulched treatment and very low biomass in the straw mulched treatment, but there was no effect on phytochemical production. In 2012, total phenolic concentrations did differ between treatments and there was a negative correlation between NO_3^- and total phenolics. In 2012, neither spinach biomass nor ORAC differed among treatments. In 2013 there were no differences seen in spinach biomass, total phenolic concentration or ORAC among treatments though there was a positive correlation between WEOM C:N and total phenolic concentration across all years. Data from the experimental plots did not support the first alternative hypothesis that higher SOM quality and the resulting increase in adequate available N positively impacts spinach phenolic concentration and antioxidant capacity. Instead, results show support for the second alternative hypothesis that nutrient stress is a larger factor in spinach total phenolic biosynthesis and antioxidant capacity.

The wide range of SOC ($10 - 60 \text{ g kg}^{-1}$) and SOM C:N and WEOM C:N across Gallatin Valley farms illustrates the effects that location, site history, and soil type can have on soil C and N even within a small geographic area. SOM is likely only one of many contributing factors in phytochemical production as we did not see ranges of spinach phenolic concentration or antioxidant capacity corresponding to differences in SOM quantity or quality across these farms. Differences in spinach biomass, total phenolics and antioxidant capacity seen across Gallatin Valley farms were likely due to a combination of the differences in location within Gallatin Valley and the microclimate variations experienced by spinach at each location, and differences in management

practices and the resulting nutrient availability. Gallatin Valley farms vary in elevation from 1.63 km at FID to 1.49 km at GVB and weather events can range dramatically from farm to farm in a growing season, despite the fact that the greatest distance between farms is 34 km. Tissue N concentration had a negative correlation with total phenolic concentration on spinach sampled from Gallatin Valley farms in 2013 further indicating that plant stress is an important driver of plant phenolic biosynthesis.

There was only moderate correlation between ORAC and total phenolic concentration in our experimental plots over all three years, though the relationship between ORAC and phenolics seen across Gallatin Valley farms was strong. A linear relationship generally exists between ORAC and total phenolics with correlations between $r = 0.5$ and $r = 0.7$, because phenolic compounds are a large family of plant compounds with high reducing capacity (2,29). Differences in the correlation between total phenolics and ORAC in our experimental plots may be the result of changes in plant chemical profiles from year to year as different reducing compounds, like vitamin C, have lower peroxy radical absorbance capacity and thus lower correlation with ORAC (64,75). Differences in plant chemical profiles from year to year are likely due to different growing conditions, including temperature, nutrient availability and interactions with soil biota (30,31). Future work could include HPLC analysis of spinach secondary compounds to investigate the relationship between quantities of individual spinach biosynthates and ORAC.

There is evidence that phenolic production is negatively correlated with available N (18,28,60), and meta-analyses have found that the most consistent difference between

organic and conventional vegetables were higher levels of tissue NO_3 in conventional vegetables (5,21,23,26). High nutrient availability often results in increased plant growth and decreased allocation to C-based compounds, including phenolic compounds (31,59,60) but the biochemical mechanisms and complexity of interactions are not clear enough to make management decisions accordingly, and different crops will have different responses to the same management practices (24,42). In the shikimate pathway, phenylalanine is deaminated by PAL, the critical enzyme in phenolic synthesis, resulting in trans-cinnamic acid and a free NH_4 (34). N-deficient conditions can increase PAL activity (30,31,34), leading to an increase in available N within the plant while also protecting existing biomass while resources are unavailable for growth (60). Spinach grown at GVB had the highest phenolic concentrations and ORAC and the lowest concentration of tissue N of sampled farms, supporting the hypothesis that available N is negatively correlated with phenolic production.

Growing Condition Factors and Dietary Antioxidant Biosynthesis

N was not the only nutrient related to spinach phenolic biosynthesis and antioxidant capacity across Gallatin Valley farms: there were also negative correlations between total phenolic concentration and K and negative correlations between ORAC and P and K. The primary role of K in plants is to maintain the osmotic potential and pH in cells for optimal enzyme activity (76). K is also a cation necessary for enzyme activation by changing the conformation of the enzyme, and K limitation can lead to interrupted metabolic pathways due to suboptimal pH in the cytosol (76). While K ranged

widely across Gallatin Valley farms, it was never deficient according to standard fertilizer recommendations for spinach (71). Therefore the relationship between phenolic concentration and ORAC and K is likely not due to K-limitation in our study. Agronomic practices optimize available nutrients in order to maintain high productivity. If plant phytochemical production is a stress response to low nutrient conditions, it is unlikely to see an increase in crop phytochemistry under any cropping system using standard fertilizer recommendations (23,77).

Though our experimental data did not support the hypothesis that SOM quantity and quality plays a role in phenolic concentration or ORAC, the regression analysis shows that total phenolic concentration is related to both of the SOM quality variables, SOM C:N and WEOM C:N, as well as available N and days above 27 °C. The model with these four variables had stronger support, given our data, than using any of those factors alone or than any other combination of edaphic factors measured in this study. Other potential stresses were minimized over the course of our experiment: there are few pests that target spinach in Montana, all the farms we sampled were irrigated – minimizing water stress over the growing season, and spinach crops missed any significant hail storms in all three years. However, the moderate explanatory power of the regression model indicates that other factors that we did not capture in our research also influence spinach phenolic production. This was also true with ORAC: though the model with the strongest support indicated that SOM quality was an important factor in ORAC, the regression only explained 15% of the variance. ORAC is a measure of the antioxidant capacity of all compounds in a sample, not just the phenolics. There are likely many

factors influencing the biosynthesis and stability of other groups of phytochemicals in spinach, and therefore ORAC, that we did not measure in our research. HPLC may be a useful tool in determining what compounds play an important role in ORAC in spinach (2,41).

Conclusions

Dietary antioxidant biosynthesis in spinach is highly dynamic and related to biotic, abiotic and edaphic factors (2,27,30). Longer-term studies can account for environmental stochasticity so that cropping system or management effects are not overwhelmed by environmental variability (9,40,78). A longer research project with more diverse rotations and amendments coupled with greenhouse studies investigating the biochemical mechanisms affected by different growing conditions may reveal patterns not found from this three-year study.

Our results support the hypothesis that increased phenolic concentration and antioxidant capacity in spinach is due, in part, to environmental stress. There is some contradiction in the goal of manipulating agricultural practices to promote phytochemical production - most agricultural production aims to minimize plant stress in order to obtain high yields. It may be more reasonable to rely on genotype, choosing varieties that have high levels of natural defense compounds, especially in organic systems that might benefit from the increased plant defense without the use of chemical alternatives (24,27,34).

Even in a relatively small valley like Gallatin Valley, soil type, soil fertility and climate can vary widely. Sustainable agriculture relies on site-specific decision-making because of variation such as this. Farmer-directed on-farm research is especially critical in sustainable agricultural systems to determine the crops and management practices that best suit the site. Understanding the site-specific conditions of an individual farm, and choosing vegetable varieties according to those conditions will result in the production of food with higher human health benefitting phytochemical contents (11,19,41,78). Moderate stress may result in the production of food crops with higher levels of dietary antioxidants. Future studies, over a wider range of farms, to examine the interactions of environmental factors and management practices that can be used to strategically stress plants without substantially reducing crop yields are necessary to reveal the driving mechanisms behind producing nutritious food.

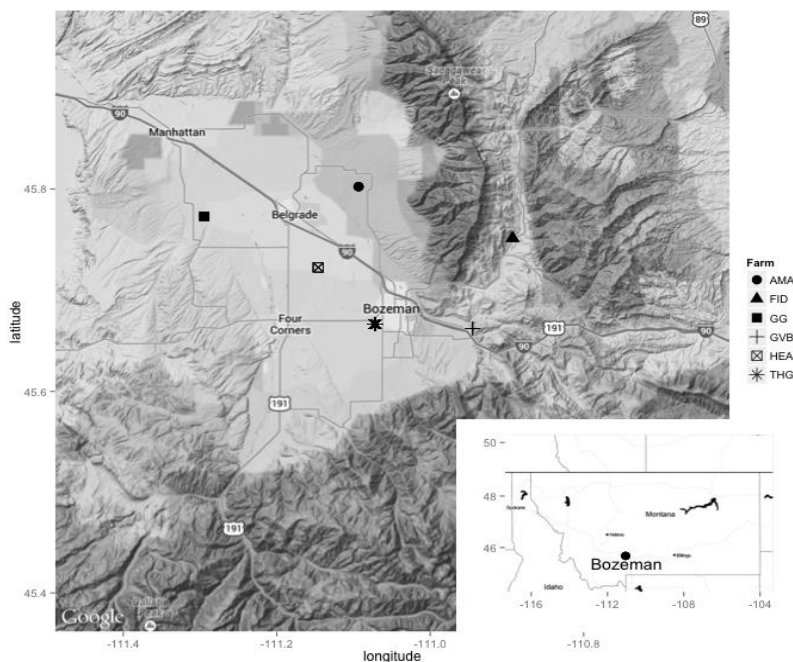


Figure 1. Map of farm locations in the Gallatin Valley around Bozeman, MT (insert: Bozeman, MT).

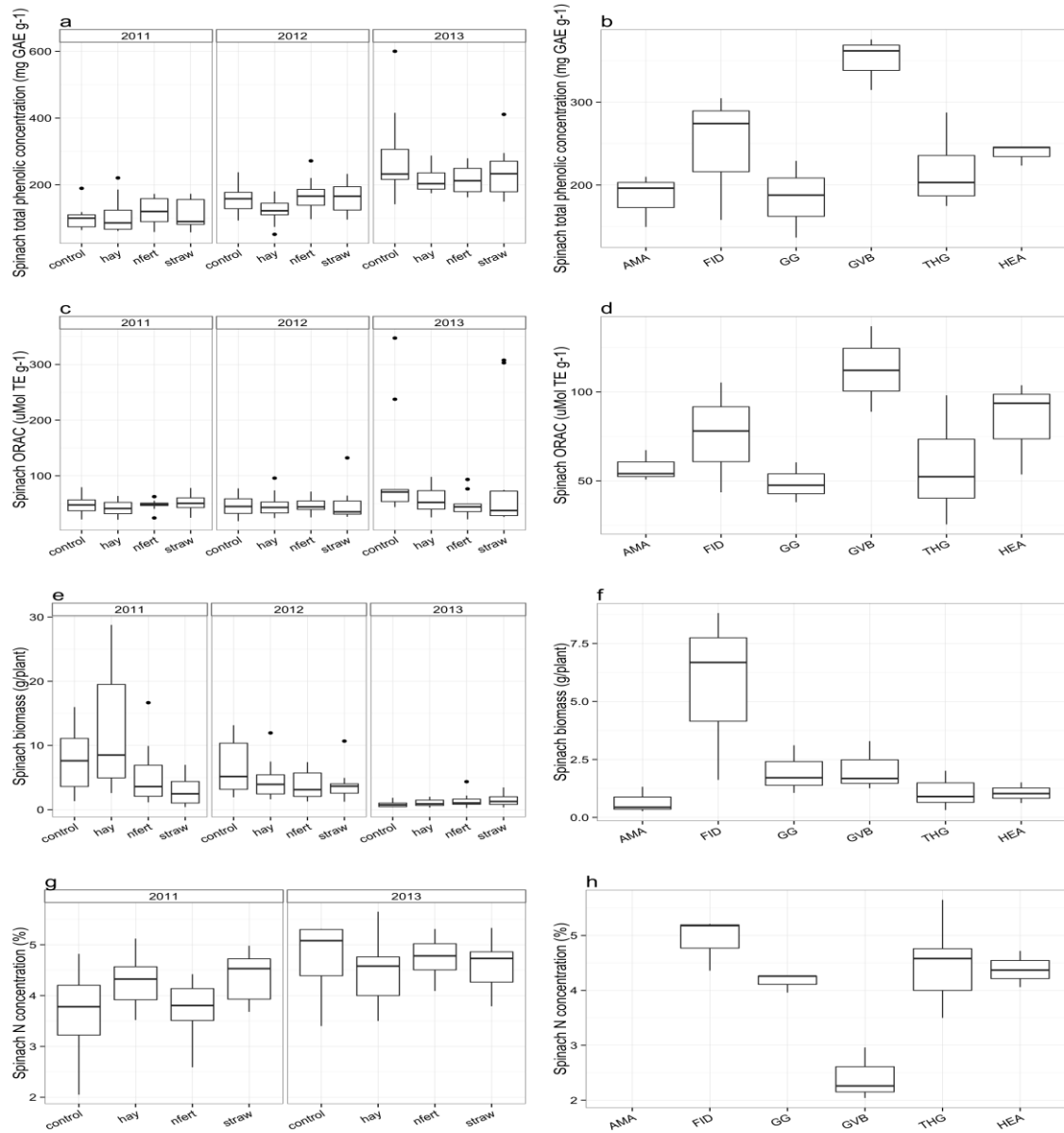


Figure 2. Spinach total phenolic concentration in each year in the experimental plots (a) and in 2013 across Gallatin Valley farms (b). ORAC in the experimental plots (c) and across Gallatin Valley farms (d). Plant biomass in the experimental plots (e) and Gallatin Valley farms (f) and plant N concentration in the experimental plots (g) and Gallatin Valley farms (h).

Table 1. Field conditions on Gallatin Valley farms, 2013. Farms include, Amaltheia (AMA), Gallatin Valley Botanical (GVB), Gallatin Grown (GG), 3-Fiddles, (FID), Three Hearts (HEA) and Towne's Harvest Garden (THG).

	latitude	longitude	Planting Date	Harvest Date	Days Above 27 °C	Sand g kg ⁻¹	Silt g kg ⁻¹	Clay g kg ⁻¹	pH	Spinach Variety
AMA	45.80	-111.09	19 Aug 2013	21 Oct 2013	27	490	300	210	7.8	Corvair
GVB	45.66	-111.95	5 May 2013	25 June 2013	11	390	370	240	6.8	Space
GG	45.77	-111.29	5 May 2013	10 July 2013	21	140	730	130	8.3	Tyee
FID	45.75	-110.89	10 June 2013	10 July 2013	20	350	340	310	7.1	Tyee
HEA	45.72	-111.15	5 May 2013	25 June 2013	5	400	370	230	7.9	Space
THG	45.67	-111.07	11 June 2013	17 July 2013	24	400	370	230	7.8	Space

Table 2. Field Dates, Towne's Harvest Garden Research Plot

	spring soil sampling date	spinach planting date	spinach harvest date	Days Above 27 °C
2011	4 June 2011	22 June 2011	20 August 2011	49
2012	5 May 2012	20 May 2012	12 July 2012	26
2013	4 April 2013	11 June 2013	17 July 2013	24

Table 3. Soil OC and nutrients in experimental plots, means and (standard errors).

	2011							2012							2013						
	Spring NO ₃ -N mg kg ⁻¹	SOC g kg ⁻¹	SOM C:N	WE OM C:N	P mg kg ⁻¹	K mg kg ⁻¹	CEC cmol kg ⁻¹	Spring NO ₃ -N mg kg ⁻¹	SOC g kg ⁻¹	SOM C:N	WE OM C:N	P mg kg ⁻¹	K mg kg ⁻¹	CEC cmol kg ⁻¹	Spring NO ₃ -N mg kg ⁻¹	SOC g kg ⁻¹	SOM C:N	WE OM C:N	P mg kg ⁻¹	K mg kg ⁻¹	CEC cmol kg ⁻¹
No- treatment	5.73 b (0.31)	20.35 b (0.03)	9.8 a (0.08)	2 ab (0.41)	84 b (2.60)	822 c (15.80)	22 a (0.17)	15.35 b (1.78)	2.21 b (0.03)	--	6 ab (1.05)	88 a (2.07)	865 b (18.97)	24 a (0.35)	14.52 a (3.20)	20.25 bc (0.04)	10 a (0.12)	9 a (2.02)	113 a (2.78)	816 b (26.37)	22 a (0.36)
N- fertilizer	5.89 b (0.33)	20.21c (0.03)	9.6 ab (0.12)	1 b (0.29)	87 b (2.84)	807 c (14.61)	22 a (0.23)	13.59 b (0.93)	2.08 c (0.03)	--	7 a (1.55)	87 a (3.33)	878 b (10.95)	24 a (0.28)	19.56 a (2.42)	20.16 c (0.03)	10 a (0.25)	9 a (1.71)	119 a (4.02)	827 b (11.40)	22 a (0.27)
Hay	13.08 a (2.16)	20.73 a (0.12)	9.4 b (0.09)	1 b (0.15)	99 a (2.60)	1045 a (38.66)	22 a (0.44)	28.81 a (7.31)	2.28 b (0.05)	--	7 a (1.23)	89 a (8.21)	998 a (26.54)	24 a (0.34)	20.30 a (3.92)	20.34 b (0.06)	10 a (0.12)	10 a (0.82)	117 a (2.20)	946 a (17.28)	22 a (0.27)
Straw	2.00 c (0.17)	20.78 a (0.07)	9.7 ab (0.12)	5 a (1.59)	83 b (3.33)	948 b (33.65)	22 a (0.30)	12.09 b (1.86)	2.39 a (0.02)	--	4 b (0.26)	86 a (3.79)	995 a (31.27)	24 a (0.36)	18.04 a (3.27)	20.48 a (0.02)	10 a (0.17)	5 b (0.64)	107 a (9.71)	935 a (20.15)	22 a (0.32)

Table 4. SOC, C:N ratios and nutrients at Gallatin Valley farms in 2013. Means and (standard errors). Farms include, Amaltheia (AMA), Gallatin Valley Botanical (GVB), Gallatin Grown (GG), 3-Fiddles, (FID), Three Hearts (HEA) and Towne's Harvest Garden (THG).

