

THE EFFECT OF FREEZE-THAW CYCLES ON MICROBIAL RESILIENCE ALONG A
CROP BIODIVERSITY GRADIENT

By

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ABSTRACT

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Freeze-thaw cycles (FTCs) are cyclical periods of soil disturbance that are increasing in number and intensity due to climate change effects on winter precipitation and temperature patterns and are not well characterized within an agroecosystem environment. First, I review the literature and discuss the effects of FTCs on soil properties, explore the nuances of characterizing FTCs in experiments, and assess the knowledge gaps of FTC studies in agroecosystems. I conducted a laboratory experiment using soils from a crop rotational diversity gradient and froze them at three distinct FTC frequencies. My results indicate that increased crop rotational diversity did not moderate FTC disturbance effects at any frequency level. Increased FTC frequencies generally increased soil organic C losses as CO₂, decreased ammonium (NH₄⁺), increased nitrate (NO₃⁻) pools, and increased extracellular enzyme activities (EEA). The respiratory burst after each freezing period was the predominant contributor to differences by FTC in cumulative CO₂ respiration by the end of the incubation. Interestingly, the medium FTC frequencies facilitated the highest EEA for select enzymes with minimal reductions in microbial biomass. This suggests that microbes and their EEA are impacted too severely with high frequency FTCs to maintain function. My study revealed that the novel microbial communities and soil processes found along a crop rotational diversity gradient are not resilient against climate change effects of FTCs in soils. Accelerated soil organic C loss and nutrient turnover are expected to occur throughout agroecosystems that experience increased FTCs.

To my family and friends who supported me when I was at my lowest.

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INTRODUCTION

The Soil System: Soil Organic Carbon Stocks

The soil system plays a role in ecological, biological, and cultural aspects of the world, from a living medium for agriculture, to governing nutrient and carbon (C) cycling across the globe between the atmosphere and land surfaces. Of the over 3000 Pg of total C in terrestrial ecosystems, almost 80% of it is stored in soil as living organisms, freely in the soil space, or locked up in C-associated minerals (Lal, 2004). C movement from terrestrial systems into the atmosphere is around 130.7 Pg C per year, where just about half of that comes from soil respiration alone, with a net gain of 4.7 Pg C per year (Lal, 2018). As a result of the slowly rising net flux of C into the atmosphere, any subtle changes in the seasonal flux of C will have cascading impacts on soil organic C (SOC) depletion, and ultimately soil functionality (Classen et al., 2015). SOC stocks are the focus of countless studies, reviews, and models linked to soil parameters such as moisture and soil texture to better predict stock trends and C sequestration, the process of capturing atmospheric C and storing it in terrestrial forms (Olson et al., 2014; Wieder et al., 2018). The status of soils as a sink for CO₂ is a complicated, ongoing discussion due to uncertainties of soil sensitivity to climatic factors that moderate C fluxes (Todd-Brown et al., 2014). Accelerated SOC loss at a global scale is predicted to be 50 Pg C from the upper horizon of soils by 2050 (Crowther et al., 2016). Terrestrial ecosystems are at great risk of elevated SOC loss due to accelerated decomposition of SOC stocks, led on by heterotrophic respiration that is stimulated by various global change factors, such as climate change (Dignac et al., 2017).

Climate Change and FTCs

Climate change is characterized as the global increase of climate temperatures that exacerbates a slew of environmental concerns. Greenhouse gases (GHG) from soils, such as carbon dioxide (CO₂) and methane (CH₄) are of major concern because large amounts of SOC is stored in permafrost soil layers, any additional permafrost melt produce a net input of CO₂ into the atmosphere. As a result, higher GHG fluxes will occur between the atmosphere and soils as warming continues, creating a climate-warming positive feedback (Koven et al., 2011). Less SOC will be available to drive multiple soil functions, microbial activity may be stunted, and soil fertility will suffer (Andresen et al. 2012; Boswell et al., 2020). Of recent concern are regions of seasonally frozen ground that encompasses over 50% of total land in the Northern Hemisphere, where freeze-thaw cycles (FTCs) are commonplace (Zhang, 2003). Because FTCs are cyclical periods of soil disturbance, characterized by a period of freezing and a period of thawing, they are responsible for large, seasonal bursts of GHGs during winter seasons and early spring thaw (Skogland et al., 1988; Sharma et al., 2006; Kurganova et al., 2007; Williams et al., 2015). Because of this, FTCs are often responsible for SOC flux and fundamental to measuring biological activity and SOC mineralization during winter periods (Nikrad et al., 2016). FTC activity and potential impacts on soils are heavily governed by snow cover that when present can insulate and protect the soil from both frigid air temperatures and warming effects. Reductions in snowfall duration and snow depth and increases of winter rain shorten the duration and depth of snow cover (Feng and Hu, 2007), which leads to an increase in the number and severity of FTCs in some regions (Wipf et al., 2015; Gavazov et al., 2017; Wu et al., 2018). Lack of snow cover and inadequate insulation generates erratic fluctuations of soil and air temperatures throughout the winter seasons, leading to greater temperature variability and susceptibility to FTCs as

temperatures cross the freezing threshold of 0 °C (Sinha and Cherkauer, 2010). Regional FTC patterns vary based on inherent snow cover, precipitation, and soil temperature conditions (Feng and Hu, 2007; Henry, 2008). Increasing FTCs are an important component of climate change as they disrupt SOC pools by accelerating SOC loss through respiratory bursts and nutrient cycling driven by microbial activity, contributing to a positive feedback loop of warmer temperatures and greater amounts of atmospheric GHGs (Andresen et al. 2012; Boswell et al., 2020).

FTCs and Respiration and Nutrient Dynamics

FTCs can be large, but often over-looked contributors to annual GHG emissions from soils. Measured fluxes of CO₂ from soils after freezing and during thawing are predominantly from surviving microbes utilizing the newly free C sources from both dead cells (necromass) and decomposing soil organic matter (Buckeridge et al., 2020). Burst respiration may decrease over time as the microbial population declines from more frequent FTCs that kill off more of the surviving microbes faster than they can recover (Han et al., 2018). The bursts may be large relative to baseline respiration rates if FTC amplitudes are large, i.e. lower freezing and higher thawing temperatures (Stres et al., 2010), and if they occur in historically undisturbed soils (Makarov et al., 2015), or in heavily managed soils (Ouyang et al., 2015).

The magnitude of FTC-related SOC loss is heavily dependent on ecosystem type and land use (Shi et al., 2014). Studies located in grassland and forest soils report higher FTC related respiration rates due to higher organic matter content than croplands (Han et al., 2018), but SOC in agricultural soils report higher respiration sensitivity to FTCs, often resulting in reductions in respiration rates over time (Table 1). Higher sensitivity to temperature changes and large releases

of C are reported in alpine and subarctic regions, where permafrost melt is of utmost concern (Davidson and Janssens, 2006; Knoblauch et al., 2013). Further confounding varying results across different ecosystems, inconsistencies in measured respiration rates exist between laboratory versus field experiments. In a meta-analysis conducted by Song et al. (2017) that recorded C and N response to FTCs, respiration responses in laboratory studies positively correlated with higher freezing temperatures and multiple FTCs; in that same study, in-situ (field) respiration showed negative responses related to multiple FTCs. In addition, some of the variation in responses is likely due to differences in labile SOC pools dissolved organic C (DOC) across ecosystems. DOC is the C pool that is directly mineralizable and accessible for microbes, and usually increases in concentration with FTCs. Some laboratory studies report reductions in DOC, likely due to the limited pool size of easily accessible labile materials as incubations progress assuming no added C sources (Henry, 2007). In-situ studies are more difficult to establish DOC relationships, where the first FTC appears the most influential to the soil system (Hentschel et al., 2008). In some systems, most of the labile C that is initially accessible may be used up while in other systems it may persist across several freeze thaw cycles (Herrmann and Witter, 2002).

During FTCs, changes in SOC dynamics are accompanied by altered nutrient cycling. During a FTC, nutrients are liberated from fractured aggregates (Kim et al., 2017), dead microbial cells and plant roots (Larsen et al., 2002; Kilpeläinen et al., 2016) and enter the soil space mostly as dissolved organic compounds. Elevated levels of N mineralization with FTCs are variable, but generally are also increased with greater intensity FTCs, similar to C mineralization trends due to microbial N release (Schimel and Clein, 1996; Hosokawa et al.,

2017; Song et al., 2017). Specific inorganic N forms, namely ammonium (NH_4^+), are noted to spike during the first FTC events, along with increased dissolved organic N (DON), to be used for plant uptake and microbial growth (Freppaz et al., 2007). While nitrification and nitrate (NO_3^-) may also increase with continual FTC disturbances like NH_4^+ , it may be underestimated in field studies due to the simultaneous nitrate leaching that occurs with snowmelt (Urakawa et al., 2014) and the limited uptake due to root mortality (Groffman et al., 2001; Tierney et al., 2001; Hosokawa et al., 2017). Precipitation events also contribute to leaching of dissolved organic materials, resulting in nutrient losses from plant soil systems in recently thawed areas (Wipf et al., 2015). Thus, tracking nutrient flow in response to FTCs is heavily reliant on the timeframe of measurement and the study type, which makes generalization of nutrient cycling dynamics with FTCs impossible with the current state of knowledge.

FTC Impacts on Soil Structure and Moisture

FTCs alter the size and distribution of soil particles (Xie et al., 2015; Lu et al., 2018), and affect soil aggregate size (Zhang et al., 2016). Aggregation occurs when intermolecular forces between mineral surfaces and clay particle surfaces are enhanced, facilitating adsorbance onto other particles to form larger aggregates (Zhang et al., 2016). During the freezing phase, moisture in soil aggregates form crystals that expand and fracture aggregates, reducing overall soil aggregate stability and liberating organic materials for microbial decomposition or nutrients for plant uptake (Lehrsch et al., 1991; Denef et al., 2001). Higher moisture content soils enable greater freezing impacts by generating larger crystals to break particle bonds, while soils with reduced moisture will form crystals small enough to occupy the pore spaces (Hohmann, 1995; Koponen and Martikainen, 2004; Zhang et al., 2016). Soils with high clay content are more

susceptible to aggregate breakdown due to the finer clay particles adsorbing structural water that silt and sand particles do not (Lehrsch et al., 1991; Zhang et al., 2016). Overall, FTCs are destructive soil disturbances that dynamically shape soil structure, releasing large portions of labile C and material from not only aggregates, but also microbes.

FTC Disturbance Impacts on the Soil Microbial Community

FTCs eliminate members of the microbial community by crystallizing water in microbial cells, causing them to expand and lyse, and spilling their contents into the soil space. The resulting material contributes to the active pool of SOC, which is then assimilated by the surviving microbes (Skoghland et al. 1988; Aanderud et al. 2013). Mortality rates after an initial freeze of the season may reach around 60% under severe (less than -20 °C) temperatures (Trofymow et al., 1983). However, entire microbial consortiums are rarely eliminated under freezing conditions, and in some cases between 60 to 90% of total biomass may still survive through physiological alterations or generation of antifreeze proteins (Kawahara et al., 2001; Walker et al., 2006). Microbial communities are understood to be both resistant (able to withstand disturbance) and resilient (able to recover from disturbance) due to inherently high biomass and prevalence of community members (Killham and Prosser, 2015) to perform functional traits, even with missing members (i.e. functional redundancy) (Allison and Martiny, 2008). A combination of destructive pressures of FTCs (Williams et al., 2015; Garcia et al., 2020) and competition for limited sources of accessible, labile C, contribute to the formation of microbial communities more capable of rapidly utilizing nutrients (Stres et al., 2010; Jansson and Hofmockel, 2020). The altered or adapted microbial communities may be more capable of maintaining their activity levels of SOC mineralization, and therefore CO₂ respiration against the

same disturbance, but whether that activity *returns* back to normal levels may depend on the new, stable community structure and how well it can retrieve the nutrients needed for growth and development.