	Spring NO ₃ mg kg ⁻¹	SOC g kg ⁻¹	SOM C:N	WEOM C:N	P mg kg ⁻¹	K mg kg ⁻¹	CEC cmol kg ⁻¹
AMA	--	20.87 b (0.03)	10.30 c (0.17)	15.03 b (0.24)	25.67 c (0.88)	536.00 c (25.70)	20.47 d (0.27)
GVB	--	30.27 ab (0.13)	11.37 b (0.22)	48.23 a (8.91)	11.00 d (0.58)	194.00 d (9.54)	24.53 b (0.18)
GG	--	10.37 d (0.03)	22.13 a (0.69)	22.10 ab (2.50)	24.67 c (2.03)	285.67 d (16.15)	34.03 a (0.12)
FID	--	50.93 a (0.59)	10.87 bc (0.28)	10.40 c (0.92)	42.00 b (5.03)	475.00 c (29.28)	33.23 a (0.19)
HEA	--	20.93 b (0.02)	10.90 bc (0.46)	48.10 a (1.71)	44.67 b (9.49)	743.67 b (117.57)	22.83 c (1.25)
THG	20.30 a (3.92)	20.34c (0.06)	10.29 c (0.12)	9.68 c (0.82)	116.6 7 a (2.20)	945.56 a (17.28)	21.99 c (0.27)

Table 5. Regression analysis of soil and environmental factors influencing total phenolic biosynthesis across all farms and research plots. The 'Model' column indicates the parameters in each model; 'K' is the number of parameters in the model; 'AICc' is Akaike's Information Criterion, corrected for small sample size; ' Δ AICc' is the difference in AICc between models; and 'w' is the relative likelihood of the model, given the entire suite of models.

Model	K	AICc	Δ AICc
spring NO ₃ + SOM C:N + WEOM C:N + Days Above 27 °C	6	27.67	0
spring NO ₃ + SOM C:N + WEOM C:N	5	33.24	5.58
spring NO ₃ + Days Above 27 °C + SOC + SOM C:N + WEOM C:N + P + CEC	9	35.92	8.25
spring NO ₃ + SOC + SOM C:N + WEOM C:N	6	36.40	8.73

Table 6. Regression analysis of soil and environmental factors influencing ORAC across all farms and research plots. The 'Model' column indicates the parameters in each model; 'K' is the number of parameters in the model; 'AICc' is Akaike's Information Criterion, corrected for small sample size; ' Δ AICc' is the difference in AICc between models; and 'w' is the relative likelihood of the model, given the entire suite of models.

Model	K	AICc	Δ AICc
spring NO ₃ + SOM C:N + WEOM C:N	5	45.38	0
spring NO ₃ + SOM C:N + WEOM C:N + Days Above 27 °C	6	48.27	2.89
spring NO ₃ + SOC + SOM C:N + WEOM C:N	6	48.55	3.17
spring NO ₃ + Days Above 27 °C + SOC + SOM C:N + WEOM C:N + P + CEC	9	59.89	14.51

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CHAPTER FOUR

ESTIMATING PLANT AVAILABLE NITROGEN IN ORGANIC MARKET GARDEN SYSTEMS

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CHAPTER FOUR

ESTIMATING PLANT AVAILABLE NITROGEN IN ORGANIC
MARKET GARDEN SYSTEMSAbstract

Nutrient management in organic systems requires stimulation of soil biological processes to supply plant available nutrients. Because the stoichiometry of organic inputs can vary widely, an accurate estimation of plant available nutrients through the growing season can increase our capacity to maximize yields and minimize nutrient losses. In this paper we investigate the potential of four soil analyses: pre-seeding soil NO_3 , water-extractable organic matter (WEOM) C:N, potentially mineralizable N (PMN), and microbial C respiration, to predict growing season N availability. Pre-seeding soil NO_3 is the most common soil nitrogen (N) test currently used by producers. WEOM is the most labile form of SOM and regarded as a primary source of microbial nutrients and energy. PMN measures mineralized-N under ideal conditions and can be used as an indicator of both available-N and biological activity, and microbial C respiration has been positively linked to PMN and available N. We also explore how the C:N of organic matter inputs can affect microbial C respiration and subsequent availability of N in organically or sustainably managed systems.

Measuring soil NO_3 is a good estimator of plant N uptake in systems using organic inputs, but soil N analyses that capture biological inputs (water-extractable organic matter) or processes (potentially mineralizable N and microbial respiration) can

provide farmers with comparable N-management information as well as additional information about how their fertility related management practices affect the biology of their system. Using microbial C respiration as a surrogate indicator of available N is a useful tool as long as input nutrient ratios are considered in parallel.

Introduction

For decades agronomists have practiced nutrient replacement management – annually supplementing mineral fertilizers for nutrients removed via harvest and the inherent “leakiness” of agricultural systems (Drinkwater and Snapp 2007). Nitrogen (N) management is especially important to agronomists as N is often limiting for plant growth and necessary for maximized yields (Marschner 1995). In the form of nitrate (NO_3), N is also mobile in most North American soils and can become an environmental hazard if leached into groundwater or removed with surface waters (Masclaux-Daubresse et al. 2010, Drinkwater and Snapp 2007, Haney et al. 2001). The NO_3 soil test measures only a snapshot of available N, because NO_3 is mobile and rapidly cycled by microbes. Soil NO_3 levels are measured prior to planting to inform fertilization rates for the growing season (Birkhofer et al. 2008). The soil NO_3 monitoring method invites a view of soils as simply a substrate – storing plant available nutrients and stabilizing roots rather than the view of soils as dynamic belowground ecosystems with intersecting physical, chemical, and biological characteristics (Magdoff and Weil 2004, FlieBbach and Mader 2000).

The common practice of using mineral N-fertilizer is beginning to change, especially in market garden systems, as fertilizer prices rise and consumers demand “sustainable” or organically grown produce (Rosen and Allan 2007, Seiter and Horwath 2004, Loveland and Webb 2003). Common organic fertility strategies include adding organic materials to the soil, such as green manures, mulches, and composted animal manure, and relying on biological processes to mineralize the organic nutrients present in the inputs (Bowles et al. 2013, Watson et al. 2002). Once incorporated into soils, organic inputs decompose to become soil organic matter (SOM). Total SOM is composed of decomposing plant residue, biotic tissues and biotic residues like polysaccharides and enzymes from both plant and microbial sources (Denef et al. 2009, Gregorich et al. 2006). Soil organic matter has many ecosystem services, including physical and chemical functions like increasing soil water holding capacity and cation exchange capacity (CEC), decreasing bulk density, and contributing to aggregate stability (Kong et al. 2007, Magdoff and Weil 2004, Six et al. 2004, Loveland and Webb 2003, Hudson 1994, Stevenson 1986). Some of these functions, like increasing CEC and water holding capacity, can influence plant available N.

Using organic inputs complicates nutrient availability calculations because organic input contributions to plant available nutrients are temporally variable and not always related to inorganic nutrient availability (Toosi et al. 2012, Clark et al. 1998). The primary influence SOM has on nutrient cycling is through biological interactions as a source of energy and nutrients for soil biota (Gregorich et al. 2006). In soils, the C and N cycles are tightly linked and SOM decomposition is the source of energy for the entire

soil food web (Frank and Groffman 2009, Schomberg et al. 2009, Kibblewhite et al. 2008). Decomposition of SOM provides up to 50% of N taken up by plants each growing season (Stevens et al. 2005) and the decomposition rate is correlated with input C:N, the microbial community composition, soil physical conditions and climate (St. Luce et al. 2014). Organic-C makes up about 58% of total SOM and can be freely available in the soil-water matrix or heavily complexed with soil minerals (Six et al. 2004, Blackwood and Paul 2003, Christensen 2001). Organic-C is an energy source of aerobic soil microbes and complex organic molecules are catabolized by microbes and extracellular enzymes (Bowles et al. 2014, Hopkins and Gregorich 2005, Weil and Magdoff 2004, Brady and Weil 2002).

Organic N becomes mineralized through various pathways, including via the soil food web (Cabrera et al. 2005, Weil and Magdoff 2004, FlieBbach and Mader 2000), and through desorption via extracellular enzymes and abiotic soil chemical reactions (Bowles et al. 2013, Schimel and Weintraub 2003). Nitrogen immobilization can occur when added organic matter has a high C:N and decomposers must find additional N from soil pools to metabolize the added C (Fierer et al. 2009, Schimel and Bennett 2004).

Soil organic matter (SOM) is heavily influenced by soil type and soil management practices, and SOM transforms rapidly once it is incorporated into soils from both biological and chemical processes (Magdoff and Weil 2004, Paustian 1992, Stevenson 1986). Physical and chemical changes to organic matter influence its quality and accessibility to microbes, further affecting decomposition and plant nutrient availability (Hopkins and Gregorich 2005). Along with these abiotic influences, input

material C:N will also affect decomposition. Organic inputs can have highly variable nutrient levels depending on the source, and can subsequently change the N pool and soil C:N in the long-term (Bhogal et al. 2009, Kong et al. 2007, Agehara and Warncke 2005). It is therefore important for farmers to accurately measure available N in their system, both to ensure predictable crop response, optimal yields and to prevent N over-application. The common practice of sampling pre-seeding available- NO_3 may not adequately measure the N available for crop use throughout the growing season in systems (e.g. organic) that rely on biological N-mineralization (Haney et al. 2012). And, because N availability can also be affected by organic input C:N and decomposition rates, it is also important for farmers to understand how these inputs contribute to the soil N pool (Haney et al. 2012, Bhogal et al. 2009, Agehara and Warncke 2005). A soil test that captures the biological processes involved in N-mineralization may be a better indicator for farmers of growing-season available N in organically fertilized agricultural systems (Gregorich et al. 2006).

Biological Soil Analyses

A variety of alternative soil tests exist for estimating soil N. The most accurate are those that capture the flux of N through the growing season (Chapin et al. 1986), but this is not often a reasonable practice for producers. Pre-season soil tests that capture biological processes may be an effective compromise or surrogate to estimate plant available N in organically fertilized or mulched systems.

Water-extractable organic matter (WEOM) is the soluble portion of SOM. It is maintained in the soil solution through equilibrium with bulk solid-phase SOM

(Chantigny 2003, Zsolnay 1996). Though it is a relatively small portion of SOM, it is the most labile portion (Xu 2011, Chantigny 2003), accessible to microbial activity and thus a good indicator of the resources available to the biological processes influencing N-mineralization (Marinari et al. 2010). WEOM is very responsive to changes in management and has been used as a predictor of soil respiration and N-mineralization (Haney et al. 2012, Blackwood and Paul 2003, Chantigny 2003, Chan 2001). Like NO_3 , WEOM is a highly dynamic single point measure that is only a snapshot of the system.

Potentially mineralizable N (PMN) is a laboratory method to quantify the biotic rates of N-mineralization. It reveals the proportion of total soil N that can be accessed and mineralized by microbes and therefore potentially become plant available through microbial recycling in the short term (Bhogal et al. 2009, Schomberg et al. 2009, Robertson et al. 1999). PMN quantifies microbial N-mineralization and can be used as an indicator of microbial activity, but it can overestimate available N as it is measured under optimal laboratory conditions (Sharifi et al. 2007, Robertson et al. 1999).

Microbial respiration has been linearly linked to N-mineralization (Spargo et al. 2011, Haney et al. 2001, Vahdat et al. 2001, Franzluebbers et al. 2000) and recently used as a predictor of crop productivity (Culman et al. 2013, de Castro Lopes et al. 2012). Microbial C-respiration is another method of determining the biological activity of soils and the active OM pool (Vahdat et al. 2010) and can be an indicator of the soil's capacity to store and cycle nutrients (Bhogal et al. 2009, Anderson and Domsch 1993).

Our primary research question was: When using organic fertility inputs, how does the soil NO_3 test compare with other soil tests that may better capture the biological

processes responsible for N-mineralization at predicting plant available N. To answer this question, we compared plant N-uptake in greenhouse-grown ryegrass with available N, quantified from each soil test described above. In a system with high SOM and increased biologic activity, we expected that microbial respiration and PMN would be more closely correlated with plant N-uptake than pre-seeding soil NO_3 . We expected that labile OM (WEOM) would be a good predictor of plant available N. We compared pre-seeding spring soil NO_3 with field-grown spinach N-uptake to determine the accuracy of using the NO_3 test to predict available N in field conditions.

We also investigated the factors that might influence available N with different organic inputs, specifically input C:N and microbial respiration, as variables related to biological decomposition and contributing to N-mineralization with organic fertility management. Our goal was to recommend the best single or combination of tests to estimate plant available N with different organic fertility inputs in market garden systems.

Methods

Field Study

The Montana State University (MSU) Horticulture Farm, located on the MSU campus in Bozeman, MT (45.66, -111.07), was under conventional cereal-fallow management for 20 years prior to mixed vegetable production. The legacy of this farm includes intensive tillage, and high phosphorus (P) and potassium (K; Table 1). The soil is a Turner loam with 400 g kg^{-1} sand, 370 g kg^{-1} silt, 230 g kg^{-1} clay.

We conducted a complete randomized block experiment of four reps of four soil treatments across 16 plots in a 2000 m² field, growing spinach (*Spinacia oleracea* var. Space (F1)). Our field study for this analysis was conducted over the 2011 growing season within a four-year experiment, from 2010-2013. Soil treatments consisted of two mulch additions, barley straw and sanfoin (legume)-grass hay, a N-fertilizer treatment and an untreated control. Straw mulch was applied at a rate of 5.5 kg m⁻² and hay mulch at 9 kg m⁻², at the recommendation of local vegetable farmers. Mulch additions differed in C:N (t-test, $p = 0.048$), and averaged 26.5 for the hay mulch and 41.6 for the straw treatment. The N fertilizer was added in the form of urea (46-0-0). Existing NO₃-N was determined by sampling the soil prior to planting (see 2.4). Urea was side-dressed following MSU guidelines (Dinkins et al. 2010) for Montana vegetable production (0.0159 kg N m⁻²), approximately 10 days after spinach emergence. The untreated control had no fertility additions over the course of the experiment.

Spinach was harvested in August, 2011, 60 days after planting. Aboveground biomass from three randomly selected spinach plants was clipped at the soil surface, dried at 75 °C for 48 hours and weighed for biomass. Biomass was used to measure plant growth and to calculate N-content. Tissue was then ground with a mortar and pestle and analyzed for N content, using a TruSpec CN combustion analyzer (LECO Corporation, St. Joseph, MI).

Greenhouse Study

Ultimately, soil N availability can be assessed by measuring plant nutrient uptake: the most accurate way to determine the conditions a plant actually experienced. It is not

appropriate for growing season nutrient estimation, but it can be a useful tool to compare various soil tests in an experimental setting (Zebarth et al. 2005). Following Liu et al. (2011), we conducted a N-uptake bioassay study in the greenhouse using ryegrass (*Lolium multiflorum*) as our bioassay species. Field soils were collected after spinach harvest from all four replicates of each of the four treatment field plots in October of 2011. Soil from each plot was used to fill three 10 cm deep x 10 cm dia pots, which were planted with 25-30 ryegrass seeds. Plants were grown for eight weeks after germination. Aboveground biomass was clipped at the soil surface, oven dried at 75 °C for 48 hours and weighed. Tissue was ground with a mortar and pestle and combustion-analyzed for N content, using a LECO analyzer.

Soil Analysis

Soils were collected in June, 2011 for nutrient analysis prior to planting spinach in the field. Ten 1-cm dia x 15-cm deep cores were collected and composited from each plot. Analysis included NO₃-N, total-N, OM (loss on ignition (LOI)), Olsen-P, K and CEC (Agvise Laboratory, Northwood, ND). NO₃ data were used in correlation analysis with spinach N-uptake.

At spinach harvest in August, 2011, 1000 g of soil were sampled from the root zones of the same three spinach plants selected for tissue analysis in order to measure the soil conditions experienced by each sampled plant. After tissue sampling, a soil core (diameter equivalent to the diameter of the outer rosette leaves and 40 cm deep) containing the root mass of the spinach plant was extracted from the soil. Soil adjacent to the roots were collected from this core and all visible roots were removed. Soils were air

dried overnight, sieved to 2mm and held at 4 °C until analyzed. Soils were analyzed for NO₃-N, total-N, OM (LOI), Olsen-P, K and CEC (Agvise Laboratory). Post-harvest soils were also analyzed for WEOM analysis, PMN, and microbial respiration for correlation with ryegrass N-uptake. Water extractable organic matter analysis was performed following Toosi et al. (2012) and extracts were analyzed on Shimadzu TOC-V analyzer (Shimadzu Corporation, Kyoto). Soils were incubated at 25 °C for 18 days to determine C-respiration and PMN, following Robertson et al. (1999). NO₃-N was extracted from soils following a modified version of Keeney and Nelson (1987), using 1M KCl, and analyzed using Lachat QuickChem FIA analyzer (Hach Instruments, CO). Soils collected in October 2011 for use in the greenhouse study were also analyzed for NO₃-N following the same method, prior to seeding with ryegrass.