The plasticity of microbial communities during dynamic FTC periods can be tracked by examining nutrient content of the microbial biomass. For example, an increase in microbial biomass C:N ratio is a trend observed in studies with single FTCs (Song et al., 2017) and has been linked to shifts in microbial community structure. Particularly in alpine conditions, Perez-mon et al. (2020) showed a large abundance of fungal OTUs representing cold-adapted genera compared to bacterial OTUs that consistently changed in response to frequent FTCs. This suggests fungal communities are more resilient to FTC effects than bacterial communities (Han et al., 2018; Perez-mon et al., 2020). Fungal diversity and biomass are expected to increase in arctic systems with gradual warming, leading to a fungal dominant community that has a larger affinity for C storage (Deslippe et al., 2011; Birgander et al., 2017). Between fungi and bacteria, fungi remain physiologically active in colder temperatures and may be less susceptible to community composition changes to FTCs (Haei et al., 2011; Kreyling et al., 2012). However, some laboratory studies that measured PLFA composition suggest strong variations in community composition among similar latitudes, suggesting climatic factors as selective drivers (Kumar et al., 2013). Our current understanding of microbial communities under-going multiple FTCs suggests that an emergence of a resilient microbial community is traceable through changes in C storage and nutrient flow, which may be adversely influenced if the microbial community is unable to recover from the disturbances.

Despite the observed FTC-induced changes in microbial community structure, these changes may not be long-lasting. In fact, the microbial composition may actually “reset” back to a previous composition once spring thaw occurs (Aanderud et al., 2013), likely either because the disturbance was not intense enough, or that compositional changes were undetectable through the measured microbial functions (Koponen et al., 2006). Microbial communities affected from previous environmental changes may acclimate more quickly, while communities in constant environments may be more sensitive, and thus respond dynamically to disturbances (Hawkes and Keitt, 2015). In essence, microbes are responsible for the ecological properties in nutrient cycling, and dynamically respond to disturbances. As mentioned previously, microbial resistance may be considered as the ability to resist changes onset by disturbance, while microbial resilience may be considered as the ability to recover and return to original levels. Knowing that climate change is adversely affecting our soil system, it is now more important than ever to understand the finer scale, dynamic microbial response to disturbance and tie it to larger, ecosystem response (Jansson and Hofmockel, 2020). The critical aspect of this thesis is to explore the mechanisms that moderate microbial resilience to soil disturbance and improve overall ecosystem resiliency against climate change.

A Need for Resilient Agroecosystems

As our climate changes and ecosystems experience warming and altered precipitation patterns, much focus is now on ecosystem resiliency. In particular, we need to design resilient cropping systems to meet the world’s food demands without compromising soil health. Under a wide range of perturbations, resilient agroecosystems are expected to provide multiple ecosystem services such as soil C and water storage and nutrient and water provisioning. Specifically, soil C

stocks and nutrient pools should remain unchanged or quickly return to original levels and provide nutrients, especially N, to support crop growth. In natural systems, we know that increasing plant community diversity is linked to system resiliency in terms of net primary productivity (NPP), and that high diversity facilitates greater stability against climatic extremes through compensatory growth in later seasons (Agg et al., 2017) and through landscape, community, and species-specific mechanisms that promote functionally diverse belowground communities (Allan et al., 2011; Oliver et al., 2015; Oehri et al., 2017). We also know that plant diversity is linked to microbial resilience and that increased microbial diversity generally leads to greater resiliency to soil disturbances through increased soil C sequestration (Jansson and Hofmockel, 2020). Species richness strongly influences belowground processes by increasing soil microbial activity, which in turn increases SOC storage capacity (Chen et al., 2018). Indeed, this effect is observed in grasslands, where plant diversity moderated warming effects and conferred positive benefits to belowground processes in the form of increased root biomass (Cowles, et al., 2016), and enhanced microbial biomass and activities (Milcu et al., 2010; Porazinska et al., 2018). However, the question remains if these diversity-resiliency relationships occur in agroecosystems.

Crop Diversity in Agroecosystems

In agroecosystems, crop rotations introduce temporal crop diversity, with different plant species grown in the same place spread over time. Landowners in the Midwest and Northeast regions of the United States utilize cover crops, such as conventional winter rye, red clover, legume, and alfalfa to address specific farmer land management goals. These goals include improved crop yields (Bowles et al., 2020), soil health (Bezdicsek and Granatstein, 1988; Snapp

et al., 2005), soil moisture retention (Marini et al., 2020), and root associations with bacteria and fungi (Yang et al., 2020). Diverse residue decomposition stimulates microbial activity to yield greater SOM content through enhanced microbial necromass production and nutrient cycling (Kumar and Goh, 2000; Smith et al., 2007; McDaniel et al., 2014b; Tiemann et al., 2015; Wang et al., 2017). Differences in microbial community structure and function may be significant enough that more diverse crop rotations have greater metabolic soil diversity and activity when compared to monoculture counterparts (Smith et al. 2016; D'Acunto et al., 2018). The question that remains, is whether these differences persist through dynamic soil disturbances like FTCs, and how we are to define what ecosystem resilience looks like and what factors contribute to it.

Agroecosystem Resiliency Against FTCs

Understanding the benefits of crop diversity are essential for assessing ecosystem resilience. The challenge is to tie the contributions of crop diversity effects to resilience in the context of specific resilience factors. Peterson et al. (2018) explores a four-parameter framework to assessing agroecosystem resilience: productivity (increased production with minimal negative impacts), stability (reduced variability), ecological resilience (resistance to growth decline), and engineering resilience (recovery to baseline performance). Discussions regarding agroecosystem resilience have since moved from comparing contributions against monoculture and conventionally managed systems and to more critical analyses in ecosystem service provisioning, particularly microbial community resilience in the face of climate change (de Vries et al., 2018; Peterson et al., 2018; Jansson and Hofmockel, 2020).

Understanding resilience specifically to FTCs is crucial, not only because FTCs are soil disturbances that may influence seasonal SOC dynamics (Matzner and Borken, 2008), but are commonplace in agricultural systems. Agricultural regions are one of the largest contributors to GHGs, particularly in the Midwest, where FTCs are a growing concern (Johnson et al., 2010; Levine et al., 2011; Ruan and Robertson, 2018; Wagner-Riddle et al., 2017). FTCs may increase aboveground crop productivity, but at the expense of root length (Kreyling et al., 2008) restricting nutrient uptake for future seasons. FTCs may also cause root injuries and encourage fungal activity (Kreyling et al., 2012). These shifts towards fungal communities may reduce overall microbial diversity and remove key members responsible for regulating GHG emissions (Levine et al., 2011), and exacerbate SOC loss. To date, most research has focused on SOC losses related to FTCs within forest, subarctic or arctic ecosystems where climate change effects and SOC dynamics are expected to be the most impactful, leaving FTC studies in croplands somewhat limited in comparison (Todd-Brown et al., 2014; Crowther et al., 2016). Many influential FTC studies have assessed microbial response in forests (Urakawa et al., 2014; Watanabe et al., 2019), in addition to arctic regions (Buckeridge et al., 2010; Walz et al., 2017; Lim et al., 2020), and conclude that there are strong links between reduced snowpacks and increased FTCs and increased GHG emissions and N transformations. The belowground impacts of FTCs may undergo a time lag on the scale of days, to even several years before measurable effects can be detected (Henry, 2007; Kreyling et al., 2010). But again, FTC effects are not well-characterized in agricultural systems.

Freeze Thaw Cycles Overview: How Experiments are Done

As mentioned briefly above, some of the variability in observed effects of increasing FTCs may be due to differences between lab versus field studies and even differences in experimental design. There is a great variety in FTC study design, with some studies conducted as in-situ/field experiments or some as lab incubations. A field study may be further classified as a snow removal experiment, artificially removing snow cover buffer to increase soil exposure, creating temperature and precipitation changes (Henry, 2007, 2008; Blankinship and Hart, 2012; Yanai et al., 2014). Other equipment such as warming coils may also be installed underneath soil beds to artificially induce different thawing temperatures and emulate spring thaw (Sanders-DeMott et al., 2018). The greatest benefit of field studies is the likelihood of results to be representative of local conditions, generating relevant, comparable results (Morrow et al., 2016). On the other hand, laboratory incubations provide greater temperature control and moderation of freezing temperatures and are able to address additional temporal parameters of FTCs, namely duration of time frozen or time thawed (Henry, 2007). However, because there are no set controls or temperature regimes for lab incubation studies, researchers are free to customize freezing temperature, freeze duration, thaw temperature and thaw duration parameters to either emulate natural conditions, or purposefully test for a specific parameter more closely.

FTC effects are characterized as the combination of factors that dictate the level of disturbance. These factors include: the amplitude of temperature extremes (freezing and thawing temperature range), frequency of FTCs (the number of FTCs over a set period), and duration (how long the soils stay frozen) of both the freezing and thawing periods (Henry, 2007; Boswell et al., 2020). Measuring the impact of a FTC involves recording the cumulative effects of

multiple FTCs on multiple parameters, such as microbial biomass, soil respiration rates, or net N mineralization, and these must be measured for comparison between early and late FTCs or comparison against unfrozen or control treatments (Larsen et al., 2002; Sorensen et al., 2016; Song et al., 2017). Generally speaking, the more “intensive” the FTC is (i.e. high frequency, large temperature amplitudes, longer durations), the more extensive the disturbance effects are on the soil’s physical structure and more damaging to the microbial community structure and functions (Freppaz et al., 2007; Joseph and Henry, 2008; Yergeau and Kowalchuk, 2008; Han et al., 2018; Sorensen et al., 2018). Selection of parameters for a FTC study may impact how its results are comparable to other similarly designed studies, but ultimately depend on the researcher to make the decision between realistic and optimal conditions for assessing FTC effects.

FTC studies are difficult to compare because of all the variability in experimental design as demonstrated in Table 1. Close examination of methods from 29 different FTC studies and details of FTC design reveal a range of freezing temperatures from -80 to -1 °C, a freezing duration range of 1 h to 145 d, and a range of the total number of FTCs from 1-82 cycles (Table 1). Out of the 29 studies, only six were conducted in cropland soils. Most freezing temperatures in most study types (laboratory and field) were low to moderate temperatures (between 0 and -10 °C). 75% of the recorded FTC studies were conducted as laboratory incubations, where most of them also established soil freezing temperatures considered to be extreme for those regions (less than -10 °C). Several studies, particularly in agricultural and grassland soils, focused on the effects of a single FTC, some of which noted significant effects on nutrient levels even after one FTC (Freppaz et al., 2007; Xu et al., 2016; Pelster et al., 2019). Of the field studies, most were

conducted with a minimum freezing temperature of -6 °C. The number of FTCs were highly variable in field studies as some studies would define an FTC as “the days when soil temperature fluctuated across 0 °C” (Urakawa et al., 2014), but most of the field studies recorded between six to 12 FTCs. Soil nutrient dynamics generally remained unchanged or decreased with FTC treatment in field studies than compared to laboratory studies. Using the info summarized from this table, I identified the key parameters of FTCs that may illicit strong disturbances to microbial communities and nutrient dynamics in a laboratory incubation.

Current Study: Experimental Design and Hypotheses

Few studies have considered assessing the impact of FTCs within the context of agroecosystem environments. As explored in Table 1, while few FTC studies have captured the FTC effects within an agricultural system, far fewer have yet to examine these effects within a crop rotational diversity gradient. The main research question that I propose: How do diverse cropping systems confer resilience to soils such that effects of FTCs on microbial communities and SOC stocks are moderated?

The primary goal of this study was to assess how cropping system diversity interacted with increasing frequency FTCs to alter microbial activities that affect SOC and soil nutrient pools. I used soils from a unique long-term experiment – the Biodiversity Gradient Experiment at the Kellogg Biological Station – to design a short-term, 60 d, laboratory incubation, during which I measured soil respiration over the course of the incubation. Afterwards, I measured microbial biomass C and N, DOC, DON, soil inorganic N (net N mineralized) and extracellular enzyme activities (EEA) related to soil organic matter breakdown.

I hypothesized that 1) Increased cropping system diversity would increase resiliency of the microbial communities, such that soils from a cropping system with a higher diversity experiencing FTCs would experience post freeze bursts of respiration, but would then return to baseline (pre-FTC levels) and overall produce less total CO₂ with little to no change in microbial biomass, net N mineralization and EEA relative to no FTC control soils. 2) As the frequency of FTCs increases, such that there is less time in between each thawing period (shortening of the interval), mechanisms for microbial resiliency are compromised. At lower frequency FTCs, microbes will have more time to recover from freezing conditions, such that respiration rates and nutrient dynamics will be similar to undisturbed soils. At higher frequency FTCs, pulses in respiration rates during thawing, baseline respiration rates, microbial biomass, EEA and nutrient mineralization will all be decreased relative to control levels. 3) Resilience conferred by increasing cropping system diversity will moderate the impacts of increasing FTC frequency, such that responses to increasing FTCs in soils from higher cropping system diversity will be less pronounced compared to responses in soils from lower cropping system diversity. For example, lower diversity cropping systems subjected to high frequency FTC conditions will experience greater differences, namely respiration rates during thawing and baseline respiration rates, relative to undisturbed soils. When higher cropping diversity systems are subjected to the same, high FTC frequency treatment, the differences relative to the control will be less pronounced.

MATERIALS AND METHODS

Site Description

Soils were collected from the Long-Term Ecological Research Cropping Biodiversity Gradient Field Experiment located at the W.K. Kellogg Biological Station in Michigan, USA (42° 24' N, 85° 24' W). Mean annual precipitation is 1005 mm/year, with mean annual temperature around 10.2 °C (Robertson and Hamilton, 2015; Tiemann et al., 2015). The soils are a sandy loam mesic Typic Hapludalfs, soil series Kalamazoo and Oshtemo. Sand and clay content average 43% and 17% respectively (Robertson and Hamilton, 2015). Total soil C ranged between 5.5 – 10.4 mg C g⁻¹ bulk soil, with significantly greater C with greater diversity crop rotations (Tiemann et al., 2015). The experimental field layout consisted of a complete, randomized block design with all crop diversity treatments, at all crop entry points across four treatment blocks. Experimental plots sized 9 by 27 m contained a gradient of rotational diversity using common cash and cover crops regimes from the Midwestern U.S. This included corn (*Zea mays L.*), soybean (*Glycine max L.*), and winter wheat (*Triticum aestivum L.*) and the cover crops red clover (*Trifolium pratense L.*), and cereal rye (*Secale cereal L.*) (Robertson and Hamilton, 2015). Established in 2000, the purpose of this experiment was to examine the extent of benefits to ecosystem functions that come from increasing crop diversity within an organic row-crop system (Smith et al., 2007). No fertilizers or pesticides were applied. The biodiversity gradient permits belowground microbial parameters such as soil microbial biomass and disease suppression to be addressed without confounding factor of differing chemical inputs (McDaniel et al., 2016; Peralta et al., 2018). The crop treatments used for this specific study are as follows: monoculture corn (MC), a corn and soy rotation (CS), monoculture corn with a red clover cover

crop (C1), and three rotations of corn, soy, wheat, one with no cover crops (CSW), one with the red clover cover crop (CSW1), and one with both red clover and cereal rye cover crops (CSW2).

Field Sampling

I sampled soil from plots for all treatments across different entry points such that all plots had been in corn the previous growing season to reduce confounding treatment comparisons between other crops in the rotations. Soil cores were collected to a depth of 10 cm using a 3/4 inch diameter metallic soil sampler corer on March 17th, 2020. Two sets of six cores within each plot across the four blocks were taken within the rows and at least 2 m from the edges of the plot to reduce potential edge effects. Soil samples were first placed in labeled gallon plastic bags and then into coolers during sampling, before their return to Michigan State University where they were stored at 4 °C.

Experimental Design

Soil Preparation

I used a 4-mm mesh sieve to sieve the soils, gently shaking and breaking along natural fracture planes to remove any stones, large organic residues (leaves, pinecones, wood), and visible roots, while preserving soil structure to some extent. 10 g triplicates of each soil sample were dried at 60 °C for 48 hours to determine gravimetric soil moisture at the time of sampling. I allocated 75 g (dry weight) of sieved field soil from each plot into glass Mason jars with rubber septa fitted into the lids of each jar to accommodate gas sampling. Soil moisture was adjusted to 60% field capacity with nanopure H₂O for each jar, which was then weighed to record a reference mass for maintaining consistent soil moisture throughout the incubation.

Description of Lab Incubation and Setup

I assigned four replicate jars (one per block) from each of the six crop treatments as either controls (no FTCs) or to one of three different FTC frequencies: 4 FTCs (Low), 8 FTCs (Med), and 12 FTCs (High) for a total of 96 jars (6 Crop treatments x 4 FTC treatments x 4 replicate blocks). Soils were frozen in a laboratory freezer set to -10 °C. To monitor inner freezer temperature, I placed both a digital temperature gauge as well as a mercury thermometer inside the freezer to verify this temperature throughout the experiment. Soils were thawed in a walk-in cooler preset at 4 °C. These temperature parameters were chosen to simulate the historical winter (Dec-Feb) climate data collected at the KBS field site over the last 30 years, with near average day (thawing) and night (freezing) temperatures (Robertson and Ruan, 2018; Robertson, 2019).

Microbial Respiration

Prior to the actual FTC incubation, I conducted preliminary freezing and gas sampling trials with soils previously sampled in July 2019 to determine how much time was needed for the soil temperature to reach 0 °C after removal from -10 °C freezing conditions, the time interval for the respiratory burst, and approximately when respiration returns to a stable, baseline rate. Briefly, I allocated 75g of soil adjusted to 60% field capacity to a subset of six glass Mason jars. An electronic temperature sensor probe was placed in the soil and fixed onto the jars to measure the soil temperature as they were frozen and thawed. It took 4 hours for soil temperatures to reach 0 °C and begin thawing. Next, I sampled gas from the jar headspace across two time periods starting 4 hours after removing each soil from freezing conditions to determine sampling timeframe for the respiratory burst. Once soils were thawed, I determined the interval for capturing the respiratory burst to be 24 hours and for soils to return to baseline respiration rates

48 hours after thawing. Therefore, I sampled headspace gases and CO₂ accumulation at two time intervals, the first from 0-24 hours after soils were thawed (T1 and T2) and the second 48 to 72 hours after soils were thawed (T3 and T4). Respiration rates were calculated for these two distinct time periods using T1 to T2 to represent the respiratory “burst” that occurs as soils are thawing and T3 to T4 to represent “baseline” respiration that occurs after the thawing burst is complete. I assumed that the respiration rate for the days between T4 sampling and the next freezing period were the same as the previous measured baseline rate, and thus used the baseline respiration rate to determine the cumulative respiration between freezing and thawing burst periods. I flushed the incubation jars with lab air to flush accumulated CO₂ after the T2 and T4 gas samplings to keep incubation conditions aerobic.

I used the following calculations to determine the cumulative respiration of the entire incubation for each sample jar:

$$\begin{aligned}
 &[(\text{burst respiration rate}) * (\text{time elapsed during burst})] + \\
 &[(\text{average of burst and baseline resp rates}) * (\text{time elapsed between burst and baseline} \\
 &\text{measurements})] + \\
 &[(\text{baseline respiration rate}) * (\text{time elapsed between baseline gas measurements})] + \\
 &[(\text{baseline respiration rate}) * (\text{time elapsed between FTCs minus the 16 hours soils were frozen})] \\
 &= \text{cumulative respiration}
 \end{aligned}$$

Baseline respiration rates are missing for the second FTC in the high frequency FTC treatment. Any calculated negative respiration rates were discarded. The total incubation time for

each FTC treatment was 60 d. The start times for each treatment were staggered to manage the high volume of gas samples taken during the incubation. To limit handling time and management of gas samples, I staggered the freezing of the treatments. The high FTC and control treatments began on the same day, while the medium FTC and low FTC treatments began 8 days later.

I collected gas samples in evacuated 13 ml glass vials capped with rubber septa and stored them room temperature for no more than one week before analysis. Because gas samples were collected at 4 °C and would be analyzed at ambient room temperature (25 °C), 12 ml of gas volume was collected from the sample jars to ensure atmospheric pressure remained at 1 atm upon removal from the low incubation temperature to room temperature conditions in accordance with the ideal gas law ($R = 0.08206 \text{ L-atm/mol-K}$). Gas samples were analyzed on a Trace 1300 gas chromatograph (Thermo Scientific, USA). Respiration rates were recorded as $\mu\text{g CO}_2\text{-C g}^{-1} \text{ soil hr}^{-1}$, and cumulative respiration as $\mu\text{g CO}_2\text{-C g}^{-1} \text{ soil}$.

Post-Incubation Sub Sampling

Once the initial 60 d soil incubation was complete for each treatment, I removed 26 g of soil from each jar and allocated it for the following soil analysis: 2 sets of centrifuge tubes with 8 g each for microbial biomass and dissolved organic C and N; 5 g frozen at -20 °C for extracellular enzyme assays; 5 g for determining end-incubation soil moisture. Soil moisture was determined by weighing before and after oven drying at 60 °C for 48 hours.