Statistical Analysis

To determine differences in crop response and N levels between treatments, 1-way Analysis of Variance (ANOVA) were performed and LSD test was used for multiple means comparison. Variables were tested for non-normality using the Shapiro-Wilk test and for unequal variance using the Fligner-Killeen test for homogeneity of variances. For variables with non-normality and unequal variance, we used Kruskal-Wallis (K-W) to test for differences between groups and used the mean rank to determine differences between groups. We ran Spearman's correlation analysis on variables with unequal variance to quantify the degree of relationships between ryegrass N-uptake and soil N as estimated with each analysis.

To identify the relationship between ryegrass N-uptake and the different soil N analyses, multiple regression analysis was performed using a generalized linear model (GLM) on the log-transformed ryegrass N contents. Regression models used combinations of WEOM-C:N, WEOM-C, WEOM-N, PMN, microbial C-respiration and soil NO_3 , as explanatory variables.

To identify the functional relationship between microbial respiration available N, we ran multiple regression analysis using a generalized linear model (GLM) on the log-transformed levels of microbial respiration. Regression models used combinations of the explanatory variables WEOM-C:N, WEOM-C, WEOM-N, a mulch indicator dummy variable and an interaction term between WEOM-C and -N. These factors were chosen because microbial respiration results from a labile food source (WEOM-C; Weil and Magdoff 2004, Jandl and Sollins 1997) but N is also necessary for microbial growth (WEOM-N; Schimel and Weintraub 2003, Manzoni et al. 2008). We also considered that OM quality (the ratio of C:N) may be important to estimate microbial respiration, and we included a mulch indicator dummy variable because of differences in quantities of C-respiration in mulched versus non-mulched treatments. The interaction term between WEOM-C and WEOM-N was included in response to patterns seen in preliminary investigations. WEOM C:N, WEOM C and WEOM N, while all likely contributing to microbial activity, have high levels of autocorrelation. The effect of these variables on microbial respiration may be indirect and/or linked through other processes.

To rank multiple candidate models for both regressions to determine the relative importance of explanatory variables, Akaike's Information Criterion (AIC_C) was used,

corrected for small sample size (Burnham and Anderson 2002). All data analysis and graphics were performed using version 2.15.2 of R (R Core Team 2012), using the following packages: ggplot2 (Wickham 2009), reshape (Wickham 2007), AICcmodavg (Mazerolle 2013), and nmle (Pinheiro et al. 2013).

Results

Plant Response to Soil Treatments

In both the field and greenhouse studies, plant biomass differed between soil treatments (field grown spinach: K-W, $H_3 = 9.54$, $p = 0.02$; greenhouse grown ryegrass: $F_{3,44} = 9.7$, $p < 0.001$). In the field, mean spinach biomass was nearly six times greater in the hay mulch treatment than spinach grown in the straw mulch treatment (Table 2). Spinach biomass was intermediate in the N-fertilized and no treatment control soils. The greenhouse-grown ryegrass biomass was also highest in soils with the hay treatment compared with all other treatments.

Nitrogen concentration differed for both species across treatments (spinach: $F_{3,28} = 2.7$, $p = 0.06$ and ryegrass: $F_{3,42} = 4.1$, $p = 0.01$). Nitrogen concentration only differed between spinach grown in the straw treatment and the control (Table 2). Ryegrass N-concentration was greater in the N-fertilized plots compared with the hay mulched treatment and no-treatment control (Table 2). Plant N-content for both spinach and ryegrass also differed across treatments (spinach: K-W, $H_3 = 10.06$, $p = 0.02$; ryegrass: $F_{3,42} = 11.23$, $p < 0.001$). N-content in spinach from the hay treatment was over five times greater than spinach from the straw treatment. Spinach N-content from plants grown in the control and N-fertilizer plots were intermediate. In the greenhouse, ryegrass N content

was nearly 50% higher in the hay treatment soil than in ryegrass grown in any of the other treatments (Table 2).

Soil Tests That Predict Available N

Field soil $\text{NO}_3\text{-N}$ prior to crop seeding (seven months post soil mulch treatment application) differed across the soil treatments (K-W, $H_3 = 26.58$, $p < 0.001$), with the hay treated soils containing nearly six times more $\text{NO}_3\text{-N}$ than soils from the straw treatment and twice as much $\text{NO}_3\text{-N}$ as the non-mulch treatments (Table 3). Soil $\text{NO}_3\text{-N}$ prior to the greenhouse study also differed between treatments (K-W, $H_3 = 21.71$, $p < 0.001$). Soils from the hay treatments had nearly two times more $\text{NO}_3\text{-N}$ than any other treatment.

Whole soil C:N differed slightly between treatments ($F_{3,44} = 2.05$, $p = 0.12$; Table 3) and also differed in WEOM C:N ($F_{3,28} = 4.2$, $p = 0.015$). The C:N of the WEOM in the soils from the straw treatment was twice as high as control soils and four times as high as the N-fertilized and hay-treated soils. Accordingly, labile WEOM-C was also different between treatments ($F_{3,28} = 6.1$, $p = 0.0025$), with values nearly twice as high in both mulched treated soils than in the N-fertilized or control soils. Labile WEOM-N also differed between treatments (K-W, $H_3 = 17.83$, $p\text{-value} < 0.001$). Soils from the hay treatment had over 50% more WEOM-N than N-fertilized soils, and three times the WEOM-N than the control and straw-treated soils. Potentially mineralizable N also differed between treatments ($F_{3,28} = 7.4$, $p < 0.001$), with twice the mineralized N in hay mulched soils than in the remaining three treatments.

Microbial respiration ranged from 157 kg-C ha⁻¹ to 669 kg-C ha⁻¹ across treatments (K-W, $H_3 = 24.48$, $p < 0.001$). Mulched treated soils had higher microbial respiration than non-mulched soils. Microbial respiration from the two mulch treatments did not differ from each other, but respiration in the two non-mulch treatments did differ from each other, with N-fertilizer treatments having the lowest microbial respiration.

Soil Parameters and Plant N-uptake

The correlation between spinach N-content and spring soil NO₃ was $\rho = 0.46$ ($p = 0.007$; Figure 1a). Ryegrass N-content had fairly similar correlation across all N measures, with a correlation of $\rho = 0.54$ with pre-seeding soil NO₃ analysis ($p < 0.001$), $\rho = 0.56$ with WEOM-N ($p = 0.001$), and $\rho = 0.42$ with PMN ($p = 0.02$, Figure 1b, 1c, 1d, respectively).

Microbial respiration has been promoted as a good indicator of plant available N (Kick 2014, Culman et al. 2013, Vahdat et al. 2010, Haney et al. 2008), however there was no correlation between microbial C-respiration and ryegrass N-uptake in our study ($\rho = 0.14$, $p = 0.4$, Figure 2a). Correlation increased between microbial respiration and ryegrass N-uptake, ($\rho = 0.40$, $p = 0.05$, Figure 2b) when straw treatment was removed from the analysis.

Generalized linear regression was employed to assess whether biological measures of soil N predict plant N-uptake more accurately than the NO₃ test. Five models shared strong support from our suite of models regressing the natural log of ryegrass N content with each of the soil N analyses, individually and in combination (Table 4). The factors included in the models with strong support, given our data, included WEOM-N,

NO₃, microbial respiration, and WEOM-C. All of the models with strong support explained a similar amount of the variation in the data.

Available N, Microbial Respiration and Input C:N

Microbial C respiration is currently promoted as an effective way to estimate plant available N in the field (Culman et al. 2013). There was no correlation between microbial respiration and NO₃ ($\rho = 0.14$, $p = 0.44$, Figure 3a) or WEOM-N ($\rho = 0.09$, $p = 0.64$, Figure 3e), though there was some correlation between microbial respiration and PMN in this study ($\rho = 0.43$, $p = 0.01$, Figure 3c). But with the removal of data from the straw treatment, correlation increased between microbial respiration and NO₃ ($\rho = 0.38$, $p = 0.07$), PMN ($\rho = 0.66$, $p < 0.001$) and WEOM-N ($\rho = 0.31$, $p = 0.14$, respectively; Figures 3b, 3d and 3f).

In both mulched treatments WEOM-C and microbial respiration were high compared with the non-mulched treatments (Table 3). However, the relationship seen between high labile C and high respiration with available N and plant N-uptake differed between the two mulch treatments. Soils from the straw mulch treatment had the highest values of microbial respiration, but very low production of plant biomass, plant N-uptake, and available N. Soils from the hay mulch treatment had high microbial respiration levels, and high available N, high plant biomass and high plant N-uptake (Tables 2 and 3).

Generalized linear regression was employed to assess how labile C and N influence microbial respiration, as modeling empirical data can be a useful tool to

determine whether expected patterns match observation. If labile-C is indeed the preferred food source for soil microbes, we expected WEOM-C should drive carbon respiration (Figure 4a; $R^2_{\text{adj}} = 0.53$). Though the plotted data appeared linear, the moderate R^2 value indicated a high degree of unexplained variance. When considering individual trend-lines for each soil treatment, overall respiration levels were higher in mulched treatments than in non-mulched treatments, and the rate of respiration was greater in the hay treatment than the other three treatments (Figure 4b). This indicates that another factor or factors influence respiration, besides labile C.

The model with the strongest support given our data was the full additive model (Table 5), with the log of microbial respiration as a function of WEOM-C:N, WEOM-C, WEOM-N, whether plots were mulched or non-mulched, and an interaction term between WEOM-C and -N. This model had a $R^2_{\text{adj}} = 0.87$, a ΔAICc of 1.30 units over the second model and an AIC weight (w) of 0.46. The second ranked model was the log of microbial respiration as a function of WEOM-C and the mulch indicator variable. The evidence ratio between the top two models was only 1.9 indicating that insufficient data exists to support only the top model.

Of all the parameters, the intercept, the mulch indicator variable and WEOM-C had the highest relative importance at 1, 1, and 0.99, respectively. The remaining parameters had lower relative importance with 0.75 for WEOM-N, 0.60 for the interaction between WEOM-C and WEOM-N, and 0.55 for WEOM C:N.

Discussion

Soil Tests and Plant N-uptake

Pre-seeding soil NO_3 remains an adequate estimate of plant available nitrogen, regardless of the fertility management strategy, in the growing conditions of this experiment. The presence of NO_3 is a reflection of biological mineralization, despite it being inorganic. Spring NO_3 measures mineralization that occurred over the fall and winter and thus remains a good indicator of plant available N, even in systems using organic fertility inputs. The temporal differences in NO_3 between sampling periods illustrate the fluctuations of NO_3 concentrations in soils.

Soil analyses that measure biological processes can provide organic farmers with more information about how their system is functioning than if they used NO_3 analysis alone. Using WEOM, C-respiration and N-mineralization as measures of soil biological function provides an indicator of how management practices impact the mechanisms driving plant-available N-mineralization. WEOM-N followed similar patterns across treatments as NO_3 and was similarly correlated with ryegrass N-uptake. Measuring WEOM-N can give farmers an idea of how their management practices are impacting the labile energy source of the soil community (Marinari et al. 2010, Chantigny 2003). WEOM can also be an early indicator of management effects on SOM (Haney et al. 2012, Marriott and Wander 2006, Doane et al. 2003, Jandl and Sollins 1997).

Input C:N has a large effect on N-mineralization and availability, measured in both the NO_3 and plant N-content. This has also been measured in a number of studies conducted in forest systems (Wardle 2002), and in agricultural systems where inputs

changed total SOM quality (Doane et al. 2003, Carpenter-Boggs et al. 2000). In our study, PMN was not highly correlated with plant N-uptake. It is possible that the timing of plant growth did not correspond with maximum N-availability. However, the correlation between PMN and ryegrass N-uptake strengthened when a single outlier was dropped ($r = 0.38$, $p = 0.04$). Soils are inherently and unavoidably heterogeneous for these variables, thus there is low certainty in generalizations (Haney et al. 2012, Manzoni et al. 2008).

Labile C provides energy for microbial respiration and has been found to be more limiting to microbial growth than N in intensively managed agricultural systems (Schimel and Weintraub 2003, Scow 1997). We found this to be true in the non-mulched treatments, with lower WEOM-C and lower microbial respiration. In the mulched treatments, high microbial respiration did not necessarily correspond with high plant-available N. When inputs with a high C:N are added to soil, microbes must seek N from existing soil N pools, limiting or delaying what is available for plant uptake.

It was inconclusive which soil N analyses best estimated plant N-uptake. NO_3 , WEOM-N, WEOM-C, C-respiration, PMN and WEOM C:N all had similar predictive strength and all explained a low quantity of the variability seen in the data. These results were contrary to our expectations that combining biological soil measures with the pre-seeding NO_3 test would increase the ability to predict plant available N. Use of a non-linear model may provide a better fit and additional insight on what soil N measures best predict plant N uptake. We would expect that WEOM-N, PMN and C-respiration would all provide additional predictive power to the NO_3 test based on relationships found in

previous studies (Chapter 2, Haney et al. 2012, Culman et al. 2013, Agren et al. 2013, Toosi et al. 2012).

Input C:N Effects on Available N

Plant available N is affected by microbial decomposition of SOM and understanding how different organic inputs influence microbial activity is important to accurately estimate decomposition and subsequent N availability (Haney et al. 2012, Chapter 5). If microbes need both N and C to grow (Bowles et al. 2013, Haney et al. 2012, Schimel and Weintraub 2003), we would expect a relationship to exist between microbial respiration, labile C and labile-N or PMN. We saw this response in the hay mulched treatment, and as a result measured both high microbial respiration and high plant N-uptake and biomass. However, the results between the two mulch treatments were not consistent with each other. Straw mulched treatments also had very high respiration, but very low plant N-uptake and biomass. The contradictory responses between plant N-uptake and microbial respiration in the two mulch treatments indicated that microbial respiration alone was not an adequate proxy measure of available N in organic input systems. The importance of input C:N in providing plant available N was confirmed with the removal of the straw treatment data from our analysis. Without the straw mulched treatment, the pattern in the data indicated that there was a relationship between plant N-uptake and microbial respiration. This confirms that the nutrient balance of inputs must be considered when estimating plant available N from microbial respiration.

In this study, high C was only an indicator of plant-available N if there was also high N present. This was measured in the distinct differences in N-availability and plant N-content between the hay treatment and the straw treatment. The regression model with the second highest support contained only the mulch indicator variable and labile C. The insignificant ΔAIC between the top two models and the relative importance of the mulch indicator parameter and labile-C both suggest that C is indeed a primary driver of microbial respiration. The greater rate of respiration in the hay treatment implies that there is a greater biological response to added C from inputs with lower C:N (Figure 4b). High soil N had a greater influence on plant-available N if there was also high C, as measured in the higher plant biomass and N-content for both plant species when grown in the hay treatment versus the N-fertilizer treatment.

These data follow patterns measured previously of decomposition and plant available nutrients with low and high quality litter additions (Doane et al. 2003, Craft 1998, Swift 1987). Though it is important to remember that the effects of input C:N on microbial respiration and N-mineralization will also vary with different management histories, soil types and the C:N of SOM already present in the system.

Conclusions

Sustainable farmers are faced with different challenges than their conventional peers, but nutrient management remains a critical consideration across all farms. Organic fertilizer inputs differ in form and function from mineral fertilizers and require soil biological processes to make the nutrients in SOM plant available. The management of

organic systems requires consideration of the entire aboveground-belowground ecosystem (Kibblewhite et al. 2008, Brussard 1994) and organic farmers must cultivate conditions that are optimal for soil biotic activity to provide adequate plant nutrients (Seiter and Horwath 2004).

Within these conditions, farmers have a variety of soil test options for estimating plant available N through the growing season but nutrient balance of inputs must be considered when choosing an appropriate test. The spring soil NO₃ test remains an informative analysis in predicting plant available N over the growing season, even under organic management. Biological assays like PMN and WEOM-N have similar predictive power and may contribute additional information about how management practices influence soil activity. WEOM-N, especially, can be a useful tool for organic farmers to monitor how management practices affect SOM stores through time (Toosi et al. 2012, Chantigny 2003). This study illustrates how the nutrient ratio of inputs can increase microbial C-respiration without a corresponding increase in plant available N. To promote biological nutrient cycling, inputs of available carbon must be a part of sustainable agricultural management in order to provide an energy source for soil biota. However, adding carbon alone will not necessarily lead to the turnover of available nitrogen; the stoichiometry of inputs must be considered when organic fertility inputs are used in N-limited systems. This necessitates a broader, systemic view of the relationship between C and N in the soil ecosystem, to satisfy the appetites of both microbes and plants.