Soil Microbial Biomass and Dissolved Organic Matter Analysis

Microbial biomass C and N were determined on sub-sampled soils using the chloroform fumigation-extraction method (Brookes et al., 1985; Vance et al., 1987; Jenkinson et al., 2004). Briefly, I added 2 ml of chloroform (CHCl_3 , 99.9%) to one set of the 8 g centrifuge tubes for 24 hours (FUM). Next, I added 40 ml of 0.5 M K_2SO_4 to the second set of 8g tubes (UNFUM) and placed them on an orbital shaker at 200 RPM for 1 hour. Afterwards, I filtered the UNFUM sets through #1 Whatman filter papers into a clean, labeled centrifuge tube. After 24 hours, I vented them and added 40 ml of 0.5 M K_2SO_4 to the FUM sets, shaking and filtering as described above and placed all extracts in a -20 °C freezer until further analysis. Extracts were thawed before analysis on a Vario Select TOC analyzer (Elementar, Germany). Microbial biomass C and N ($\mu\text{g MBC-C g}^{-1}$ dry soil) are calculated as the difference between the fumigated and non-fumigated DOC and TN, multiplied by a fumigation efficiency factor of 0.45 (Franzluebbers et al., 1999; Jenkinson et al., 2004; Setia et al., 2012). The same unfumigated K_2SO_4 extractions were used to estimate the dissolved organic carbon (DOC) and total dissolved nitrogen (TN) with an extraction. DON was determined by subtracting the total sum of NH_4^+ and NO_3^- (method below) from TN (Jones and Willett, 2006).

Total Inorganic Nitrogen Analysis

Ammonium

Ammonium assays were performed based on (Sinsabaugh et al., 2000). Three 100 μL replicates of the UNFUM K_2SO_4 extract for each sample and standards were added into a clear 96-well plate. 40 μL of ammonia salicylate was added to each well first, lightly tapping the edge of the plate to mix, and left to sit for 3 minutes. Afterwards, 40 μL of ammonia cyanurate was

gently mixed into the wells and left for 20 min for color development. Ammonium concentration was determined spectrophotometrically using a Synergy HT1 microplate reader (Biotek, Vermont, USA) at an absorbance of 610 nm and reported in $\mu\text{g NH}_4\text{-N g}^{-1}$ dry soil.

Nitrate

I performed nitrate assays based on the nitrate reductase manufacturer's protocol (www.nitrate.com/node/164). Notable reagents are as follow: Nitrate Reductase (AtNaR2 from *Arabidopsis thaliana*, EC 1.7.1.1), 25 mM Na₂EDTA, phosphate buffer (pH = 7.5), β -Nicotinamide adenine dinucleotide (NADH, reduced form, salt) stock solution, 1% Sulfanilamide (SULF) solution, N-1-naphthylethylenediamine dihydrochloride (NED), and 100 ppm NO₃-N Stock. Working solutions of AtNaR2 and NADH were made the day of the assay. I pipetted triplicates of 25 μL of UNFUM K₂SO₄ sample extracts into a clear 96-well plate. Next, I added 50 μL of AtNaR2 50 μL of NADH working solutions to the plates and incubated them for 10 minutes in the microplate reader. Finally, 50 μL of SULF and 50 μL of NED were pipetted into the plates and incubated for 2 minutes at 30 °C. Nitrate concentration was determined using the same microplate reader at a wavelength absorbance 540 nm. Nitrate concentration was reported in $\mu\text{g NO}_3\text{-N g}^{-1}$ dry soil.

Description of Extracellular Enzyme Assay

Extracellular enzyme activity is a versatile tool for measuring microbial response to an assortment of experimental treatments (Henry et al., 2012). I selected six hydrolytic enzymes and two oxidative enzymes relevant to SOM degradation to perform assays on the post-incubation soils. β -1,4-glucosidase (BG) is responsible for hydrolyzing short chain cellulose polymers and

glucose chains into glucose. β -D-1,4-cellobiohydrolase (CBH) is responsible for hydrolyzing links of cellulose. Together, BG and CBH reflect labile organic C turnover and fundamental decomposition of soil organic matter (Turner et al., 2002; German et al., 2011). Leucine aminopeptidase (LAP) hydrolyzes leucine and amino acid residues (Lauber et al., 2008). β -1,4-N-acetyl-glucosaminidase (NAG) targets N-linked glucosaminidase residues and chitin, and with LAP indicate N acquisition (German et al., 2011). Acid phosphatase (PHOS) hydrolyzes phosphomonoester bonds and mineralize organic P into releasing phosphate, a prime indicator for P acquisition (German et al., 2011; Jian et al., 2016). Phenol oxidase (PHENOX) and peroxidase (PEROX) determine recalcitrant C acquisition and oxidative decomposition due to its non-specific oxidative properties. PHENOX is capable of targeting phenol groups using Cu-functional groups, while PEROX relies on Fe-functional groups associated with hydrogen peroxide (H_2O_2) to target aromatic compounds (Sinsabaugh, 2010; German et al., 2011).

Fluorescent indicators linked to substrates, namely 4-Methylumbelliferone (MUB, Millipore Sigma, 90-33-5) and 7-Amino-4-methylcoumarin (MC, Millipore Sigma, 26093-31-2) were used for determining activity of the respective enzymes. Enzyme commission numbers and substrates for each enzyme are as follows: BG, 4-MUB- β -D-glucopyranoside, 18997-57-4; CBH, 4-MUB- β -D-cellobioside, 72626-61-0; NAG, 4-MUB-N-acetyl- β -D-glucosaminide, 37067-30-4; PHOS, 4-MUB-phosphate, 3368-04-5; and LAP, L-Leucine 7-amido-4-MC, 62480-44-8. First, I removed 1 g of soil from the 5 g sub sample frozen at -20 °C freezer earlier and left it to thaw at room temperature. Using an immersion blender, I homogenized 125 ml of nanopure H_2O and for 30 seconds to form a slurry. Next, I placed the slurry onto a stir plate and continually stirred it under a low-level RPM to keep soil in suspension. While stirring, I pipetted eight 200 μ L

replicates of slurry systematically into a black 96-well plate along with 50 μL of the respective MUB (BG, CBH, NAG, and PHOS) or MC (LAP) labeled substrates. Standard curves were determined by pipetting 50 μL , 25 μL , and 10 μL of 50 μM MUB or MC with 200 μL , 225 μL , and 240 μL of nanopure H_2O into a separate plate. PHENOX and PEROX activity were determined by monitoring L-3,4-dihydroxyphenylalanine (L-DOPA) oxidation. Similar to above, I aliquoted eight 200 μL replicates of slurry into a clear 96-well plate along with 50 μL L-DOPA solution. 10 μL of 0.3% H_2O_2 was added to the PEROX plates. I recorded the exact date and times of substrate additions to soil slurry plates. All plates were covered and incubated in darkness overnight at 24 $^{\circ}\text{C}$. All black plates were read the following day on a microplate reader. Enzymes were read at the following wavelengths: MUB-associated enzymes (BG, CBH, NAG, PHOS) at 370 nm excitation and 455 nm emission and MC-associated enzymes (LAP) at 350nm excitation and 430 nm emission. PHENOX and PEROX were read at 460 nm. Enzyme activity was reported in $\text{ng substrate hr}^{-1} \text{ g}^{-1} \text{ soil}$.

Statistical Analyses

All absolute data were assessed following general normal distribution assumptions. PEROX and PHENOX data were combined to result in a single variable to represent total oxidase activity. Histogram, quartile, residual and studentized residual plots for each variable were examined first for normality (Shapiro-Wilk). Variables were transformed appropriately to meet assumptions. For the absolute data, log transformations were done on BG, CBH, PHOS, and LAP; square root transformations on DON, MBN, 1 d and 60 d cumulative respiration, total cumulative respiration, and average burst and baseline respiration. The response variables NAG, total oxidase, NO_3^- , NH_4^+ , and microbial C:N could not be transformed to satisfy normality

assumptions and were subject to non-parametric median and rank analysis. Results for these variables were divided by the largest value, and ANOVA was performed on the resultant rank ordering. In the case where multiple transformations yielded acceptable normality parameters, skewness and kurtosis values were examined in conjunction with residual plots to determine appropriate transformation (Kozak and Piepho, 2018).

Percentage of control data were calculated from the absolute data and used for ANOVA analyses and in all graphs. For percentage of control data not normally distributed, I used log transformations (BG, CBH, NAG, LAP, NH_4^+ , DON, MBN, C:N, and 60 d Burst), square root transformations (1 d burst and 60 d baseline), and reciprocal transformation (NO_3^-). Three response variables (PHOS, Total Oxidase, 1 d baseline) were subject to non-parametric median and rank analysis as described above.

Statistics were conducted using the SAS Enterprise Guide 7.1. I analyzed the absolute data using an analysis of variance (ANOVA) on the FTC frequency (Low, Med and High) and Dunnett's test to compare the FTC treatments against control soils. I used a reduced maximum likelihood approach and the Kenwardroger2 approximation function. I modeled covariance structure using a spatial power model, sp(POW), assigning each crop treatment within each block with a coordinate ID created by the block ID (1-4) and specific treatment ID as assigned by KBS.

For the percentage of control data, I analyzed all data using a two-way mixed model analysis of variance (ANOVA) with crop diversity (MC, C1, CS, CSW, CSW1, CSW2) and FTC

frequency (Low, Med, and High FTC) as fixed main effects and blocking as a random effect. The same covariance structure and model parameters mentioned previously were used. Five variables (total cumulative respiration, CBH, PHOS, NH_4^+ , and microbial C:N) did not fit the model (e.g. G matrix error, hessian matrix not positive definite, infinite t scores) and were analyzed as a general linear model (GLM), with no random blocking effect. Differences of least square means (LSM) of pairwise comparisons of FTC frequency differences within crop treatments were made to compare data results. In addition, I performed a one-way ANOVA on the soil parameters on the control soils, with crop diversity as the only main effect, and the random blocking effect. Log transformations were necessary for CBH, NAG, MBN, and microbial C:N. All figures were generated in RStudio 3.6.3.

RESULTS

All the variables and explanation of trends are presented as a percentage of the control (unfrozen soils) treatment unless otherwise noted. In most of the following parameters, crop diversity treatment was not a significant main effect, nor was the interaction between FTC and crop diversity treatment significant. However, graphs of data by cropping system diversity are included to illustrate the relative resilience, or lack thereof, and trends described as needed.

CO₂ Respiration Dynamics

As expected, 1 d respiration rates generally did not vary across FTCs except for baseline respiration which was greatest in the medium compared to low and high FTC frequencies (Fig. 1a, c; Table 2). The range of absolute respiration rates for the burst and baseline respiration rates on this day were 0.00234 to 0.115 $\mu\text{g CO}_2\text{-C g}^{-1}\text{ soil hr}^{-1}$ and 0.00233 to 0.0763 $\mu\text{g CO}_2\text{-C g}^{-1}\text{ soil hr}^{-1}$, respectively. The low, medium, and high FTC treatments were all above control levels ($P = 0.0014$, $P < 0.0001$, $P = 0.0135$, respectively; Table 3). Only baseline respiration for the medium FTC was above control levels ($P = 0.0085$). Since this was the first FTC for all soils, it was not expected that there would be differences. However, I did expect to see differences in respiration rates by crop diversity and did find respiration generally increased along the crop diversity gradient (Fig. 1b). Differences in baseline respiration (Fig. 1d) were less pronounced and overall, no 1 d respiration rates varied significantly by crop diversity treatment (Table 2).