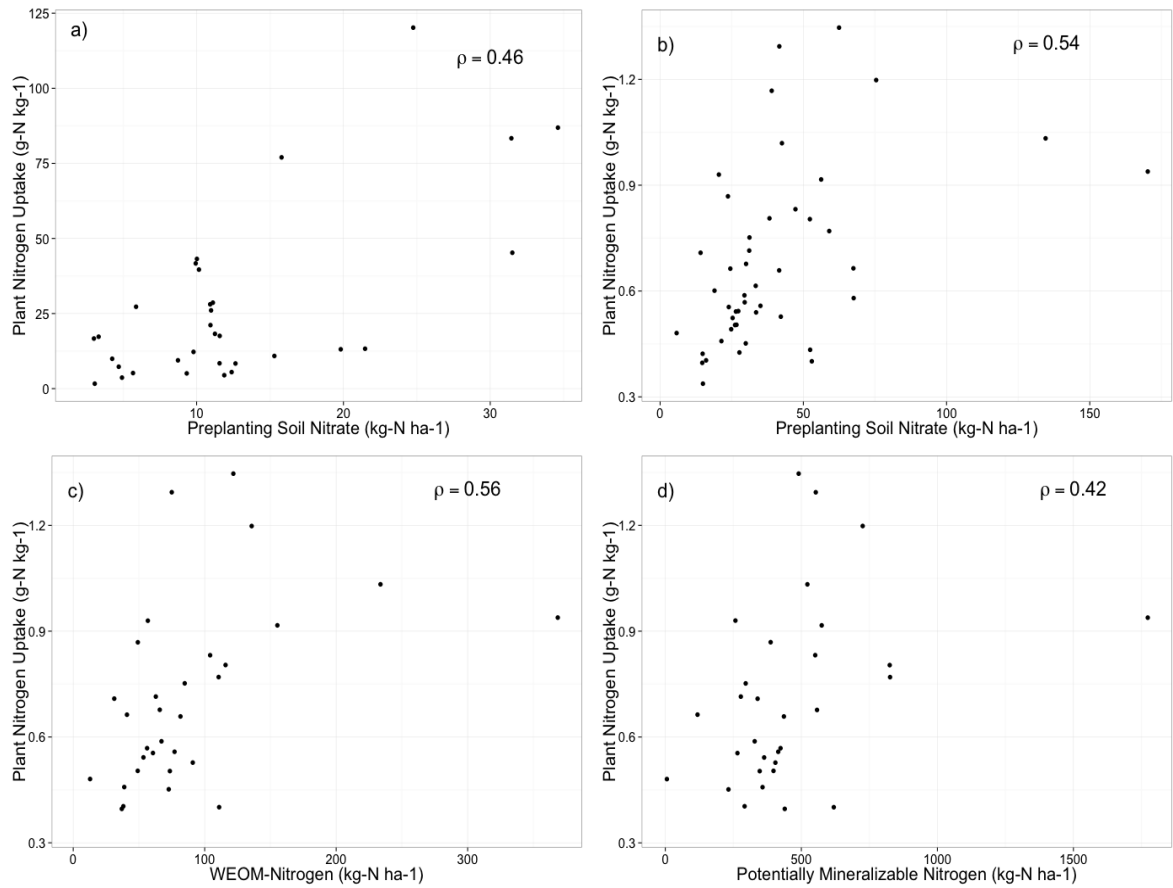


Figure 1. The relationship between plant N uptake with pre-seeding soil NO₃ for spinach (a) and ryegrass (b), as well as the relationship between ryegrass N uptake with WEOM-N (c) and PMN (d). Spearman correlation (ρ) shown for variables with unequal variance.

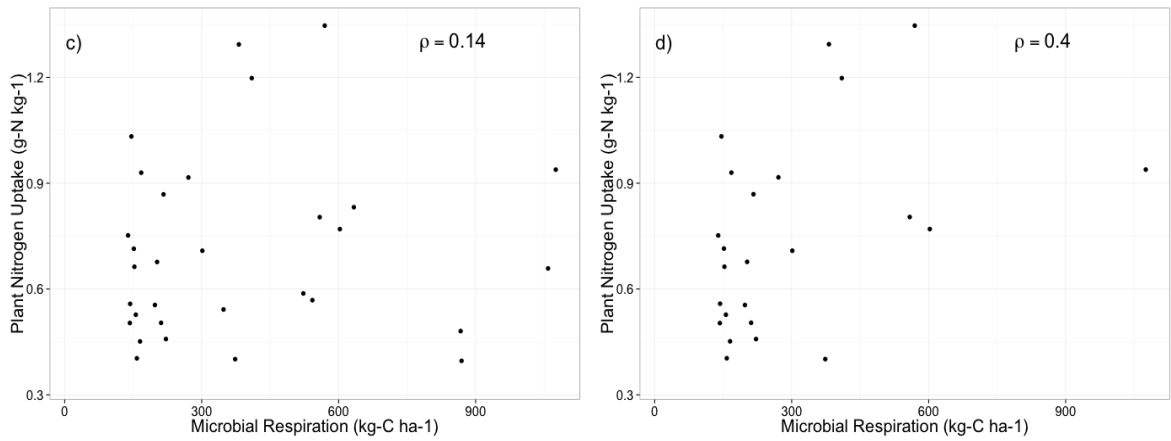


Figure 2. The relationship between plant N-uptake and microbial respiration is not strong when all treatments are considered for both plant species, spinach (a) and ryegrass (c). The relationship improves when the straw treatment is removed from the analysis, spinach (b) and ryegrass(d). Spearman correlation (ρ) shown for variables with unequal variance.

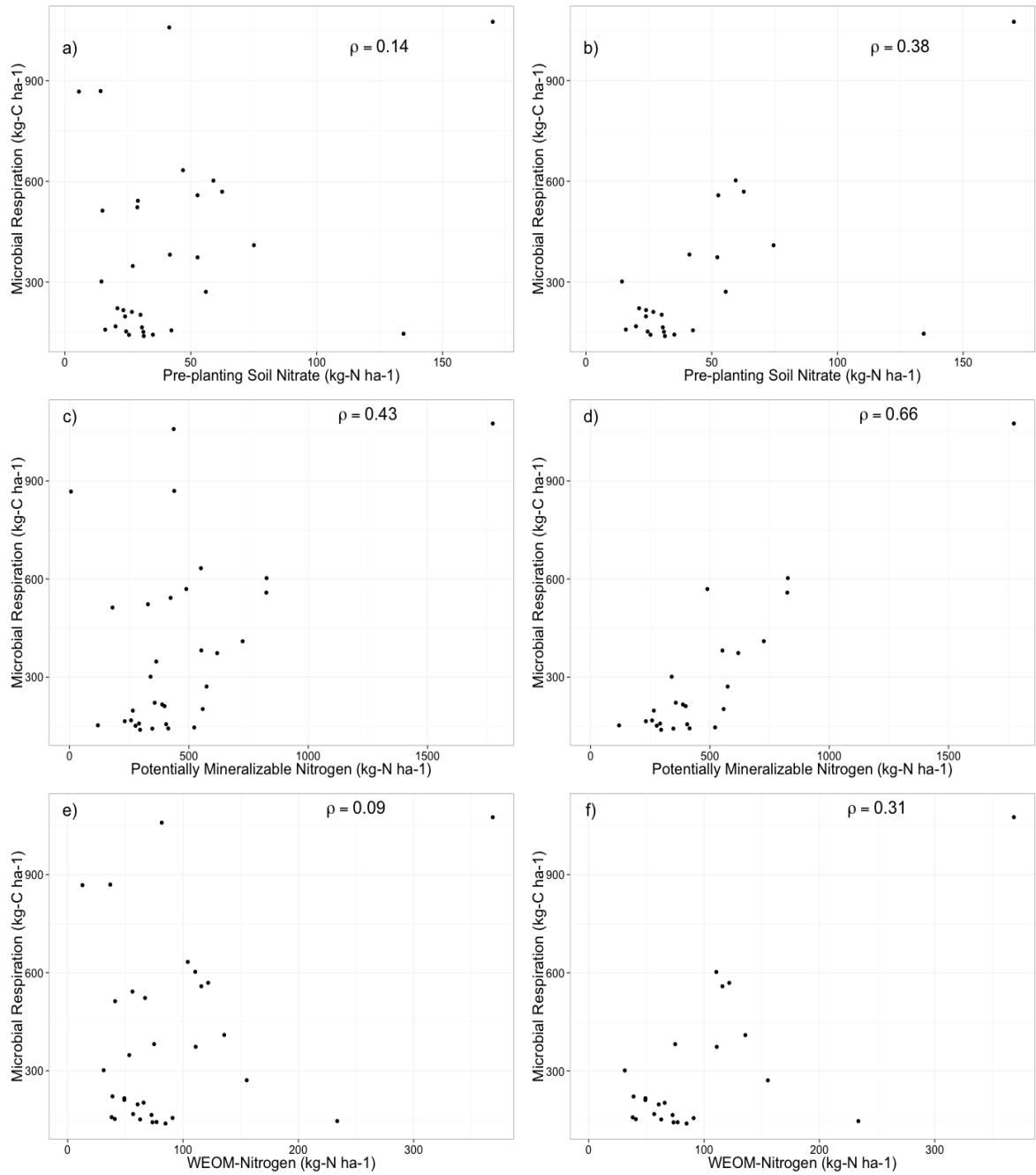


Figure 3. When all treatments are considered, there is little relationship between microbial respiration and pre-planting NO₃ (a), WEOM-N (c) or PMN (e). However, when the straw treatment was removed, the relationship strengthened between microbial respiration and NO₃ (b), WEOM-N (f) and PMN (d). Spearman correlation (ρ) shown for variables with unequal variance.

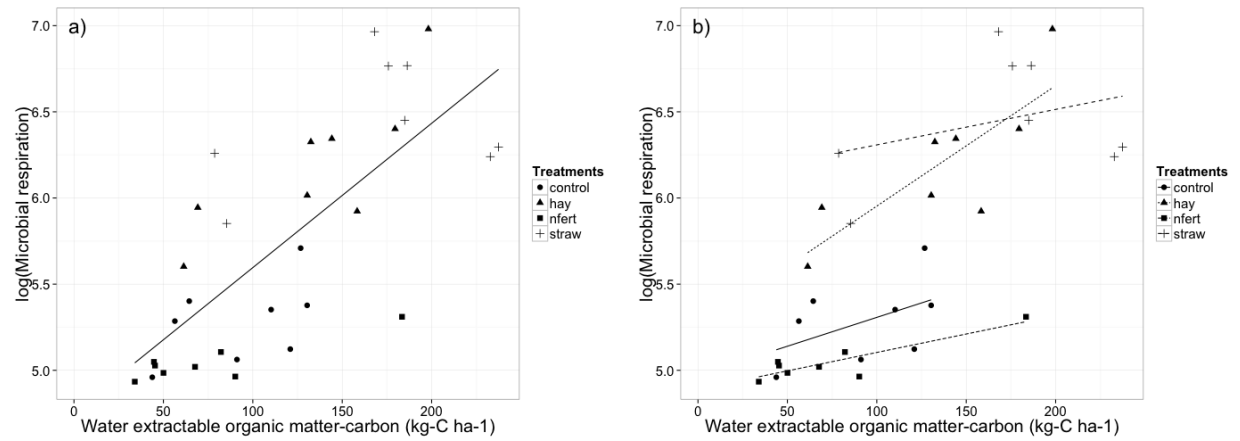


Figure 4. The relationship between microbial respiration and WEOM-C is linear (generalized linear regression model, plot a). Individual trend lines for each treatment shows a clear differentiation between mulched and non-mulched treatments and a difference in slope between the hay treatment and the other three treatments (b), indicating factors other than just labile C influence microbial respiration rates.

Table 1. Soil characteristics sampled June 2011, mean and (standard error), n = 16

pH	Olsen P mg kg ⁻¹	K mg kg ⁻¹	Total N g kg ⁻¹	OC g kg ⁻¹
7.6 (0.09)	115 (2.9)	968 (18.4)	1.7 (0.02)	23 (0.28)

Table 2. Plant characteristics across treatments, mean and (standard error)

	Spinach Biomass g plant ⁻¹	Ryegrass Biomass g plant ⁻¹	Spinach N- concentrat ion g kg ⁻¹	Ryegrass N- concentrat ion g kg ⁻¹	Spinach N-content mg plant ⁻¹	Ryegrass N-content mg plant ⁻¹
Control	6.9 ab (1.56)	0.3 b (0.03)	36.34 b (1.65)	17.45 b (0.19)	23.02 ab (4.49)	0.56 b (0.02)
N- fertilizer	5.09 bc (1.90)	0.4 b (0.02)	37.36 ab (1.05)	17.79 b (0.47)	16.70 bc (5.01)	0.63 b (0.02)
Hay	13.41a (3.61)	0.5 a (0.04)	43.02 ab (0.93)	20.30 a (0.29)	56.24 a (14.67)	0.97 a (0.04)
Straw	2.63 b (0.76)	0.3 b (0.02)	43.78 a (0.85)	18.73 ab (0.2)	11.12 c (3.07)	0.57 b (0.02)

Table 3. Soil analysis results, mean and (standard error).

	Pre-seeding NO ₃ Field kg-N ha ⁻¹	Pre-seeding NO ₃ Green-house kg-N ha ⁻¹	Soil C:N g g ⁻¹	WEO M C:N g g ⁻¹	WEO M C kg-C ha ⁻¹	WEO M N kg-N ha ⁻¹	PMN kg-N ha ⁻¹	Microbial Respiration kg-C ha ⁻¹
Control	10.9 b (0.28)	22.7 c (1.67)	9.8 a (0.07)	2.1 a (0.42)	93.0 bc (14.84)	49.7 c (4.92)	330.5 b (23.77)	202.1 b (23.08)
N-fertilizer	10.8 b (0.47)	40.9 b (8.86)	9.6 ab (0.12)	1.1 b (0.34)	74.7 c (20.27)	91.1 b (20.39)	353.0 b (54.94)	157.0 c (9.28)
Hay	24.4 a (2.62)	61.4 a (10.38)	9.4 b (0.09)	1.0 b (0.15)	134.2 ab (17.87)	149.2 a (31.32)	798.3 a (143.68)	530.2 a (87.90)
Straw	4.3 c (0.33)	32.01 bc (5.82)	9.7 ab (0.13)	4.4 a (1.46)	168.7 a (25.50)	56.7 c (9.63)	341.0 b (61.99)	669.3 a (82.41)

Table 4. Model selection criteria for regression analysis determining N measures that are good predictors of plant N uptake. The 'Model' column indicates the parameters in each model and their effect (+ or -); 'K' is the number of parameters in the model; 'AICc' is Akaike's Information Criterion, corrected for small sample size; 'ΔAICc' is the difference in AICc between models; and 'w' is the relative likelihood of the model, given the entire suite of models.

Model	K	AICc	ΔAICc	w	R ² adj
WEOM-N	3	19.47	0	0.21	0.20
NO ₃ - WEOM C:N	4	20.14	0.67	0.15	0.23
NO ₃ - C-RESPIRATION	4	20.53	1.06	0.12	0.22
NO ₃ - PMN	4	20.65	1.16	0.12	0.21
NO ₃ - WEOM-C	4	20.68	1.21	0.11	0.21
WEOM-N - PMN	4	22.05	2.58	0.06	0.18

Table 5. Model selection criteria for regression analysis determining the labile C and N factors influencing microbial respiration. The 'Model' column indicates the parameters in each model and their effect (+ or -); 'K' is the number of parameters in the model; 'AICc' is Akaike's Information Criterion, corrected for small sample size; ' Δ AICc' is the difference in AICc between models; and 'w' is the relative likelihood of the model, given the entire suite of models.

Model	K	AICc	ΔAICc	w	R²adj
WEOM C:N + WEOM-C - WEOM-N + MULCH INDICATOR + WEOM-C*WEOM-N	7	10.61	0	0.46	0.87
WEOM-C + MULCH INDICATOR	4	11.91	1.30	0.24	0.84
WEOM-C - WEOM-N + MULCH INDICATOR + WEOM-C*WEOM-N	6	12.95	2.34	0.14	0.86
WEOM C:N + WEOM-C + WEOM-N + MULCH INDICATOR	6	14.16	3.55	0.08	0.85

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CHAPTER FIVE

THINKING LIKE A MICROBE: THE BIOLOGICAL MECHANISMS OF FERTILITY
IN SUSTAINABLE MIXED-VEGETABLE PRODUCTION

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CHAPTER FIVE

THINKING LIKE A MICROBE: THE BIOLOGICAL MECHANISMS OF FERTILITY
IN SUSTAINABLE MIXED-VEGETABLE PRODUCTIONAbstract

Agricultural production covers a large portion of the landscape and has the potential to significantly contribute to CO₂ emissions from SOM decomposition. As farmers increasingly use plant based fertility inputs in organic and sustainable systems, understanding decomposition of organic matter (OM) for soil fertility is needed to estimate agricultural CO₂ efflux and C sequestration. To examine this question, we implemented a small-plot experiment using two different quality (C:N) mulches and N-fertilizer to compare how different fertility inputs affect soil biotic functions and how the decomposition of different substrates contribute to N mineralization and C-respiration. Large differences in microbial community indicators (extracellular-enzymes, AMF colonization, microbial biomass) and cumulative C-respiration and N mineralization were measured in soils from the mulched treatments. Decomposition rate did not appear to differ greatly between treatments, though there was considerable variation from year to year. Understanding input effects on soil community function and nutrient availability will help small-scale farmers plan crop rotations to maximize the benefits from their inputs. These findings could direct future research on how to manage fertility inputs in agroecosystems to provide adequate available nutrients for crop growth, but limit excess CO₂ efflux.