The range of absolute respiration rates for the burst and baseline respiration rates on the final day were 0.000406 to 0.0558 $\mu\text{g CO}_2\text{-C g}^{-1} \text{ soil hr}^{-1}$ and 0.00134 to 0.0481 $\mu\text{g CO}_2\text{-C g}^{-1} \text{ soil hr}^{-1}$, respectively. Like the 1 d respiration rates, only 60 d baseline respiration rates varied across FTC frequencies with high FTC significantly greater than medium and low FTC frequencies (Fig. 2a, c; Table 2). Low and medium FTC treatments were both lower than control levels ($P < 0.0001$). In addition, burst respiration was similar to control soils and baseline respiration lower than control levels in the high and medium FTC treatments (Fig. 2a, c). I again found no effect of crop diversity on respiration rates on 60 d.

The range of the absolute, average respiration rates for the burst and baseline respiration rates were between 0.0156 to 0.0719 $\mu\text{g CO}_2\text{-C g}^{-1} \text{ soil hr}^{-1}$ and 0.0156 to 0.0309 $\mu\text{g CO}_2\text{-C g}^{-1} \text{ soil hr}^{-1}$, respectively. Respiratory bursts averaged across the entire incubation varied by FTC treatment, being highest in the low compared to medium and high frequency FTC treatments (Table 2, Fig. 3a). Only the low FTC treatment was significantly above control levels ($P < 0.0001$, Table 3). Despite a greater number of respiratory bursts in the high FTC treatment, average size of the respiratory bursts was similar between the medium and high FTC treatments (8 FTCs compared to 12 FTCs, respectively). I found that all FTC treatments averaged greater than control level respiration rates during bursts, but low and high frequency FTCs had lower than control average baseline respiration rates, while medium frequency FTC was similar to controls (Fig. 3c). The low and med FTC levels were significantly lower than control levels ($P = 0.001$, $P < 0.0001$, respectively). There was a marginally significant ($p = 0.051$) effect of crop diversity on average burst, but no effect on average baseline respiration rates. I found that average burst respiration rates (Fig. 3b) generally increased along the crop diversity gradient.

The range of the 1 d and 60 d cumulative C respired were between 0.758 to 4.97 $\mu\text{g CO}_2\text{-C g}^{-1}$ soil and 0.105 to 3.47 $\mu\text{g CO}_2\text{-C g}^{-1}$ soil, respectively. In addition, the range of the absolute, total cumulative C respired was between 18.5 and 63.6 $\mu\text{g CO}_2\text{-C g}^{-1}$ soil. Total $\text{CO}_2\text{-C}$ respired or cumulative respiration on 1 d of the incubation was surprisingly different by FTC treatment, even though this was only the first FTC for all treatments. For 1 d cumulative respiration, low and high FTC treatments were significantly below control levels ($P = 0.0034$, $P = 0.0143$, respectively). Similarly, 60 d cumulative respiration for all three FTC treatments were significantly below control levels ($P < 0.0001$). I found significantly greater cumulative C respired from the medium compared to the low frequency FTC treatment (Fig. 4a; Table 2) It was also surprising to find that 1 d cumulative respiration was below no freeze control soil levels in the low and high frequency FTC soils. Again, I saw no significant effects of crop diversity on day 1 cumulative respiration (Fig. 4b). 60 d cumulative C respired was not different by FTC or crop diversity treatment, but the former exhibited lower cumulative C respired than no freeze control soils (Fig. 4 c, d).

Total C respired over the whole incubation (60 days) was significantly affected by both FTC frequency and crop diversity. Not only was cumulative C in the low, medium, and high FTC treatments significantly below control levels ($P = 0.0041$, $P = 0.0002$, $P < 0.0001$, respectively), cumulative respiration from soils in the high frequency FTC treatment was significantly less than the low and medium frequency FTC treatments (Table 2, Table 3, Fig. 5a.). I found that the MC and CSW1 crop diversity treatments had lower cumulative C respired compared to the CSW treatment (Table 2, Fig. 5b).

Inorganic Nitrogen

NH_4^+ ranged between 0.0548 to 2.86 $\mu\text{g NH}_4\text{-N g}^{-1}$ dry soil. NH_4^+ concentration generally decreased with increasing FTC frequency (Table 2). Average NH_4^+ levels for the medium and high FTC treatments were relatively lower than the control at roughly 61.9% and 57.5% of the control, respectively, while only NH_4^+ levels in the low FTC treatment were similar to control levels (Fig. 6a). The medium and high FTC treatments were lower from the control ($P = 0.0202$, $P = 0.049$, respectively). In general, lower FTC frequency led to higher concentrations of NH_4^+ relative to the control within FTC treatments.

NO_3^- absolute values ranged between 0.192 and 94.8 $\mu\text{g NO}_3\text{-N g}^{-1}$ dry soil. Only the main effect of FTC frequency affected NO_3^- results, where NO_3^- in the low FTC was lower than the high FTC (Table 2, Fig. 6c). NO_3^- levels in the high FTC treatments were significantly higher than the control levels ($P < 0.0001$, Fig. 6c). An inverse relationship is present when comparing NO_3^- concentrations to the previously discussed NH_4^+ concentrations between FTC treatments.

Dissolved Organic C and N and Microbial Biomass

In all soils and treatments, absolute values of DOC ranged from 0.0257 to 0.0796 $\mu\text{g DOC-C g}^{-1}$ dry soil. FTC frequency was significant in determining DOC concentration (Table 2), marginally increasing with increasing FTC frequency, where the high FTC was significantly higher than both FTC treatments (Fig. 7a). Furthermore, DOC levels in the low and high FTC treatments were significantly above control levels ($P = 0.0009$, $P < 0.0001$, respectively), but surprisingly the medium FTC treatment was not (Table 3). DOC concentration was consistent along the crop diversity gradient (Fig. 7b).

Absolute values of DON ranged from 0.000928 to 0.0211 $\mu\text{g DON-N g}^{-1}$ dry soil. FTC frequency was significant, with DON levels in low FTC treatments lower than the others (Table 2, Fig. 7c). DON results were all higher than control levels. In particular, the medium and high FTC treatments reported well over 150% of the control ($P < 0.0001$, $P = 0.0061$, respectively, Fig. 7c). Large variances in DON concentration are notable within FTC treatment replicates.

MBC absolute values ranged from 0.0276 to 0.268 $\mu\text{g MBC-C g}^{-1}$ dry soil. Strong FTC frequency effects on MBC were present. MBC levels were significantly lower for the high FTC treatment when compared to the low and medium FTC treatments, as well as the control ($P = 0.0148$). MBC levels were also above control levels in the low FTC treatments, suggesting consistently reduced MBC in more frequently disturbed soils (Table 2, Fig. 8a).

Absolute values for MBN ranged between 0.000213 and 0.0262 $\mu\text{g MBN-N g}^{-1}$ dry soil. MBN in the low FTC treatments were several times larger than the other treatment values, nearly double compared to the medium FTC treatment (Fig. 8a). Despite the large variation of results in the low FTC treatment and statistical insignificance, MBN levels generally decreased with increasing FTC frequency, where the results under medium and high FTC treatment were similar to control levels and low FTC was significantly above it ($P = 0.0253$, Table 2, Table 3, Fig. 8c). This trend was also present in the MBC values as reported previously, but at considerably smaller magnitudes with less variation, and statistical significance.

Microbial C:N ratio was the only dataset not represented as a percent of the control, and instead as the ratio of the absolute MBC and MBN for clearer data interpretation. The C:N ratio

generally remained consistent between the medium and high FTC treatments relative to the C:N ratio in the control soils, while the low FTC treatment reported a ratio less than half of the other treatments and significantly below control levels ($P = 0.0237$, Table 3, Fig. 9). Wide variances were observed for both the high and medium FTC treatments, particularly for the CS treatment.

In general, FTC frequency affected mineralized C and N differently dependent on its location as either in the soil space or within microbes, such that higher FTC frequencies led to greater DOC and DON in soils. The opposite trend is observed for MBC and MBN, where lower FTC frequency encouraged greater microbial biomass C and N. Along the crop gradient, DOC and DON were generally increasing, but not continuously.

Extracellular Enzyme Activity

Labile C acquisition enzymes (BG and CBH) were significantly affected by FTC frequency, but followed a distinctive, centralized trend, and were nearly identical in trends and responses. Absolute BG activity ranged from 63.11 to 444.61 ng substrate $\text{hr}^{-1} \text{g}^{-1}$ soil, and between 15.46 to 167.73 ng substrate $\text{hr}^{-1} \text{g}^{-1}$ soil for CBH. Both BG and CBH activity followed a centralized trend, where EEA in the medium FTC treatment was significantly the highest amongst the other FTC treatments and nearly twice that of the low FTC treatment (Table 2, Fig. 11a, Fig. 11b). As visible in the figures, activity in the medium and high FTC treatment were both significantly higher than control levels ($P < 0.0001$, $P = 0.0110$, respectively, Table 3). Both BG and CBH activity remained relatively consistent along the crop gradient (Fig. 11c, Fig. 11d).

Absolute values of NAG activity were between 13.40 and 109.91 ng substrate hr⁻¹ g⁻¹ soil, while for LAP activity it was 1.55 to 69.36 ng substrate hr⁻¹ g⁻¹ soil. N acquisition, as measured by NAG activity, was the highest in the soils subjected to medium FTC frequency treatment, dominating the other FTC treatments and significantly above the control level ($P < 0.0001$). FTC frequency was highly significant in determining NAG activity, where the medium FTC levels were consistently higher amongst the low and high FTC treatments (Table 2, Fig. 12a). The largest mean difference in activity between FTC treatments was in comparing the medium and low FTC treatments, with the former resulting in almost twice as much as the latter (Fig. 12a). Similarly for LAP activity, the main effect of FTC frequency was highly significant (Table 2). LAP activity in the low, medium, and high FTC treatments were above control levels ($P < 0.0001$, $P < 0.0001$, $P = 0.0086$, respectively, Table 3), and among the FTC treatments was lowest in the high FTC treatment (Fig. 12c).

P acquisition generally measured by PHOS activity, was the most variable enzyme measured across FTC treatments. Range of absolute PHOS activity was between 1.25 to 66.49 ng substrate hr⁻¹ g⁻¹ soil. PHOS activity in the medium and high FTC treatments, in addition to being above control levels ($P < 0.0001$), were by far the most influenced by FTCs, yielding well over double the PHOS activity in the high FTC treatment and quadruple in the medium FTC treatment (Table 2, Table 3, Fig. 13a). In addition, I found that the MC crop diversity treatment had higher PHOS activity compared to the moderate crop diversity treatments (C1 and CS), but were similar to higher diversity rotations (CSW) (Table 2, Fig. 13b).

Total oxidase activity varied between crop treatments and within FTC treatments, representing a significant interaction of FTC frequency and crop treatment (Table 2). Absolute values of total oxidase activity were large, with a range between 766.29 and 4513.59 ng substrate $\text{hr}^{-1} \text{g}^{-1}$ soil. Similar to previous EEA trends, noticeable separations between FTC treatments were visible, but this time with both high and medium FTC treatment reporting highest average oxidase activity consistently between each crop treatment. Oxidase activity within the low FTC treatment consistently hovered around control levels along the crop gradient. The only significant differences between crop treatments were in the high FTC, higher diversity crop treatments (CSW1 and CSW2). In both treatments, higher FTC frequencies led to greater oxidase activity, but the high and medium FTC treatments were similar to one another (Fig. 14).