Introduction

From a bird's eye, the earth is a patchwork of different land uses, with nearly 40% in agricultural production (World Bank 2014). Agricultural lands are a large part of the global C and N cycles: mineral soils store about 2200 Pg of C and 135 Pg of N, and respiration from soil microbial decomposition regulates the turnover rates of C from soils (Batjes 2014, Bradford et al. 2013, Schmidt et al. 2011, Fierer et al. 2005, Hopkins and Gregorich 2005). In systems relying on organic production methods, decomposition regulates the availability of mineralized N and other nutrients important for crop growth. Decomposition rates can vary between agroecosystems, depending on duration and variety of plant cover, soil organic matter (SOM), tillage practices, soil moisture, temperature, soil nutrients and soil type (Hopkins and Gregorich 2005, Weil and Magdoff 2004). Though microbes act on individual molecules at a micron scale, the effects of decomposition accumulate across landscapes and can have a large impact on global CO₂ emissions (Anderson and Domsch 2010).

From a microbe's perspective, SOM has numerous benefits. Not only a primary source of energy and nutrients, SOM is also an integral structural component of soil architecture, contributing to water and nutrient retention, pore networks, microbial habitat and the movement of gasses and water through the soil system (Kibblewhite et al. 2008, Young and Ritz 2005). Below-ground ecosystems are a complicated web of relationships between soil biota, soil chemical processes and the soil physical structure; microbial communities change and are simultaneously changed by their physical surroundings (Young and Ritz 2005). Microbial community structure and function can be altered by

changes in SOM substrate quality (Blagodetskaya et al. 2009) and changes in soil community function can lead to changes in resource allocation, soil structure and nutrient availability (Schimel and Schaeffer 2012). At the same time, microbial use of substrates alters the chemical structure of SOM: changing the reactivity and accessibility of molecules and the overall C:N of soil (Agren et al. 2013, Joffre et al. 2001).

Most microbially accessible SOM is found in the liquid matrix of soils and can be measured using water-extracted organic C (WEOC) and dissolved organic N (DON) (Haney et al. 2012, Toosi et al. 2012, Zsolnay 1996). WEOC and DON are used to calculate water-extractable organic matter (WEOM) C:N, an indicator of SOM quality that is more sensitive to management than solid-state SOM C:N (Haney et al. 2012, Toosi et al. 2012). Soil microbes rely on extracellular enzymes to depolymerize complex organic molecules into simple, assimilable components (Conant et al. 2011, Schimel and Bennett 2003). The most labile energy in soils come from simple, reduced C molecules, suspended in soil water, accessible to microbes and oxidizable to CO₂ (Hopkins and Gregorich 2005). As molecule complexity increases, more enzymes – and therefore energy – are needed to break it into simpler subunits (Deng and Popova 2011, Shi 2011, Bosatta and Agren 1999). Complex molecules, like cellulose and chitin are significant sources of C (cellulose) and N (chitin) in soil organic matter (Deng and Popova 2011, Shi 2011) and require multiple enzymes for depolymerization. The soil activity of the final enzyme in each reaction can be used as an indicator of overall microbial cellulase and chitinase activity (Shi 2011). However, it can be difficult to use enzyme activity as a predictor of nutrient availability as both nutrient abundance and deficiency can promote a

spike of enzyme production and because extracellular enzymes can persist in soils for many years (Bowles et al. 2014, Burns et al. 2013). Similarly, microbial populations themselves are poor indicators of overall microbial activity in soils because microbial biomass is temporally dynamic and a single measure does not give a good indication of the microbial community size through time (Lauber et al. 2013). Soil microbial biomass can, however, represent a relative measure of the energy and nutrient storage and cycling potential of soils (Bhogal et al. 2009).

One of the primary goals of agricultural management is to optimize nutrient availability to meet crop needs and maximize net returns. In conventional agriculture, this usually means adding mineral fertilizer to boost existing quantities and prevent nutrient deficiencies. In organic systems, organic material is added with the objective of stimulating microbial decomposition and nutrient cycling (FlieBbach and Mader 2000). Different types of agricultural inputs can have varying effects on N mineralization, making it difficult to estimate potential available plant nutrients (Haney et al. 2012). Mineralizable N and C-respiration are representative measures of biologic decomposition of SOM. Mineralizable N is the proportion of soil N that is accessible to microbial mineralization and potentially plant available in the short term (Bhogal et al. 2009). C-respiration measures can indicate the accessibility of short- and longer-term C pools to microbial degradation over time (Robertson et al. 1999). Both can be useful measures to assess the long-term implications of fertility inputs on N-mineralization and CO₂ emission from agricultural systems under different management.

The complexity of below-ground systems and the temporal and spatial scales that govern below-ground processes make it difficult to determine the structure of the relationships regulating soil functions (Hopkins and Gregorich 2005). In agricultural systems it can also be difficult to measure the impact of management practices on soil community functions because the annual cycles of crop management, including frequent tillage, the removal of plant material each year, and nutrient additions can mask soil functions (Weil and Magdoff 2004). The goal of this research was to measure how soil fertility management practices impact the soil processes facilitated by soil microbial communities, especially organic matter decomposition and N-mineralization. To achieve this goal we implemented a three year strip-plot research study at the Montana State University (MSU) research vegetable farm, Towne's Harvest Garden (THG), using four different fertility inputs. The inputs included a barley-straw mulch: added as a high C:N, lower quality input; a legume-grass hay mulch: added as a lower C:N, higher quality input; urea fertilizer: a conventional mineral fertilizer; and a no-treatment control.

We hypothesized that 1) cumulative C-respiration and N mineralization will differ between years for each treatment due to a change in substrate quality and quantity through time from the different fertility inputs added in the first year; 2) high OM systems will have a greater microbial response, measured by C-respiration, N mineralization, microbial biomass, mycorrhizal infectivity potential, and enzyme activity, than lower OM systems; and 3) C-decomposition will differ between treatments and years due to microbial utilization of different quality available substrates.

Methods

Field Study

The THG research vegetable farm, is located at the Montana State University (MSU) Horticulture Farm in Bozeman, MT (45.66, -111.07). The research plots were under conventional cereal-fallow management for 20 years, prior to this experiment. The soil is a Turner loam with 40% sand, 37% silt and 23% clay, 7.3 pH and an initial organic matter content of 3.9% (LOI). We conducted a randomized strip-plot experiment of four replicates of four soil treatments across 16 plots in a 2000 m² field, growing spinach (*Spinacia oleracea* var. Space (F1)), tomato (*Solanum lycopersicum* var. Juliet), corn (*Zea mays* var. Vision) and broccoli (*Brassica oleracea* var. Arcadia) over three growing seasons, from 2011-2013 (Appendix A).

Soil treatments consisted of two mulch additions, barley straw and grass-legume hay, a N-fertilizer treatment and an untreated control. At the recommendation of local vegetable farmers, straw mulch was applied at a rate of 5.5 kg/m² and hay mulch at 9 kg/m². Mulch additions differed in C:N (t-test; $t = 2.5$, $p = 0.049$; Appendix A), and averaged 26.5 for the hay mulch and 41.6 for the straw treatment. Both mulches were applied only once over the course of the experiment, in October 2010. The mulches overwintered on the surface and were incorporated into the soil via tilling in June 2011. The N fertilizer was added in the form of urea (46-0-0). Existing NO₃-N was determined by sampling the soil prior to planting. Urea was side-dressed each year following MontGuide (Dinkins et al. 2010) recommendations for Montana vegetable production

(0.0159 kg N m⁻²), approximately 10 days after spinach emergence. The untreated control had no fertility additions over the course of the experiment.

Soil Analysis

Soils were collected each spring for NO₃-N analysis prior to planting spinach in the field (Agvise Laboratory, ND). Ten randomly selected 1-cm dia x 15-cm deep cores were collected and composited from each plot.

Soil samples were collected from the root zones of spinach plants at spinach harvest for analysis of soil function. We chose to focus analysis on soil from spinach plots because it was the most consistently successful crop over all three years and because the timing of spinach harvest best fit the logistics of the long-term soil incubation. Soils were air dried overnight at 20 °C, sieved to 2mm and held at 4 °C until analyzed. Soils were analyzed for NO₃-N, OM (LOI), Olsen phosphorus (P), and potassium (K) (Agvise Laboratory, ND). Post-harvest soils were also used for WEOM analysis and SOM C and N concentration. WEOM analysis was performed following Toosi et al. (2012; Appendix A) and extracts were analyzed with a TOC-V analyzer (Shimadzu Corporation, Japan) for WEOC and DON. WEOM C:N was also derived from these measures. Dried soils were milled using a ball-mill and analyzed for organic C and N using a TrueSpec CN combustion analyzer (LECO Corporation, USA).

Each year, soils were incubated at 25 °C for 32 weeks to determine C-respiration and N mineralization, following Robertson et al. (1999; Appendix A). CO₂ was sampled weekly by drawing off headspace from the incubation jars and analyzed on a Varian CP-3800 gas chromatograph (Agilent Technologies, USA). NO₃-N extractions occurred at

the beginning of the incubation, and at weeks 1, 2, 4, 8, 16 and 32. NO₃-N was extracted from soils following a modified version of Keeney and Nelson (1987, Appendix A), using 1M KCl, and analyzed using Lachat QuickChem FIA analyzer (Hach Instruments, CO).

The physiological method of substrate-induced respiration was used to estimate microbial biomass in 2013 following Anderson and Domsch (1978; Appendix A). AMF colonization potential was determined following Brundrett et al. (1996) by growing sudan grass (*Sorghum bicolor*) in intact 12 cm deep x 8 cm dia. soil cores for 6 weeks in the greenhouse. Soils for AMF colonization potential were collected from the field in November 2011. β -glucosidase and N-acetyl- β -glucosaminidase activities were determined colorimetrically in 2013, following Deng and Popova (2011; Appendix A) using *p*-nitrophenol standard.

Statistical Analysis

To measure differences in OM quality and quantity between treatments across the three years of the experiment, two-way Analysis of Variance (ANOVA) was performed. The least significant differences (LSD) test was used for multiple means comparison (de Mendiburu 2014). Variables were tested for non-normality using the Shapiro-Wilk test and for unequal variance using the Fligner-Killeen test for homogeneity of variances. For variables with non-normality and unequal variance, we used Kruskal-Wallis (K-W) to test for differences between groups and the mean rank of groups to assign differences between treatments. To determine differences between treatments for biological measures, one-way ANOVA was used. Variables were tested for normality and homogeneity of variance as above. We used Pearson's correlation analysis to determine

the relationship between OM measures, soil nutrients and soil biotic variables.

Following Robertson and Paul (2000) and Collins et al. (2000) we fit exponential decay models independently to estimate the active (C_a) and stable (C_s) pools of C from the respiration data of the 32-week soil incubation and to determine decay rates for each pool. Models were fit independently for each treatment in each year using the equations:

$$\text{C-respiration} = C_a * (\exp(-k_1 * t)) \text{ for active C}$$

$$\text{C-respiration} = C_s * (\exp(-k_2 * t)) \text{ for stable C}$$

where C_a and C_s are estimates of C in each pool, $-k_1$ and $-k_2$ are decay rates for each pool and t is sampling time. To locate the appropriate cutoff between C_a and C_s , we ran preliminary linear models through subsets of the data to determine the location of the inflection point. In comparisons using weeks 1, 2 and 3 as the cutoff, all plotted linear models intersected at week 2.5. Thus, k_1 is the slope of the curve from weeks 1 to 3, and k_2 is slope of the tail from weeks 4 to 32. In 2013, the incubation was 4 weeks in total, so we only calculated k_1 .

We also used non-linear model fitting to explore whether two decay curves were actually necessary to describe C decay through time. We let R choose a random distribution for C_a , C_s , k_1 and k_2 from the decay model estimates and standard deviations. We ran the model 10,000 times and calculated the number of times that the second decay model (C_s) more accurately estimated respiration through the tail of the curve compared with just using the first decay model (C_a) to estimate respiration through the whole curve.

Results

Cumulative C-respiration and N Mineralization

Being the most labile portion of SOM, WEOC and DON can be good indicators of microbial activity and nutrient cycling (Haney et al. 2012, Toosi et al. 2012). WEOC differed between treatments in 2011 (ANOVA $F_{3,28} = 7.876$, $p = 0.006$), with over twice as much labile C (WEOC) in the straw mulched treatments than in either of the non-mulched treatments, and about 30% more in the hay than the non-mulched treatments (Figure 1a). In 2012, WEOC increased in all treatments relative to 2011, and was about 25% higher in hay mulched plots than the no-treatment control plots (ANOVA $F_{3,16} = 2.7$, $p = 0.08$). DON also differed between treatments in 2011 and 2012 (ANOVA $F_{3,28} = 4.34$, $p = 0.01$; $F_{3,16} = 8.75$, $p = 0.001$, respectively, Figure 1b). In 2011, hay mulched treatments had nearly twice the DON as the other treatments, but in 2012 straw mulch treatments had the greatest amounts of DON. By 2013, both WEOC and DON were comparable across all treatments.

Microbial response to differences in available C and N was apparent in the cumulative values of C-respiration and N mineralization from the 32-week incubation (Figures 1c and 1d). Cumulative C-respiration differed by treatment and by year, with highest respiration in both mulched treatments in 2011 and 2012 (K-W $H_3 = 24.67$, $p < 0.001$; K-W $H_3 = 21.65$, $p < 0.001$, respectively). By 2013, only the hay mulched treatment was higher than the non-mulched treatments (K-W $H_3 = 6.69$, $p = 0.08$). Mineralized N also differed by treatment and by year, following similar patterns as C-respiration (K-W $H_3 = 17.1$, $p < 0.001$ in 2011; K-W $H_3 = 21.84$, $p < 0.001$ in 2012; K-W

$H_3 = 5.97$, $p = 0.1$ in 2013). In 2011 and 2012, both mulched treatments were higher than the non-mulched treatments. In 2013, only the straw mulched treatment had higher mineralized N than the no-treatment control.

Biological Response to Different OM Inputs

A greater quantity of available energy and nutrients can support a greater quantity of biological activity (Weil and Magdoff 2004). In 2013, we saw persistent, though small, differences in total SOM among treatments (ANOVA $F_{3,35} = 29.79$, $p < 0.001$, Figure 2) with the highest SOM in the straw mulched treatments. SOM C:N did not differ between treatments, but WEOM C:N did differ between treatments (ANOVA $F_{3,35} = 2.59$, $p = 0.07$). The lowest C:N was measured in the straw mulch treatments even though there were no measurable differences in either WEOC or DON among any of the treatments.

Though we primarily saw similarities among treatments in OM quality measures in 2013, we did see differences in most of the biological parameters between treatments. AMF colonization differed between treatments with higher colonization rates in the mulched treatments than in the control (ANOVA $F_{3,29} = 2.35$, $p = 0.09$). Microbial biomass did not differ between treatments (ANOVA $F_{3,28} = 1.058$, $p = 0.38$, respectively, Figure 3). β -glucosidase and N-acetyl- β -glucosaminidase activity also differed between treatments (ANOVA $F_{3,11} = 26.6$, $p < 0.001$; K-W $H_3 = 10.3$, $p = 0.01$, respectively, Figure 3). Hay treatments had over twice as much β -glucosidase activity than the N-fertilizer and no-treatment plots and both mulched treatments had higher N-acetyl- β -glucosaminidase activity than either non-mulched treatments.

Links between biological functions and available C and N should be clear as soil microbes need both elements to live. Like most soil relationships, however, the patterns between SOM quality and biological functions are not linear (Table 1). N mineralization was most strongly correlated with all SOM quality parameters except SOM-C and -N. C-respiration was moderately correlated with SOM quality, but surprisingly was not correlated with WEOC. Though, illustrating the complicated covariance in soils, both microbial biomass and AM infectivity were correlated with WEOC. β -glucosidase activity and N-acetyl- β -glucosaminidase activity were both correlated with microbial biomass ($r = 0.48$, $p = 0.07$ and $r = 0.75$, $p = 0.001$, respectively), but neither were correlated with AM infectivity. β -glucosidase activity was not correlated with any SOM quality parameters and N-acetyl- β -glucosaminidase was only correlated with total SOM. Of the soil nutrient factors measured, WEOC, OM, SOM-C and DON were most often correlated with biologic functions.