DISCUSSION

Overview

Increasing crop rotational diversity increases SOM and microbial biomass (McDaniel et al. 2014b) and can enhance the diversity of microbial communities (Tiemann et al. 2015; Smith et al. 2016; Schmidt et al. 2019). I hypothesized that these positive impacts of rotational diversity would lead to increased resilience in the face of stress and disturbance for soil systems under high crop rotational diversity. Specifically, I examined how crop rotational diversity interacts with stress and disturbance caused by different levels of FTC frequency on SOC and microbial functioning. My results contrast my original hypotheses, such that most SOC and nutrient pools, as well as microbial activities, were not resilient to FTCs, regardless of crop rotational diversity. Also contrary to my hypotheses I did not find that increasing FTC frequency resulted in overall greater potential for soil C losses. Also contrary to my hypotheses, I found that microbial respiration dynamics and most enzyme activities were not impacted by crop diversity. Interestingly, EEA were greatest in the medium compared to both high and low FTC frequency treatments. Overall, I was surprised to find that crop rotational diversity had a limited role in influencing nutrient dynamics and microbial functioning while being subjected to different levels of FTCs, especially when the control soils (no FTC) showed greater respiration, microbial biomass C and enzyme activities in the high diversity (CSW1 and CSW2) compared to low diversity crop rotation treatments (MC) (Table 3).

FTC Effects on Respiration, C and N Dynamics

Increased freezing disturbance is the underlying mechanism for elevated DOM levels, as well as the moderator for the CO₂ dynamics observed between FTC treatments. DOC is

primarily composed of simple and microbially accessible C sources and may be the largest contributor to the FTC respiratory bursts (Haei et al. 2011; Song et al. 2017; Wu et al. 2020). The importance of calculating the respiratory burst is highlighted by Schimel and Clein (1996) who reported different respiratory burst sizes for tundra soils that influenced seasonal respiration dynamics. When soils freeze, new DOM can become available from lysed microbial cells or as aggregates are broken open, however, the source of the DOM that influences subsequent soil CO₂ efflux during thawing is difficult to track. As explored by Walz et al. (2017), increased de-aggregation resulting from FTCs may confound sources of organic matter released either from fractured aggregate structures, or from microbial necromass, where the latter may contribute around 65% of the CO₂ generated from the respiratory burst (Herrmann and Witter, 2002). Importantly, the release of DOC and DON during FTCs is tightly coupled, particularly in forest studies, where significant changes in DOM or substrate quality were attributed to FTCs enhancing N cycling through increased NH₄⁺ availability (Shibata et al. 2013; Watanabe et al. 2019), which I discuss later. In my study, all of the imposed FTC treatments generated respiratory bursts that decreased in size by the end of the incubation. Reductions in DOC, and therefore respiratory bursts with increasing FTCs may be explained by limited aggregate breakdown in later FTCs (Gao et al. 2021), or the emergence of resilient microbes with greater nutrient efficiency.

Contrary to my original hypothesis, the average baseline respiration was the highest and most resilient in the medium compared to high and low FTC treatments; this would be a trend later in the discussion where the medium FTC treatment would be either closest to control levels or generate the highest concentration or activity. Cumulative respiration in the high FTC

treatment was lower than control levels by the 60 d mark of the incubation period, suggesting that the respiratory bursts were only somewhat important contributors to cumulative CO₂ respiration, where I saw no FTC treatment differences.

These results suggest a consistent decline in the contributions of respiratory bursts to cumulative respired C that becomes more pronounced with increasing FTC frequency, corroborating with respiration dynamics from other studies (Herrmann and Witter, 2002; Larsen et al. 2002; Henry, 2007; Wu et al. 2021). In addition, of the 29 studies previously examined in-depth, 17 of them documented CO₂ respiration rate dynamics (burst, baseline respiration, total respiration) after FTCs. Nine of these studies, primarily from forest and alpine systems, showed general increases to baseline respiration, while the other eight studies, primarily in croplands and grasslands, showed declines in respiration rates, including baseline respiration. These differences between ecosystem types highlight the importance of standardizing methods and not overgeneralizing effects of FTCs across different study sites. It also highlights the importance of capturing effects of FTCs at different time points as early winter versus late winter respiration dynamics in relation to FTCs may be quite different. Only measuring at one time point can result in either over or under estimating winter soil respiration.

FTC Effects on Inorganic N

In support of my hypothesis, NH₄⁺ and NO₃⁻ concentrations remained somewhat similar to controls in the low frequency FTC treatment, and deviated from it with increasing FTC frequency. Total inorganic N pools may decrease with FTCs (Pelster et al. 2019), but specific parameters beyond FTC frequency may influence inorganic N pools such as freezing temperature

severity (Liu et al. 2016; Jiang et al. 2018) and freezing duration (Han et al. 2018). I saw decreasing NH_4^+ levels with increasing FTC frequency, suggesting elevated nitrification with FTC disturbance, which has been frequently observed in laboratory soil drying-rewetting and FTC incubations and observed in field studies. Bhowmik et al. (2016) reported a gradual decrease in NH_4^+ but significant spike in NO_3^- concentrations after a single FTC in field soils. In addition, Groffman et al. (2011) reported increased soil DOC concentrations from soil freezing and greater amounts of NO_3^- left in the soil as I found in my study. These results are somewhat surprising because denitrifying bacteria may be more cold-resistant than nitrifying bacteria (Smith et al. 2010) and should therefore take advantage of both the DOC and NO_3^- spike in cold or freezing conditions. Although nitrous oxide (N_2O) emissions, a result of denitrification, were not the focus of this study, others have shown increased N_2O release after FTCs suggesting quick use of NO_3^- and DOC by heterotrophic denitrifiers (Liu et al. 2016, Sharma et al. 2006).

In addition to increased NO_3^- with increasing FTC frequency, I also found increasing DON. While both dead roots and microbes are typically the largest N sources during winter seasons (Tierney et al. 2001), the current study design suggests that the increase of available N with concomitant decreases of MBN as FTC frequency increased. This can be largely attributed to microbial mortality. Because crop diversity had no significant effect on inorganic N dynamics, only FTC frequency is the driver of the observed NH_4^+ and NO_3^- trends. These trends of decreasing NH_4^+ and increasing NO_3^- are surprising as few laboratory studies have demonstrated similar patterns (Joseph and Henry, 2008; Liu et al. 2016). Particularly in field studies, FTCs may exacerbate NO_3^- leaching events due to water additions immediately after thawing, leading to lower NO_3^- with more FTCs. Although I found no effects of crop diversity on N trends with

FTCs, it may be that plants are more important influencers of N dynamics *in situ* compared to when FTCs are isolated in a lab study (Joseph and Henry, 2008). Based on results from field studies (Kreyling et al., 2010; Urakawa et al., 2014; Hosokawa et al., 2017) and meta-analyses that show N dynamic differences between vegetation type (Blankinship et al., 2012; McDaniel et al., 2014a) it is likely that if this experiment were conducted *in situ* I would find some moderating effects of crop diversity on N dynamics.

FTC Effects on Microbial Biomass and Microbial Functioning

In line with my hypothesis, MBC decreased with increasing FTC frequency. Han et al. (2018) reported similar results, citing time spent frozen as the moderator for reduced microbial recovery and survival. In my study, the high FTC frequency treatment experienced both additional FTCs and collectively more time spent frozen relative to the other treatments, but it is difficult to determine if the duration or number of FTCs is more important in my study. Reduced MBC in the high FTC treatment may suggest too little time in between freezing periods for adequate recovery or establishment of a new stable community (Williams et al., 2015). FTCs at lower frequencies may not experience reductions in microbial biomass if the disturbance regime was not “disruptive” enough, especially if the disturbance is within expected seasonal parameters. For example, alpine and arctic studies contain microbes that have adapted to the cold climates that, regardless of a change in FTC intensity, eventually recovered in microbial functionality (Grogan et al. 2004; Freppaz et al. 2007). A similar trend may be occurring in my data, as the low and medium FTC treatments are similar to each other; the same trend can be seen in the MBN data. MBN trends in FTC studies have mostly been covered extensively in forest studies, where microbial biomass levels were typically unchanged by FTCs regardless of

microbial community composition (Hosokawa et al. 2017). This is an important consideration because based on general principles of ecological stoichiometry, the ratio of MBC to MBN may regulate heterotrophic soil respiration rates in response to warming temperatures (Li et al. 2017).

The ration of MBC to MBN may tell us something about microbial community structure, as bacteria have generally narrower C:N ratios than bacteria. However, it is important to note that the biomass C:N ratio may be “flexible” in fungal communities when nutrient supplies are dynamic (Camenzind et al. 2021). If not accompanied by increased N release, labile C released from FTC-driven de-aggregation could facilitate greater immobilization of C in fungal structures, resulting in fungal dominance and a wider overall microbial biomass C:N ratio. In my study, microbial biomass C:N ratios in the low FTC treatment were nearly half of the medium and high FTC treatment, suggesting MBN was high relative to MBC and that perhaps there was less fungal biomass, while the medium and high FTC frequency treatments, with wider microbial biomass C:N ratios may have had greater fungal dominance. This makes sense as fungi are often more active and presumably more resilient in cold and freezing temperatures (Lehto et al., 2008; Kreyling et al., 2012; Perez-Mon et al., 2020). The microbial biomass C:N ratios in my study are with the relatively wide range of 5:1 – 40:1, previously reported from this study site (McDaniel et al. 2014b, McDaniel et al. 2016). This is important to note because this suggests not only that my data falls within a reasonable range, but also that there is a high degree of plasticity in microbial biomass C:N ratios, and so the shifts in microbial biomass I observed may not necessarily represent significant shifts in microbial functioning under soil disturbance.

FTC Effects on EEA

EEA is responsive to climate change factors, such that substrate breakdown is slower in colder temperatures (Koch et al. 2007; Henry, 2012). As hypothesized, EEA in the low FTC treatment was closest to control levels for most enzymes such that EEA were less resilient as FTC frequency increased, suggesting that FTCs enhance the release of usable SOM substrates. When nutrient pools are limited, microbes use labile substrates to synthesize more enzymes to break down SOM and liberate usable nutrients. This priming effect is associated with FTCs (Feng et al. 2017), and responsible for the respiratory bursts and rapid nutrient use. Enzyme formation and activity is a high energy process that may be constrained by insufficient C and N levels (Allison and Vitousek, 2005), and especially when under physiological stress where enzyme formation is best saved for when substrate is available (Tiemann and Billings, 2011). However, the elevated labile C and N acquisition enzymes, as well as the NAG and PHOS activity in the medium FTC treatment consistently yielded activity above control levels. These results are different from my original hypothesis, and actually provide some support for the intermediate disturbance hypothesis. That is, diversity remains high at intermediate levels of disturbance due to tradeoffs between niche re-establishment and competition after each disturbance (Santillan et al., 2019). Specifically, the medium FTC frequency generated enough soil disturbance to liberate SOM and other enzyme substrates, but not enough to reduce microbial biomass significantly from control levels, as discussed previously. Increased microbial biomass but EEA similar to the control levels in the low FTC frequency treatment may be explained by two different mechanisms. First, the low frequency FTC caused enough disturbance to release trapped or mineral bound enzymes that had been accumulated prior to disturbance (Allison and Jastrow, 2006; Olagoke et al., 2020). Second, the greater microbial biomass was

utilizing the released substrates from the FTCs at a rate that at the time of assay, was not different from control levels. The EEA in the medium and high FTC treatment were elevated at the time of analysis, but whether these levels are long-term or will return to control levels is uncertain. Miura et al. (2019) reported a brief lapse in EEA that returned to baseline levels after two weeks from a single FTC, the same period between FTCs for the low FTC treatment in my study. Surprisingly, my study results suggest that frequent FTCs will increase EEA, likely because extracellular enzymes are released from mineral surfaces and the newly released labile materials from previous FTCs encourage enzyme synthesis from surviving microbes. These EEA increases were mostly observed in the medium and high FTC treatments, where they were well above control levels.