Decomposition Rates and Soil C Stores from Different Inputs

By fitting double-decay curves to the full long-term incubation data, we were generally able to explain the data better than by only fitting a single curve. In 2011 the no-treatment control data fit only moderately well with the C_s curve. In 2012 using two decay curves fit all the data better than by just using one (Table 2). In 2013 the incubation was only run 4 w so only the C_a pool was calculated. The explanatory power of including a second decay model for C_s was more variable nearer the inflection point than in the tail of the data (Table 2). We saw differences in the estimated quantities of C in the active and

stable C pools (Table 3) that followed patterns seen in the other biological responses. Higher quantities of active and stable C were estimated in both mulched treatments compared with the non-mulched treatments in all years. C decay rates differed annually, but did not appear to differ greatly among treatments (Figure 4).

Discussion

Microbial Use of Labile C and N

By contrasting two mulch treatments with N-fertilizer and an untreated control treatments, we were able to measure changes in OM substrate quality over three years and the subsequent differences in microbial use of those substrates. Our measurements captured changes in cumulative C-respiration and N mineralization as available substrate WEOC and DON varied between treatments over the course of the experiment. Higher amounts of C-respiration and N mineralization were apparent in the mulched treatments compared with the non-mulched treatments throughout the experiment. Because microbes exist and carry out functions within the soil's liquid matrix, WEOM is a sensitive measure of microbially available C and N (Kaiser et al. 2014, Hopkins and Gregorich 2005). Though the data support our first hypothesis that C-respiration and N mineralization would differ from year to year with changing available substrate, labile C and N alone do not adequately explain the patterns in N mineralization and C-respiration among treatments from year to year. Microbial biomass itself provides a temporary pool of C and N with more rapid turnover times than most measured C pools (Bhagal et al.

2009, Ros et al. 2009). While WEOM is a more sensitive measure than solid-state SOM, it likely does not capture the variations due to microbial death and consumption.

Though most differences among treatments in soil quality metrics had diminished by the end of the experiment, higher biological responses persisted in the mulched treatments, indicating a possible residual effect from the two mulches and supporting our second hypothesis. Laboratory preparation of soils removes the most fresh, labile portion of organic material by sieving (Hopkins and Gregorich 2005), but the residual effect seen in biological responses in the mulched treatments may stem from a portion of the mulch materials being small enough to fit through the 2 mm sieve, contributing more accessible C to those soils. Enzyme activity is linked to both substrate abundance and limitations (Burns et al. 2013, Shi 2011). N-acetyl- β -glucosaminidase activity is strongly associated with mineralizable N in longterm N-fertilizer studies (Shi 2011), however in our experimental plots, N-acetyl- β -glucosaminidase activity was more strongly correlated with C-respiration, β -glucosidase and microbial biomass. Both β -glucosidase and N-acetyl- β -glucosaminidase were high in the hay mulched treatment and both were correlated with microbial biomass, though microbial biomass did not differ among treatments. Surprisingly N-acetyl- β -glucosaminidase was not correlated with mycorrhizal infectivity. Higher mycorrhizal infectivity in the mulched treatments compared with the no-treatment control indicates a larger population of mycorrhizal propagules in those treatments and likely a greater quantity of chitin in soils (Nichols and Wright 2004). Increases in mycorrhizal community richness and abundance have been observed in organic agricultural systems (Verbruggen et al. 2010). Contributions of organic N from

mycorrhizal structures can impact soil organic N and, ultimately, plant available N (Shi 2011). Both N mineralization and C-respiration were moderately correlated with total SOM, as was N-acetyl- β -glucosaminidase, though N-acetyl- β -glucosaminidase was not correlated with either mineralized N or C-respiration. The relationships that exist in soils are often not direct (Kaiser et al. 2013). The abundance of indirect relationships (Table 1) indicate a need for alternative analyses of soil data, including structural equation modeling (SEM), to better estimate the relationships between soil functions and develop more precise hypotheses to test predictions (Schimel and Schaefer 2012).

Effect of Inputs on Decomposition Rate

Model-derived estimates of C in both the active and stable pools followed the patterns we saw in the other biological responses in this experiment. Greater quantities of active and stable C were estimated in both mulched treatments in all years, compared with the non-mulched treatments. The high variation in respired C and available C each year in all the treatments suggests that the dynamic qualities of C in agricultural soils could have large implications for agricultural systems and the global environment. Maintaining SOM and biologic activity is necessary in organically managed agriculture to maintain optimal plant nutrients, but that same process can have significant effects on agricultural contributions to atmospheric CO₂ (Bradford and Fierer 2012). Field studies that measure *in situ* use of C pools are necessary to fully understand the microbially mediated mechanisms that regulate C flux and to predict the dynamics of stored C in a changing world (Bradford et al. 2013). Preliminary C-respiration data collected in the field from our plots in 2012 show similar patterns as those seen in the laboratory

incubation, indicating that a large amount of microbial respired C may be emitted from agricultural soils as a result of organic matter inputs (Hartshorn 2014, personal communication). Integrating field studies with technologies like metabolomics and FTIR/NMR will also improve our understanding of how microbes utilize different resources and how those resources are impacted through agricultural management, helping to explain the patterns that are not visible or expected just by looking at available nutrients or microbial responses alone (Ritz 2011).

We expected that decomposition rate, measured by C-respiration in the laboratory incubation, would differ between treatments due to the difference in C and N availability from the different inputs. While C-respiration rates varied from year to year within treatments as available resources changed with time, we saw little differences in C-respiration rate between treatments in any year. Decomposition rates can be affected by environmental, edaphic and management factors as much as by biological activity and in many cases temperature and accessibility play the greatest roles in determining decomposition rate (Schimel and Colman, Fierer et al. 2005). The many physical, chemical and biological interactions in the soil matrix can decrease the probability of access of microbes to substrates and thus reduce the rate of decomposition (Schmidt, et al. 2011). In laboratory incubations both temperature and accessibility are manipulated by using sieved soils in optimal temperature and moisture conditions. Imbalances between substrate nutrient availability and microbial nutrient requirements varies within soil micro-sites, depending on available substrate and microbial community composition, making decay rates difficult to predict from either litter stoichiometry or microbial

physiology alone (Kaiser et al. 2014, Schimel and Schaffer 2012). Again, because laboratory methods remove the most fresh particulate OM through sieving, patterns present under more natural conditions may be disguised (Bradford et al. 2013, Mahli et al. 2011, Hopkins and Gregorich 2005).

The many circular relationships in soils makes it difficult to tell a linear story about SOM availability, soil biotic response and decomposition from one or two soil parameters. To fully understand below-ground processes, it is necessary to measure a complement of soil physical, chemical and biologic metrics. Microbes both use SOM and contribute to it with the production of extracellular exudates and with their own demise. Disentangling process from product in soils is one of the most challenging and exciting frontiers in science today, requiring interdisciplinary collaborations to fully utilize the technological and statistical tools available.

Regardless of the challenge, the need to understand how human activity affects global C cycling is only growing. And because agriculture covers over one-third of the global landmass (World Bank 2014), it is important to understand how soil processes respond to agricultural management practices. The simultaneous actions of decomposition and abiotic chemical processes cloud C and N dynamics in agroecosystem soils and impede predictions of both soil nutrient availability and CO₂ emissions (Blankinship et al. 2014, Ostle and Ward 2012, Ryan and Law 2005). In future conversations about agricultural sustainability, perhaps an additional goal will be on-farm responsibility to minimize efflux of C and N. In order for this to be an achievable goal, we must first better understand how different agricultural practices contribute to

atmospheric C and assist farmers in developing management plans that best incorporate this knowledge within their specific locale.

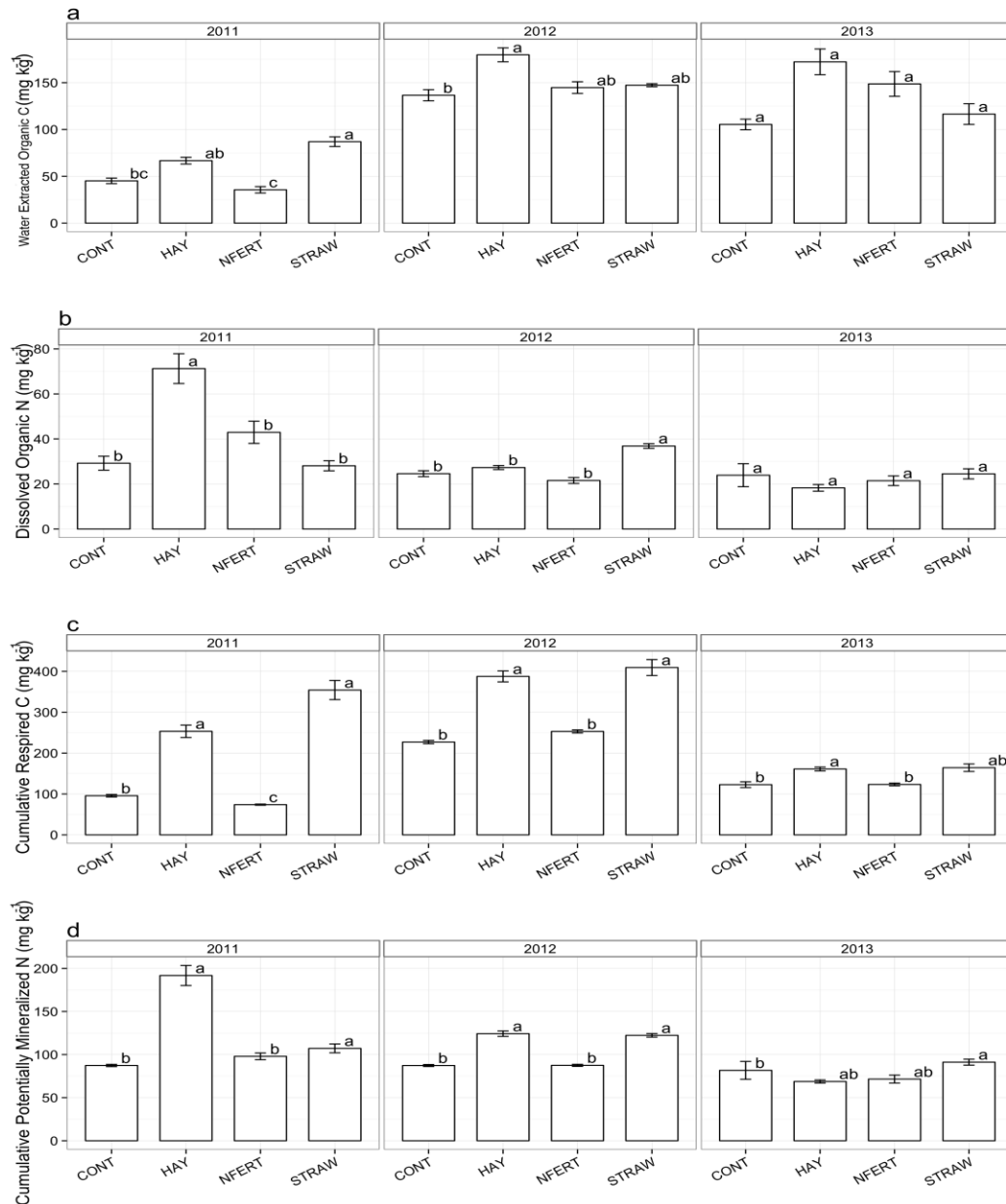


Figure 1. Labile soil C (a) and N (b) from field soils collected at spinach harvest and microbial C-respiration (c) and N mineralization from a long-term laboratory incubation, using the same soils collected at spinach harvest. Bars represent means with $\pm$ se and letters represent significant differences at $p = 0.05$.

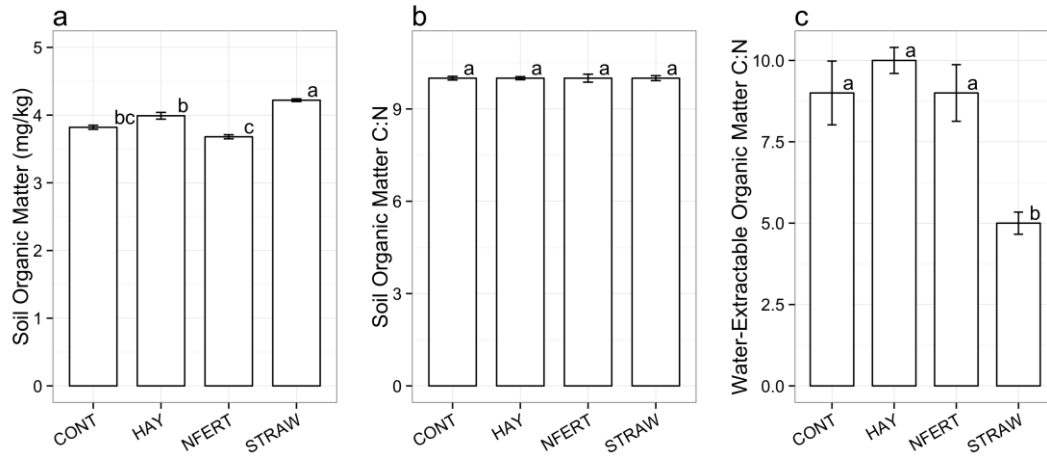


Figure 2. SOM quantity and quality measures, including total SOM (a), SOM C:N (b), and WEOM C:N (c). Bars represent means with $\pm$ se and letters represent significant differences at $p = 0.05$.

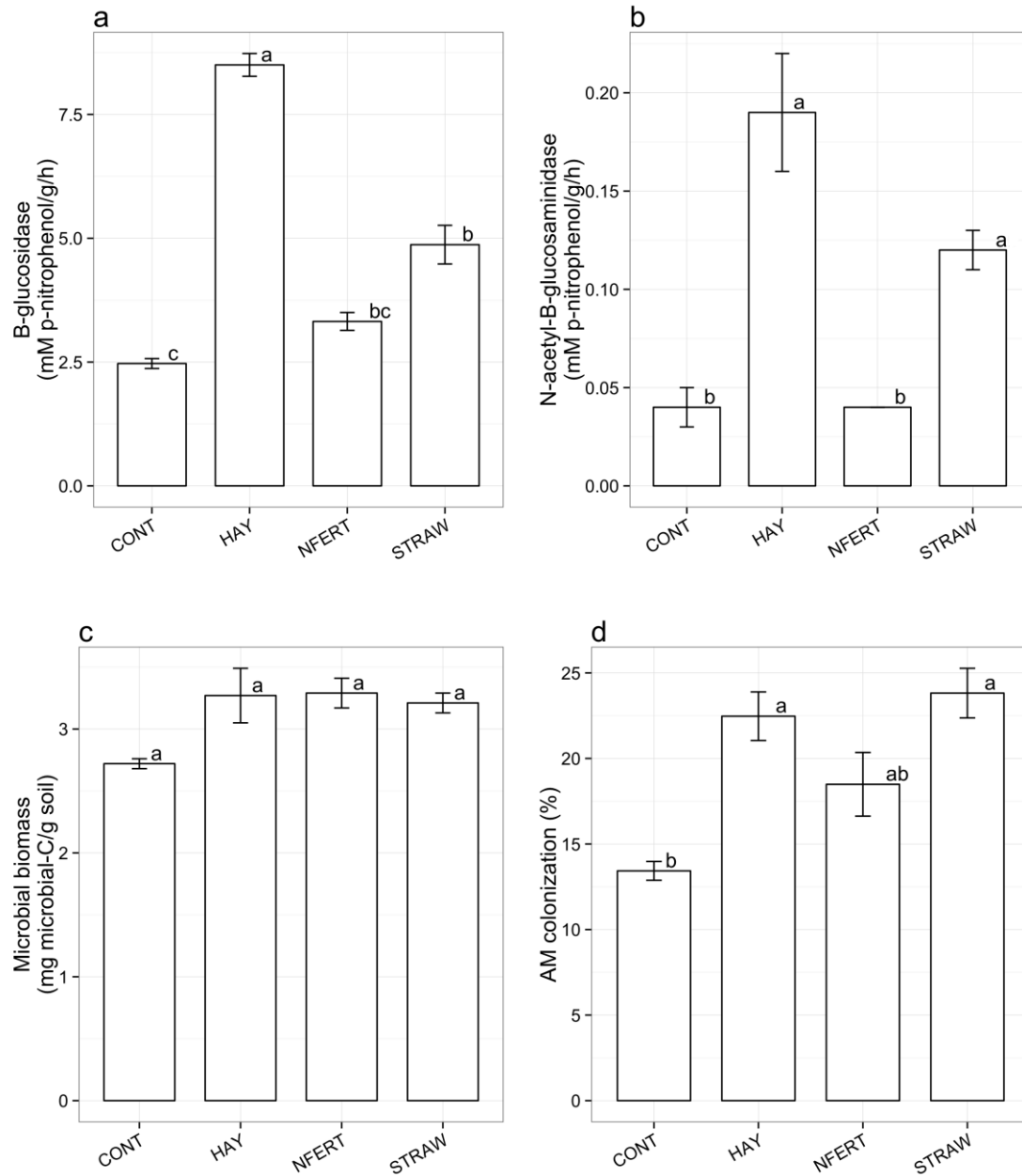


Figure 3. Soil biological metrics including B-glucosidase activity (a), N-acetyl-B-glucosaminidase activity (b), microbial biomass (c) and AMF colonization (d). Bars represent means with $\pm$ se and letters represent significant differences at $p = 0.05$.