PHOS activity was significantly higher in the medium FTC frequency, at levels well over 400% of control levels. Bell et al. (2010) also found PHOS activity highly sensitive to temperature treatments (warming), so it is possible that freezing may have elicited a similar effect. Regardless of the magnitude, PHOS activity still exhibited the same trends in the FTC treatments as most of the other hydrolytic enzymes.

Total oxidase activity was the only parameter where crop rotational diversity and FTC frequency interacted. The increased total oxidative activity results in the higher diversity treatments (CSW1, CSW2) exhibit a metabolic shift towards greater breakdown of recalcitrant C substrates (Sinsabaugh, 2010). McDaniel et al. (2014b) reported similar findings of high oxidase activity in the CSW1 and CSW2 crop treatment, suggesting higher mineralization potential in more diverse crop treatments and larger contributions to labile C pools in these rotations. Meta-

analyses of FTC studies in croplands show general increases in nutrient dynamics, namely inorganic N and DOM and reductions in microbial biomass (Song et al. 2017; Gao et al. 2021). Interestingly, Sorensen et al. (2018) reported reductions in enzyme activity and extractable C and N pools in field forest soils, but noted that declines in peroxidase and phenol oxidase activity were present into the following winter season. While this difference in EEA response may be attributed to different study type, it brings into question whether EEA in the medium and high FTC frequencies will return to baseline levels prior to FTCs over time in laboratory studies. Kreyling et al. (2008) conducted a similar FTC frequency study along various grassland communities, however they did not find enzymatic activities to be different after FTC manipulation. Jiang et al. (2018) showed that lower freezing temperatures (-15 °C) may reduce EEA in the short-term, while warmer freezing temperatures (< -5 °C) have limited or no effect on overall EEA relative to undisturbed soils. My study shows elevated EEA with -10 °C freezing, suggesting that soils that are being disturbed at a frequency that promotes, and not hinders, nutrient cycling and microbial functioning regardless of climatic factors that would normally hinder these processes. This is an important consideration for qualifying potentially long-term changes to EEA, since certain FTC parameters may stunt microbial biomass more easily. The FTC parameters in the medium FTC frequency treatment encouraged microbial functioning to the degree that microbial biomass ultimately remained unchanged, with net increases to microbial functioning and SOM mineralization through respiration, as explored earlier.

Limited Crop Diversity Effects

Contrary to my hypothesis, I found few consistent trends to suggest increased crop rotational diversity treatments effectively moderated FTC frequency effects. While I did find notable increases of nutrient levels and EEA across crop rotations, most results were not distinguishable between crop treatments and were ultimately disproven by post-hoc testing. These results contrast the results from previous studies conducted on this site. Through phospholipid fatty acid analysis, Tiemann et al. (2015) found greater bacterial biomass in SOM and microbial metabolism in higher crop diversity treatments. McDaniel et al. (2014b) reported enhanced belowground functions and nutrient cycling along under increasing crop rotations. A meta-analysis conducted by McDaniel and Grandy (2016) reported functionally distinct soil microbial communities in simpler crop rotations and large changes to biodiversity and microbial functioning when introducing cover crops. My original hypothesis was based on the knowledge that the inherent, higher quality of SOM residues and microbial community complexity found in higher crop rotations would be better off at the mineralization and acquisition of materials liberated by FTCs (Venter et al. 2016), which was partially explored in select enzymatic activities.

Respiration and microbial functional differences were evident in the control soils, so the lack of a crop diversity treatment effect after FTC treatment suggests that FTCs were too disruptive on the parameters I measured. On the other hand, it is possible that the crop diversity effects could have been detectable if I measured the parameters after each FTC, rather than only at the end of the incubation. Degens et al. (2001) reported that both drying-rewetting cycles and FTCs caused greater changes in crop soils than pasture soils, but noted that microbial processes

are so deeply intertwined with the chemical and physical environment, that defining a disturbance would be difficult. Future FTC studies should monitor the nutrient dynamics explored in this study after each subsequent FTC, or periodically within FTC frequencies.

Study Limitations

Conducting a laboratory FTC incubation limits effective study comparisons to only other laboratory incubations due to the environmental factors present in field studies that must be emulated or omitted, such as mycorrhizal root interactions and other ecological interactions (Henry, 2007). CO₂ Calculations were made under the assumption that the second respiration rate represented the new baseline for the entire period between freezing events. The assumption may have conflated the cumulative respiration results. However, midpoint respiration measurements made in the low FTC frequency treatment did not suggest any notable differences of the pre-established baseline respiration rate or the need to take another one prior to the next freezing condition (data not shown).

CONCLUSIONS

Developing resilient agroecosystems require a holistic understanding of the mechanisms that confer resilience against soil disturbances. FTCs play a significant role in seasonal soil nutrient dynamics and microbial turnover, and are a rising topic of soil disturbance in SOC loss models. FTCs studies are so variable in design, generalizations of nutrient responses cannot be made. Even so, I conducted a 60 d laboratory incubation in an attempt to capture how crop rotational diversity may confer resilience against increasing FTC frequency by assessing microbial response through nutrient dynamics and microbial functioning and comparing the results to undisturbed soils. I found that increasing FTC frequency increases C and N pools by shortening the amount of time between freezing events, disturbing the microbial communities responsible for nutrient dynamics. These disruptions on the acting microbial community are measurable by tracking CO₂ respiration, soil nutrient and microbial nutrient dynamics. Initial respiratory bursts largely contribute to the total CO₂ respiration, and are responsible for cumulative CO₂ and DOM differences between FTC frequency treatments, even when they subside over time. NH₄⁺ concentrations decreased, while NO₃⁻ concentrations increased with increasing FTC frequency, suggesting elevated nitrification and reduced microbial uptake as indirect results caused by FTCs. Medium FTC frequencies may facilitate net increases in microbial activity and C and N cycling without significant changes to microbial biomass, but whether this is due to a new stable microbial community, and if these levels do return to control levels is uncertain. Unlike previous studies that reported overall increased microbial functioning and microbial diversity (Tiemann et al., 2015; Peralta et al., 2018) at my study site, crop rotational diversity effects were limited in conferring resilience against FTC stress regardless of frequency of FTCs, and had little effect in moderating C and N pools across the crop diversity

gradient except on total oxidase EEA. If the trends observed in this study hold true across other FTC-afflicted cropland soils, microbial communities may be resilient enough against FTCs to influence short-term changes to soil C and N pools, which may lead to long-term changes in soil C and N transformations regardless of crop rotational diversity in agroecosystems.

APPENDIX

Table 1. Freeze-thaw cycle (FTC) study design with respiration and nutrient response observations. Minimum soil temperature is defined as the lowest temperature recorded or established that would generally represent "freezing" conditions and categorized into the following groups: Extreme (Less than -10 °C), Moderate (Between -10 and -5 °C) and Low (Between -5 and 0 °C). Grouped FTC frequency is defined by the number of measured FTCs elapsed within the study period: Single (1), Low (Less than 3 FTCs), Moderate (4-10 FTCs), and High (Greater than 10 FTCs).

Land Type	Type of Study*	Minimum Soil Temperature (°C)	Grouped Temp. Rating	Thaw Temp. (°C)	Freeze Duration (h)	Thaw Duration (h)	No. of FTCs	Grouped FTC Frequency	Overall 1 CO ₂	DOC/ DON	NH ₄ ⁺ /NO ₃ ⁻	MBC/ MBN	EEA
Alpine	Lab ¹	-9	Moderate	4	12	12	4	Moderate			+	+	0
Alpine	Lab ¹	-9	Moderate	4	12	12	1	Single			+	+	0
Alpine	Lab ²	-15	Extreme	2	144	24	1	Single			-	+	-
Alpine	Lab ²	-15	Extreme	5	144	24	1	Single			-	+	-
Alpine	Lab ²	-3	Low	2	144	24	1	Single			+	-	+
Alpine	Lab ²	-3	Low	5	144	24	1	Single			+	-	+
Alpine	Lab ²	-9	Moderate	2	144	24	1	Single			-	+	+
Alpine	Lab ²	-9	Moderate	5	144	24	1	Single			+	+	+
Alpine	Lab ³	-4	Low	4	9		50	High	-				
Alpine	Lab ³	-4	Low	4	9		50	High	-				
Alpine	Lab ⁴	-5	Moderate	5	120	48	3	Moderate	-		-	+	
Alpine	Lab ⁴	-5	Moderate	5	120		1	Single	+		+	+	
Alpine	Lab ⁴	-5	Moderate	5	456		1	Single	+		+	+	
Arctic	Lab ⁵	-15	Extreme	10	9	15						0	
Arctic	Lab ⁶	-4	Low	2	15	9	18	High	-			+	-/+
Arctic	Lab ⁷	-18	Extreme	4	168	720	1	Single	+				
Crop	Field ⁸	-1	Low	1	2404.8		20	High	+	+	+	+	0
Crop	Lab ⁹	-5	Moderate	10	1680	720	1	Single	-	+		-/+	
Crop	Lab ¹⁰	0, -1, -2, -5, -10	Low	3	1			Moderate			+	-	
Crop	Lab ¹¹	-2	Low	3	192	192	3	Moderate	-		+	+	-
Crop	Lab ¹¹	-2	Low	3	96	96	6	Moderate	-		+	-	-
Crop	Lab ¹²	-2, then -5	Low	2, then 5	6, then 16	4, then 22	20	High	+				-
Crop	Lab ¹³	-5	Moderate	5	24	24	15	High		+		-/+	0
Crop	Lab ¹³	-10	Moderate	10	24	24	15	High		+		-/+	-
Crop	Lab ¹⁴	-3	Low	1	576	48	1	Single	0	0		-	
Crop	Lab ¹⁴	-1	Low	1	1152	48	1	Single	0	0		0	
Crop	Lab ¹⁴	-1	Low	1	1152	48	1	Single	0	0		+	

Table 1 (cont'd)