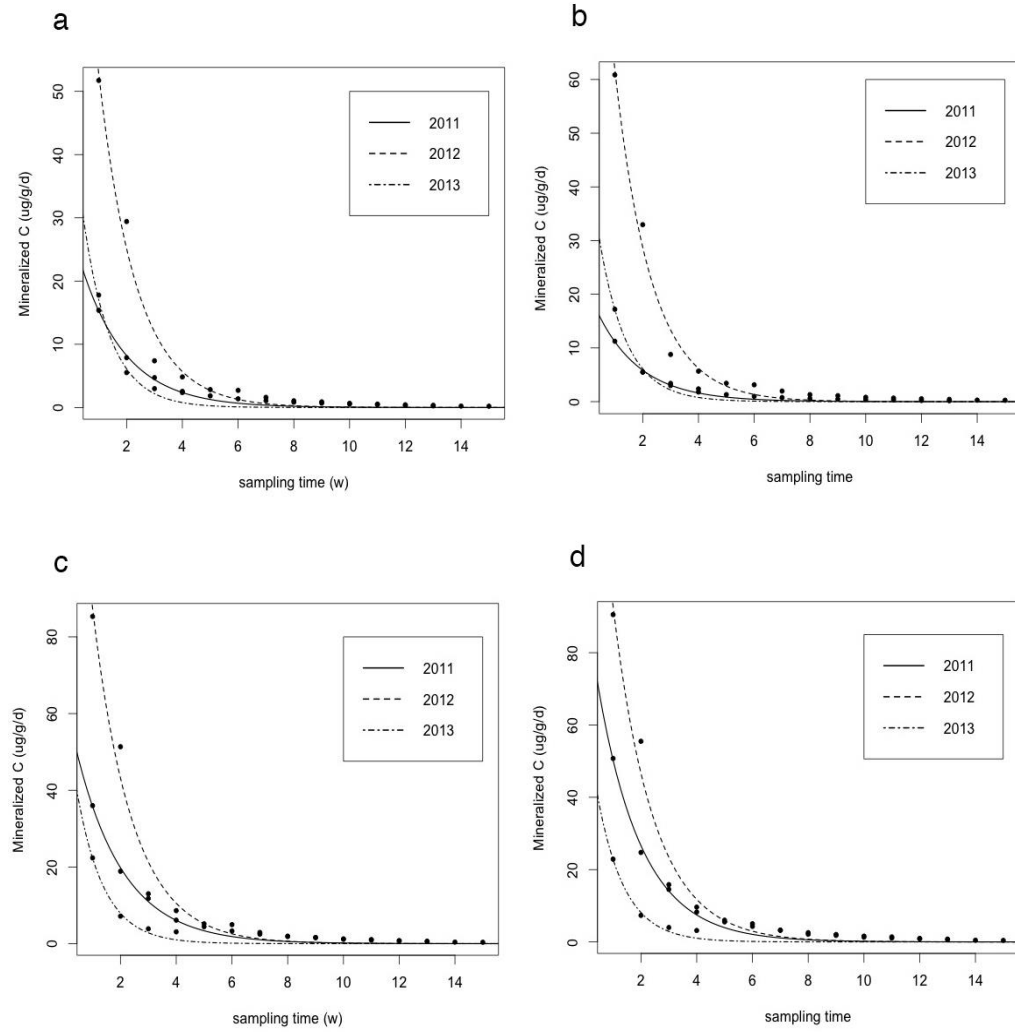


Figure 4. Model derived C decay rates for the no-treatment control (a), N-fertilizer treatment (b), hay mulch treatment (c) and straw mulch treatment (d) in all three years of the experiment.

Table 1. Correlation and (significance) between soil quality and biological metrics.

	Mineralized N	C Respiration	Microbial Biomass	AM Infectivity	β - glucosidase	N-acetyl- β - glucos- aminidase
fall-sampled	0.69					
NO ₃	(< 0.001)					
TOC	0.39 (0.03)		0.44 (0.07)	0.48 (0.004)		
DON	0.81 (< 0.001)	0.29 (0.1)				
WEOM C:N	-0.55 (0.001)					
SOM-C		0.31 (0.08)		0.32 (0.07)		
SOM-N		0.47 (0.007)				
total SOM	0.35 (0.05)	0.33 (0.07)				0.6 (0.02)

Table 2. Using a double-decay equation better described the stable C pool than a single decay curve. P-values are calculated from non-linear model fitting and represent the number of runs where using a single decay model (Ca) for the entire decay curve more accurately represented the data than if two decay models (Ca and Cs) were used. Using two decay models was superior in most cases and especially in the tail portion of the model (weeks 9 - 30).

	2011		2012	
	<u>week 2 - week 30</u>	<u>week 9 - week 30</u>	<u>week 2 - week 30</u>	<u>week 9 - week 30</u>
No treatment	p = 0.55	p = 0.27	p = 0.11	p = 0.003
N-fertilizer	p = 0.05	p < 0.001	p = 0.07	p = 0
Hay	p = 0.17	p = 0.08	p = 0.14	p = 0.03
Straw	p = 0.14	p = 0.06	p = 0.05	p = 0

Table 3 Estimates of C in the active and stable pool, calculated from a 32-week laboratory incubation. Incubation did not run long enough in 2013 to calculate C_s .

	2011		2012		2013
	C_a	C_s	C_a	C_s	C_a
No treatment	28.44 (4.89)	0.96 (0.09)	110.91 (13.54)	17.57 (1.45)	47.43 (10.82)
N-fertilizer	21.28 (2.35)	5.02 (0.46)	133.11 (12.34)	19.92 (1.07)	47.88 (5.33)
Hay	64.55 (14.19)	17.94 (2.59)	176.82 (29.70)	31.14 (3.12)	62.34 (7.63)
Straw	94.92 (20.71)	24.9 (4.04)	184.70 (35.92)	34.97 (5.03)	63.72 (14.2)

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CHAPTER SIX

CONCLUSION

Sustainable agriculture relies on site-specific adaptive management practices developed from place-based knowledge and a system-wide perspective, from microbial functions to the greater landscape (Francis et al. 2003). Management practices like the use of cover crops, IPM and maintaining SOM, either by OM inputs or tillage reduction, can increase agroecosystem species functional redundancy across scales (Shennan 2008, Altieri 1999, Matson et al. 1997). In sustainable agricultural systems it is especially important to understand how management practices affect microbial processes because below-ground processes supply plant available nutrients for crop production (Wardle 2002). The web-like connections below-ground complicate our comprehension of soil functions but are ultimately necessary to grasp if we are to fully understand the effects our management practices have on soil ecosystem services.

Designing Cropping Systems to Grow Nutritious Foods

My dissertation was in part inspired by complementary research performed on a long-term agricultural research site in Davis, CA. Tomatoes grown under organic management had higher phenolics than when grown with conventional management (Mitchell et al. 2007). Simultaneously, soil research from the same sites found greater aggregate stability (Six et al. 2004), and differences in the timing of nutrient availability without any sacrifice of yield (Doane et al. 2004). I became curious about what, from a

soil perspective, might contribute to the differences seen in vegetable antioxidant phytochemicals. A fundamental difference between organically managed soils and conventionally managed soils are the fertility input restrictions that organic farmers face - adding some form of organic material is a commonly used option to replace nutrients removed with the previous year's crop.

In our investigation into the relationship between SOM and vegetable phytochemical production, there was a correlation between soil N availability and spinach phenolic concentrations in one out of three years of the study and the results from regression analysis indicated that SOM had some influence on phenolic production, along with growing period temperature stress.

Agricultural Management Across Ecosystem Scales

With contemporary technologies that improve our ability to 'see' into the soil refining our ideas of microbial community structure (Kaiser et al. 2014, Lauber et al. 2013, Conant et al. 2011), the residence time of different C compounds (Schimel and Schaeffer 2012) and root-soil interactions (Ritz 2012, Fitter and Hodge 2011), we are better able to understand the biophysicochemical interactions occurring below-ground. Part of my dissertation research focused on microbial decomposition of SOM. Decomposition of labile substrates both fuels the below-ground foodweb and mineralizes nutrients necessary for optimal crop growth (Powlson et al. 2011, Wardle 2002). Understanding microbially mediated mechanisms that regulate input decomposition and

nutrient cycling is necessary to predict C emissions and nutrient availability (Bradford et al. 2013).

In this dissertation, soil fertility differences were measured among the four soil treatments. Hay mulch had a shorter residence time in an intensively tilled mixed-vegetable production system due to its lower C:N and faster decomposition and the higher C:N straw mulch contributed delayed but more persistent soil fertility effects. In 2013, using a wide complement of analyses to determine biological activity and functional response to the four soil treatments, we were able to measure patterns in biological activity that were not visible from using soil analyses alone. The practical applications of this work are underscored by the interest of Montana small vegetable farmers in understanding the temporal dynamics of mulch additions (personal communication).

We also investigated whether traditional spring soil N analysis is an adequate predictor of growing-season available N in agricultural systems using organic fertility inputs. Testing pre-seeding soil NO_3 can be a good predictor of available N, but other tests like WEOM, PMN and C-respiration give farmers a better idea of the biological functioning in their system. Understanding soil biological function is necessary for optimizing management practices in sustainable management. The nutrient ratio of inputs should be considered to accurately predict available N and to avoid overestimating available N in high C:N soils. The results from this paper are relevant across a wide group of stakeholders as many farmers are seeking convenient tools for assessing soil

biological function. State agencies, crop advisors, farmers and other researchers can all benefit from these results.

Soils are notoriously heterogenous leading to large variability in soil data. Most investigations of soil processes attempt to describe the factors that influence process rates within the system, but the number of covariants in soils can make it very challenging to determine the influence of the factor of interest (OM quality or quantity, moisture) amidst the influence of covarying factors (Colman and Schimel 2013). The indirect relationships between soil and biological metrics can be difficult to tease apart, especially with a small sample size. The covariance and non-linear relationships in soils are what lead to emergent patterns (Kaiser et al. 2014, Schmidt et al. 2011, Peters et al. 2007) and seemingly simple changes in management can lead to unanticipated results (Kibblewhite et al. 2008, Vandermeer 1997). In the future, designing experiments with the expectation of large variability will result in data sets large enough to use complex simulations and statistical tools like factor analysis, principle components analysis and structural equation modeling that can better reveal the interconnections behind soil processes (Colman and Schimel 2013; Figure 1).

Sustainable Agriculture in a Changing World

It would be challenge enough to manage agricultural land towards a sustainable endpoint in a static world. Managing towards sustainability in an uncertain world requires predictions to estimate how systems will respond to global change (Powlson et al. 2011). Further investigation on the management of SOM for crop nutrient availability could

reveal how SOM management affects the intermediate processes leading to C-respiration and N-mineralization and whether timing or intensity of management can manipulate the resulting nutrient availability. Measuring biological responses under different crops, presumably supporting different rhizosphere microbial communities, could begin to illuminate the effects that plants species have on soil biotic functions and the influence of crop rotations on below-ground resource allocation and microbial community dynamics.

More focused research will also increase our understanding of management effects on crop phytochemical production. Many uncontrolled variables exist in field experiments and many different environmental factors can influence the synthesis of vegetable phytochemicals (Agati et al. 2013, Lester et al. 2013). Another way to address this question would be look in a more focused way at the synthesis of phytochemicals under different conditions and in a wider variety of crops with a combination of field and greenhouse studies. Phytochemicals are dynamic within plants and a greater understanding of the factors that contribute to the biosynthesis of phytochemicals important in human health which can be manipulated through agricultural management practices will assist in developing management plans to produce more nutritious foods.

As an organic fertility input, mulches can be an effective tool to increase nutrient availability and soil biological activity through time. However, we reported crop yields and soil fertility for a single crop and in the absence of commonly used crop rotations. Future work could look at the best way for mulches to be incorporated into a full crop rotation and what the optimal rotation would be to take best advantage of the varying soil conditions as mulch additions decompose. Longer-term research could also investigate

any positive or negative accumulating effects of using mulch on soil community composition, above- and below-ground pest pressures, and weeds. Understanding the variation of biological responses throughout a growing season as well as from year to year would improve our understanding of soil biological processes and the functional responses to different management practices.

One of the biggest challenges in understanding soil processes and management effects on soils is the difficulty in visualizing what occurs under our feet. Contemporary visualization and animation technologies allow for a greater intersection of art and science to aid visual comprehension of multi-scale processes. Interdisciplinary collaborations between analytical soil scientists, soil ecologists, modelers, programmers and graphic designers could result in educational and predictive tools that increase the ability of farmers to make optimal management decisions on their site.

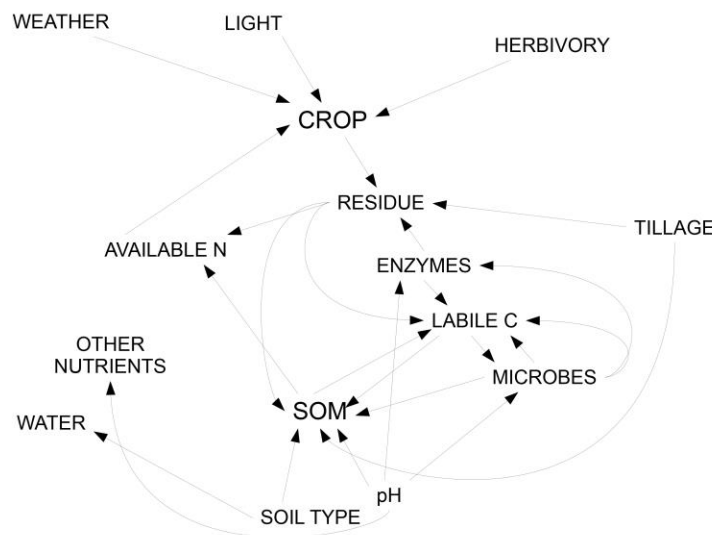


Figure 1. Diagram illustrating the complexity of above-belowground systems. Arrows indicate driving effects of one parameter on another. Many feedback loops exist in soil systems, with parameters affecting and being affected by other parameters.

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APPENDICES

APPENDIX A

DETAILED METHODS

Detailed Methods

Field Study

The Montana State University (MSU) Horticulture Farm is located 1.6 km from the MSU campus in Bozeman, MT (45.66, -111.07). Our research plots were under conventional cereal-fallow management for 20 years prior to mixed vegetable production. The soil is a Turner loam (NRCS 2013) with 400 g kg⁻¹ sand, 370 g kg⁻¹ silt, 230 g kg⁻¹ clay, 20.3 g kg⁻¹ organic carbon, a cation exchange capacity (CEC) of 22 cmol kg⁻¹ and a pH of 7.6. The legacy of this farm includes intensive tillage, and high phosphorus (P) and potassium (K).

We conducted a randomized strip-plot experiment of four replicates of four soil treatments across 16 plots in a 2000 m² field. Plot rows were 1 m wide. Plot lengths were 13 m. We grew four crops, spinach (*Spinacia oleracea* var. Space (F1)), tomato (*Solanum lycopersicum* var. Juliet), corn (*Zea mays* var. Vision) and broccoli (*Brassica oleracea* var. Arcadia) over three growing seasons, from 2011-2013. All seed came from Johnny's seed (Winslow, Maine). All planting rates followed vegetable production recommendations from Johnny's seed for each variety. Corn and spinach were direct seeded each spring using an Earthway seeder with the appropriate disk for each crop. Two rows per plot were planted for corn, broccoli and spinach. Tomatoes were planted in one row per plot.

Soil treatments consisted of two mulch additions, barley straw and sanfoin (legume)-grass hay, a N-fertilizer treatment and an untreated control. The hay mulch had a seeding ratio of approximately 60% grass seed to 40% sanfoin seed. Straw mulch was

applied at a rate of 5.5 kg m^{-2} and hay mulch at 9 kg m^{-2} , at the recommendation of local vegetable farmers. Mulch additions were sampled for C and N combustion analysis at the time of application. Four subsamples of each straw and hay were collected randomly across the mulched treatments, dried at 95°C overnight and ground with mortar and pestle for combustion analysis using TruSpec CN combustion analyzer (LECO Corporation, USA). The two mulches differed in C:N (t-test, $p = 0.048$), and averaged 26.5 for the hay mulch and 41.6 for the straw treatment. The N fertilizer was added in the form of urea (46-0-0). Existing $\text{NO}_3\text{-N}$ was determined by sampling the soil 10 d prior to planting each year. Urea was side-dressed following MontGuide (Dinkins et al. 2010) recommendations for Montana vegetable production (159 kg-N/ha), approximately 10 days after spinach emergence. Spinach plants had true leaves 3 - 6 cm long at the time of N fertilizer application. The untreated control had no fertility additions over the course of the experiment.

Plots were irrigated with T-tape drip irrigation (DripWorks, USA) for the first two years of the experiment and with overhead sprinkler the third year. Mulches were incorporated into the soil by rototilling. In subsequent years a spader was used to prepare plots for planting. Rows and plots were maintained from year to year to within 0.5 m of the original location. Plots were maintained manually each year with intensive weeding up to spinach harvest and weekly thereafter.

Plant Sampling

Spinach was harvested in July and August each year. Three spinach plants were randomly chosen from plots each year using a random number table and the plants

selected were all at the 9-12 leaf stage. Seedling spinach, very large plants and damaged plants were avoided. Three 1-g samples of leaf tissue were snipped from each selected plant and frozen in the field in liquid N. Frozen tissue was held at -80 °C until extraction, within 60 days of sampling. Aboveground biomass was then clipped at the soil surface, dried at 75 °C for 48 hours and weighed. Dried spinach tissue was ground with a mortar and pestle and 0.2 g were combustion analyzed for N content, using a TruSpec CN combustion analyzer (LECO Corporation, USA).

Gallatin Valley Farm Sampling in 2013

Spinach tissue and rhizosphere soils were sampled from five Gallatin Valley farms in June, July and October of 2013. All sampled farms use crop rotations, meaning the same family of crops were not planted in the same part of the field the previous or following years. Amaltheia Farm (AMA) has been producing vegetables since 2012. Between 2012 – 2009 it was in a mixed forage rotation and in alfalfa hay production prior to 2009. In 2012 and 2013 it transitioned to mixed-vegetable production. Fertility was managed in 2012 with a winter pea, vetch, winter wheat cover crop mixture following fall-applied pig compost at 45 t ha⁻¹. Root crops were grown in 2012 prior to spinach in 2013. AMA is on the windward side of the Bridger Mountain range, resulting in a high frequency of summer storms. Gallatin Valley Botanical (GVB) has been in production since 2008. Prior to vegetable production, the land was used for pasture and hay since the 1980's. Fertility is managed by manure application in alternate years, last applied in the spring of 2012. The preceding crop in 2012 was lettuce and salad greens. The location of GVB results in late, cool spring conditions. Gallatin Grown (GG) has been in vegetable

production since 2012. Prior to that the land was used for conventional potato production in a 7-year rotation with wheat and alfalfa. Fertility is managed with winter cattle grazing. GG is farthest west from Bozeman and has hotter summers compared with GVB. 3-Fiddles Farm (FID) has been producing vegetables in Bridger Canyon since 2008. Prior to vegetable production the land was used as grazing forage since the 1990's. Fertility is managed using a fall-seeded legume green manure. The preceding crop is unknown. FID has the highest elevation (1.6 km) and a very short growing season (approximately 60 days). Three Hearts Farm (HEA) has been in production since 2008. Prior to vegetable production it was perennial pasture since the 1970's. Fertility is managed with fall-applied manure and legume cover crop. The preceding crop was lettuce and salad greens. HEA is most similar to THG in location and environmental conditions. Three plants were sampled, following the same methodology used at THG, selecting plants from across the plot using a random number table. Spinach tissue and soils were handled in the same way as samples obtained from THG for all analyses.

Phytochemical Analysis

Samples were extracted following Prior et al. (2008). Briefly, spinach tissue was blended with acidified acetonitrile (5:1 v:w, pH 3.5) for 30 seconds using a Polytron Brinkmann homogenizer (Switzerland) on setting 3. Samples were handled to prevent thawing prior to homogenization. Homogenized samples were incubated at 20 °C for 30 minutes, shaken end-over-end every three minutes. Samples were then centrifuged at 13,000g and -4 °C for 15 minutes. An aliquot of the supernatant was reserved and stored at -20 °C until antioxidant analysis. The storage length varied each year as analysis timing

was dependent on the schedule in the BioTek plate reader's home lab. Samples from the same year were analyzed within a week of each other.

Total phenolic concentration was quantified using the Folin-Ciocalteu colorimetric assay, using gallic acid as a standard, following Singleton et al. (1999). Reaction mixtures contained 0.2 ml of either a gallic acid standard or sample. Samples were diluted 3:1 with de-ionized water (v sample:v de-ionized water), 1 mL Folin-Ciocalteu Reagent, and 0.8 mL of Na₂CO₃. The sample blank used 0.2 ml acetonitrile solution instead of sample. Mixtures were incubated at 20 °C for 2 hours and the absorbance at 765 nm was determined using a Hitachi U-2000 UV/Vis Spectrophotometer (Hitachi High-Technologies Corporation, Japan; Figure 1).

ORAC concentration was quantified following Held (2006) and Gillespie et al. (2007), using Trolox (R) as a standard (Figure 2). Interior wells of a black-sided 96-well plate contained 0.15 mL diluted sodium fluorescein solution, and 0.025 ml of phosphate buffer (blank wells), Trolox standard or sample. Samples were diluted 1:100 with phosphate buffer (v sample:v buffer). External wells held 0.3 mL de-ionized water. Plates were incubated at 37 °C for 30 minutes. After incubation, 0.025 mL of AAPH (reactive oxygen source) was added. Plates were read kinetically every minute from the bottom for one hour, using a KC4 fluorescence plate reader (BioTek, USA) with excitation/emission wavelengths of 485/20 and 528/20, respectively.

Soil Analysis

Soils were collected each spring nutrient analysis prior to planting spinach in the field. Ten 1-cm dia x 15-cm deep cores were collected and composited from each plot.

Analysis included $\text{NO}_3\text{-N}$, total-N, OM (loss on ignition (LOI)), Olsen-P, K and CEC (Agvise Laboratory, Northwood, ND).

At spinach harvest, soils were sampled from plant root zones. 1000 g of soil were sampled from the root zones of the same three spinach plants selected for tissue analysis in order to measure the soil conditions experienced by each sampled plant. After tissue sampling, a soil core (diameter equivalent to the diameter of the outer rosette leaves and 40 cm deep) containing the root mass of the spinach plant was extracted from the soil. Soil adjacent to the roots were collected from this core and all visible roots were removed. Soils were air dried overnight at 20 °C, sieved to 2mm and held at 4 °C until analyzed. Soils were analyzed for OM (LOI), Olsen-P, K and CEC (Agvise Laboratory) and were sent immediately post-sieving, within 20 days of soil sampling.

Post-harvest soils were also used for WEOM analysis and SOM C and N composition. Both analyses were completed immediately post-sieving, within 20 days of sampling. Water extractable organic matter analysis was performed following Toosi et al. (2012) using 0.001M CaCl_2 as the extractant (1:2 w:v) and extracts were analyzed on a TOC-V analyzer (Shimadzu Corporation, Japan). Soils were dried at 95 °C overnight and milled using a ball mill for 15 h. 0.2g samples were analyzed for organic C and N using a TruSpec CN combustion analyzer (LECO Corporation, St. Joseph, MI).

C-respiration and N Mineralization Incubation

For the long-term laboratory incubation to measure C-respiration and N mineralization, two replicates of six 5 g samples of each soil were prepared at uniform moisture and density in autoclaved scintillation vials. Each set of six vials were placed in

an autoclaved 0.95 L Mason jar, fitted with a septa. 10 mL of sterilized water was added to the Mason jars to maintain humidity over the incubation. Jars were stored at 25 °C in the dark for the entire 32 week incubation period. Each week, aliquots of headspace gas were drawn of using a 10 mL syringe and transferred to vacuumed exetainers (Labco, UK). Exetainers were then autosampled on a Varian CP-3800 Gas Chromatograph (Agilent Technologies, USA). Scintillation vials were removed at week 1, 2, 4, 8, 16, and 32 for N extraction. In the first year of the experiment both NO_3^- and NH_4^+ were analyzed, but NH_4^+ levels were very low to non-existent. For the remaining years we measured only NO_3^- . Soils were carefully washed out of the scintillation vials into 50 mL Falcon tubes using 25 mL 1M KCl. Tubes were shaken for 30 min at 180 oscillations min^{-1} . Extractions were refrigerated overnight to allow soil to settle out of solution. Samples were filtered using Whatman filter 45 and frozen until analysis on a Lachat QuickChem FIA analyzer (Hach Instruments, CO). Lab replicates were averaged prior to statistical analysis to avoid pseudoreplication.

Microbial Biomass

Two replicates of 10 g of soil for each sample were mixed with 0.5 g glucose:talc mixture (3:7 w:w) and added to an autoclaved scintillation vial. Vials for all samples were brought to uniform moisture using sterilized water and placed in an autoclaved 0.95 L Mason jar. Jars rested, uncapped, at 20 °C for two hours, then were sealed and incubated at 20 °C for two additional hours. Head space samples were drawn using a 10 mL syringe and analyzed using a Varian CP-3800 Gas Chromatograph (Agilent Technologies, USA).

Arbuscular Mycorrhizal Infectivity

Intact soil cores were randomly sampled from the rhizosphere of corn roots. As a monocot, corn is a good host for AMF. Soil cores were approximately 12 cm deep by 8 cm dia. and were set into sterilized 10 cm deep by 10 cm dia. pots in the field. Pots were seeded with 10-15 Sudan grass seeds (*Sorghum bicolor*) and maintained in the greenhouse for 6 w. At harvest, aboveground tissue was removed and roots were washed to remove organic matter and mineral soils. Roots were cleared using 2.5% KOH and stained using 0.05% Trypan Blue. Stained roots were then used to make two slides for each sample with 12 root segments per slide. Slides were read using microscopy with eight transects across each slide and infectivity potential calculated as percent colonization of transects.

β -glucosidase and N-acetyl- β -glucosaminidase Activity

To measure β -glucosidase activity, 0.2 mL of toluene was vortexed with 1 g soil and allowed to sit, uncapped, for 15 minutes. Then 4 mL of modified universal buffer (MUB) pH 6.0 and 1 mL p-nitrophenyl- β -D-glucopyranoside substrate (PNG) were added and vortexed and samples incubated for one hour at 37 °C. Upon removal from incubation, 1 mL 0.5M CaCl₂ and 4 mL 0.1M Tris(hydroxymethyl)aminomethane (THAM) buffer, pH 12 were added to stop the reaction. Samples were vortexed and filtered through Whatman 2 filters. Color was measured at 405 nm on U-2000 UV/Vis Spectrophotometer (Hitachi High-Technologies Corporation, Japan) using p-nitrophenol as standard (Figure 3). The same method was followed for N-acetyl- β -glucosaminidase

except p-nitrophenyl-N-acetyl- β -D-glucosaminide substrate was used instead of PNG and MUB was replaced with acetate buffer, pH 5.5.

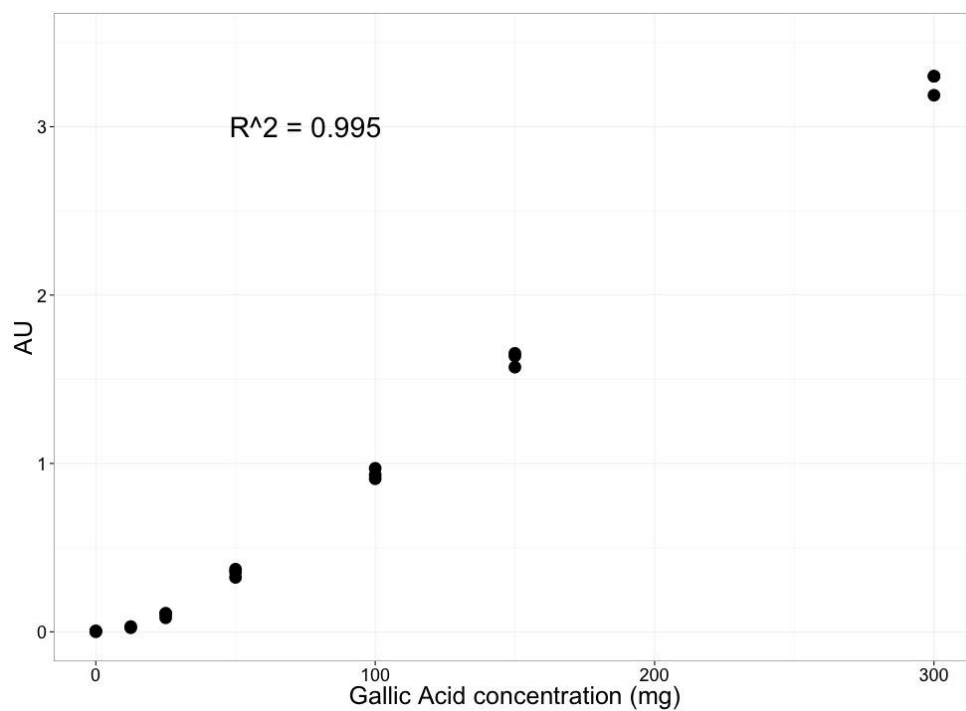


Figure 1. Standard curve used to calculate total phenolic concentration

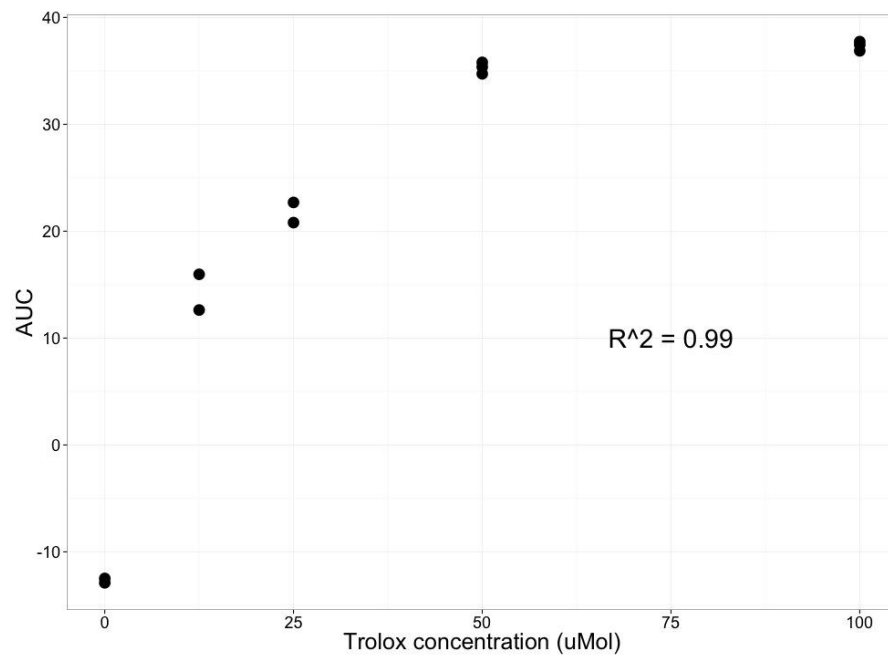


Figure 2. Standard curve used to calculate oxygen radical absorbance capacity values.

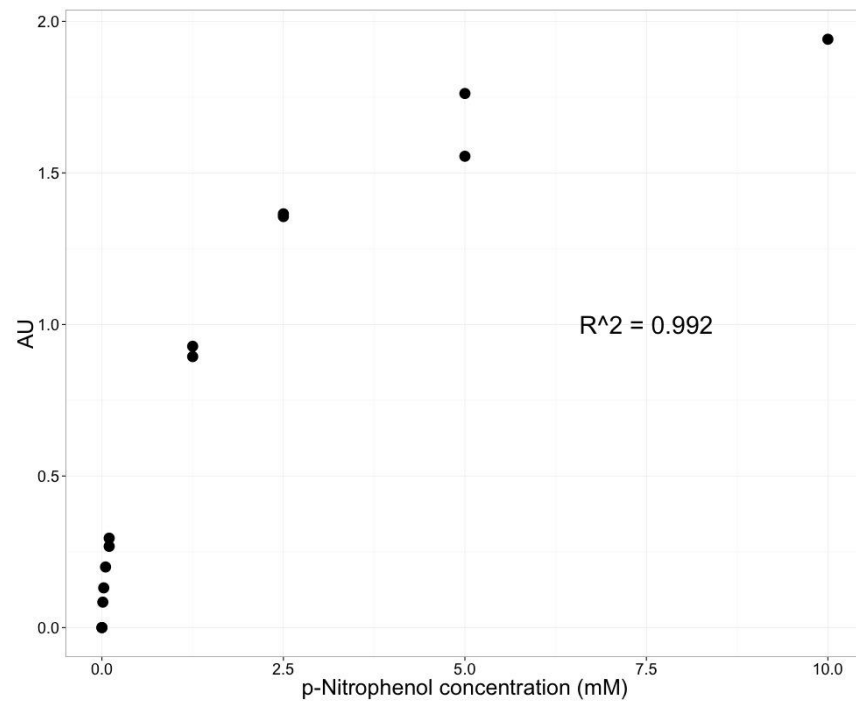


Figure 3. Standard curve used to calculate β -glucosidase and N-acetyl- β -glucosaminidase activity

APPENDIX B

DATA AND META-DATA

Data and Meta-Data

Seedbank Location: THG

Field Conditions

Meta-Data: Planting rates, seed varieties and source, plot management

Data: Mulch

Spring nutrients; all plots

Fall nutrients; all plots

Soil C and N

Yield: spinach and broccoli

Soil Incubation

Meta-Data: Methods, set-up

Data: Temperatures

PMN

PMC

Soil Analyses

Meta-Data: Methods, set-up, recommendations

Data: WEOM

Enzymes

AMF

Microbial Biomass

Tissue Analyses

Meta-Data: Methods, chemical sources, recommendations

Data: ORAC

F-C

Spinach C and N

Sampled plant biomass and average plot biomass