Land Type	Type of Study*	Minimum Soil Temperature (°C)	Grouped Temp. Rating	Thaw Temp. (°C)	Freeze Duration (h)	Thaw Duration (h)	No. of FTCs	Grouped FTC Frequency	Overall 1 CO ₂	DOC/ DON	NH ₄ ⁺ /NO ₃ ⁻	MBC/ MBN	EEA
Crop	Lab ¹⁴	-1	Low	1	576	48	1	Single	-	0		-	
Crop	Lab ¹⁴	-1	Low	1	576	48	1	Single	-	0		0	
Crop	Lab ¹⁴	-3	Low	1	576	48	1	Single	-	0		-	
Crop	Lab ¹⁴	-1	Low	1	1152	48	1	Single	+	0		-	
Crop	Lab ¹⁴	-1	Low	1	576	48	1	Single	+	+		-	
Crop	Lab ¹⁴	-1	Low	1	1152	48	1	Single	+	0		-	
Crop	Lab ¹⁴	-3	Low	1	576	48	1	Single	+	+		-	
Crop	Lab ¹⁴	-3	Low	1	1152	48	1	Single	+	+		0	
Crop	Lab ¹⁴	-3	Low	1	1152	48	1	Single	+	0		-	
Crop	Lab ¹⁴	-1	Low	1	576	48	1	Single	+	+		0	
Crop	Lab ¹⁴	-3	Low	1	576	48	1	Single	+	+		0	
Crop	Lab ¹⁴	-3	Low	1	1152	48	1	Single	+	0		-	
Crop	Lab ¹⁴	-3	Low	1	1152	48	1	Single	+	0		-	
Crop	Lab ¹⁵	-20	Extreme	10	24		1	Single	+	+			0
Forest	Field ⁸	-4	Low	1	2932.8		8	Moderate	+	+	+	+	-/+
Forest	Field ¹⁶		Low									-	
Forest	Field ¹⁷		Low								+	0	
Forest	Field ¹⁸	-2	Low	2			12	High			0		+
Forest	Field ¹⁸	-2	Low	2			12	High			0		0
Forest	Field ¹⁸	-2	Low	2			12	High			-		0
Forest	Field ¹⁸	-2	Low	2			12	High			+/-		0
Forest	Field ¹⁹	-4.14	Low	1	72	72	4	Moderate	-			+	0
Forest	Field ²⁰	-0.2	Low	13.7	12	12	16	High				0	
Forest	Field ²⁰	-4.1	Low	27.1	12	12	82	High				-	
Forest	Field ²⁰	-2.6	Low	10.6	12	12	29	High				-	
Forest	Field ²⁰	-1.9	Low	6.3	12	12	7	Moderate				-	
Forest	Field ²⁰	-0.3	Low	15.3	12	12	5	Moderate				-	
Forest	Field ²⁰	-1.2	Low	13.7	12	12	7	Moderate				-	
Forest	Field ²⁰	-5.7	Moderate	11.2	12	12	58	High				0	
Forest	Field ²¹		Low					Moderate			+	+	+
Forest	Lab ¹	-9	Moderate	4	12	12	4	Moderate			+	+	0
Forest	Lab ¹	-9	Moderate	4	12	12	1	Single			+	+	0
Forest	Lab ²²	-13	Extreme	5	336	168	3	Moderate	+		0	+/-	
Forest	Lab ²²	-3	Low	5	336	168	3	Moderate	+		-	+/-	
Forest	Lab ²²	-8	Moderate	5	336	168	3	Moderate	+		+/-	+/-	

Table 1 (cont'd)

Land Type	Type of Study*	Minimum Soil Temperature (°C)	Grouped Temp. Rating	Thaw Temp. (°C)	Freeze Duration (h)	Thaw Duration (h)	No. of FTCs	Grouped FTC Frequency	Overall 1 CO ₂	DOC/DON	NH ₄ ⁺ /NO ₃ ⁻	MBC/MBN	EEA
Forest	Lab ²³	-5	Moderate	5	12	12	7	Moderate			+	+/-	
Forest	Lab ²³	-10	Moderate	10	12	12	7	Moderate			+	+/-	
Forest	Lab ²⁴	-8	Moderate	10	1440	240	1	Single	+	+	+	+	+
Forest	Lab ²⁵	-2	Low	10	12	12	6	Moderate	-				+
Forest	Lab ²⁵	-5	Moderate	10	12	12	6	Moderate	-				+
Forest	Lab ²⁶	-18	Extreme	10	240 , 3480	48	1	Single	-	+	+	+/-	-
Forest	Lab ²⁶	-80	Extreme	10	240 , 3480	48	1	Single	-	+	+	+/-	-
Forest	Lab ²⁶	-8	Moderate	10	240 , 3480	48	1	Single	-	+	+	+/-	-
Grass	Field ²⁷	-2	Low	10	15		4	Moderate			-	-/+	
Grass	Lab ¹	-9	Moderate	4	12	12	4	Moderate			+	+	0
Grass	Lab ¹	-9	Moderate	4	12	12	1	Single			+	+	0
Grass	Lab ¹¹	-2	Low	3	192	192	3	Moderate	-		+	+	-
Grass	Lab ¹¹	-2	Low	3	96	96	6	Moderate	-		+	-	-
Grass	Lab ²⁸	-20	Extreme	5	24			Moderate	-		+		
Grass	Lab ²⁸	-5	Moderate	5	24		1	Single	-		+		
Grass	Lab ²⁹	-10	Extreme	10	12	12	20	High	+	+	+	+	
Grass	Lab ²⁴	-10	Moderate	5	168	168	3	Moderate	+	+		-	

*References: **1.** Freppaz et al., 2007; **2.** Jiang et al., 2018; **3.** Stres et al., 2010; **4.** Wipf et al., 2015; **5.** Yergeau and Kowalchuk, 2008; **6.** Larsen et al., 2002; **7.** Walz et al., 2017; **8.** Chen et al., 2020; **9.** Bhowmik et al., 2017; **10.** Elliot and Henry, 2009; **11.** Han et al., 2018; **12.** Hermann and Witter, 2002; **13.** Liu et al., 2016; **14.** Pelster et al., 2019; **15.** Sharma et al., 2006; **16.** Chen et al., 2020; **17.** Durán et al., 2016; **18.** Fuss et al., 2016; **19.** Hosokawa et al., 2017; **20.** Sorensen et al., 2018; **21.** Urakawa et al., 2014; **22.** Watanabe et al., 2019; **23.** Hentschel et al., 2008; **24.** Shibata et al., 2013; **25.** Wu et al., 2021; **26.** Xu et al., 2016; **27.** Joseph and Henry, 2008; **28.** Miura et al., 2019; **29.** Song et al., 2017.

Table 2. Summary of two-way ANOVA results with Crop Diversity and FTC frequency as main and interactive effects, with LS Means *post hoc* test results. Treatments separated by < or > indicate significant differences, while those separated by a comma are not different. Significant P-values, at $P < .05$ are emboldened. If a treatment is not listed, then it is not significantly different from any of the treatments listed e.g. MC < CSW implies all other crop treatments are not different from either MC or CSW. See main text for crop diversity treatment abbreviations.

Parameter	ANOVA Main Effects and Interaction						Post hoc Tests	
	Crop Diversity		FTC Frequency		Crop Diversity * FTC Frequency		Crop Diversity	FTC Frequency
	F	P Value	F	P Value	F	P Value		
Total Cumulative Respiration	3.11	0.015	5.62	0.006	0.39	0.947	MC < CSW, C1 > CSW1, CSW > CSW1	Low > High, Med > High
Day 1 Burst Respiration	1.81	0.182	1.32	0.281	1.66	0.311		
Day 1 Baseline Respiration	2.07	0.135	7.86	0.002	0.82	0.612		Low < Med, Med > High
Day 1 Cumulative Respiration	0.2	0.958	4.33	0.021	1.28	0.277		Low < Med
Day 60 Burst Respiration	1.44	0.263	0.51	0.606	0.68	0.736		
Day 60 Baseline Respiration	0.31	0.902	10.43	0.0003	1.02	0.446		Low < High, Med < High
Day 60 Cumulative Respiration	1.22	0.360	1.18	0.321	0.37	0.951		
Average Burst Respiration	3.08	0.051	10.03	0.0003	0.46	0.904	MC < CSW, CS > CSW1, CSW > CSW1	Low > Med, Low > High
Average Baseline Respiration	2.12	0.114	17.00	<.0001	0.7	0.715		Low < Med, Med > High
Ammonium	0.27	0.926	9.15	0.0004	0.97	0.482		Low > Med, Low > High

Table 2 (cont'd)

Parameter	ANOVA Main Effects and Interaction						Post hoc Tests	
Nitrate	0.24	0.937	16.55	<0.0001	2.14	0.051		Low > Med, Low < High
DOC	1.2	0.353	36.96	<0.0001	36.1	0.987		Low < High, Med < High
DON	1.48	0.277	9.69	0.001	2.27	0.054		Low < Med, Low < High
PHOS	2.67	0.032	109.94	<0.0001	0.52	0.869	MC > C1, MC > CS, MC > CSW	Low < Med, Low < High, Med > High
LAP	1.99	0.143	35.69	<0.0001	1.78	0.104		Low > High, Med > High
Total Oxidase	2.83	0.049	95.79	<0.0001	2.21	0.039	See Fig. 14	See Fig. 14

Table 3. Summary of one-way ANOVA results of control (no FTC) soils only with Crop Diversity as the main effect and Dunnett's *post hoc* test results of control versus all FTC treatments. Treatments separated by < or > indicate significant differences, while those separated by a comma are not different. Significant P-values, at $P < .05$ are emboldened. If a treatment is not listed, then it is not significantly different from any of the treatments listed e.g. MC < CSW implies all other crop treatments are not different from either MC or CSW. See main text for crop diversity treatment abbreviations.

Parameter	ANOVA Results Crop Diversity		<i>Post hoc</i> Comparisons	Control vs. FTC	Dunnett's <i>Post-hoc</i> FTC
	F	P Value		P Value	
Total Cumulative Respiration	4.66	0.0066	MC < CSW1, C1 < CSW1, CS < CSW1, CS < CSW2, CSW < CSW1, CSW < CSW2	<0.0001	Control < Low, Control < Med, Control < High
Day 1 Burst Respiration	4.87	0.0076	MC < C1, C1 > CS, C1 > CSW, C1 > CSW1, C1 > CSW2	<0.0001	Control < Low, Control < Med, Control < High
Day 1 Baseline Respiration	4.87	0.0076	MC < C1, C1 > CS, C1 > CSW, C1 > CSW2	0.0085	Control < Med
Day 1 Cumulative Respiration	0.96	0.469		0.0046	Control > Low, Control > High
Day 60 Burst Respiration	3.66	0.0185	MC < C1, MC < CSW1, C1 > CSW, CS < CSW1, CSW < CSW1	0.0413	Control > High
Day 60 Baseline Respiration	3.66	0.0185	MC < C1, MC < CSW1, C1 > CSW, CS < CSW1, CSW < CSW1	<0.0001	Control > Low, Control > Med, Control > High
Day 60 Cumulative Respiration	6.37	0.0014	MC < CSW1, C1 < CSW1, C1 < CSW2, CS < CSW1, CS < CSW2, CSW < CSW1, CSW < CSW2	<0.0001	Control > Low, Control > Med, Control > High
Average Burst Respiration	5.28	0.0037	MC < CSW1, C1 < CSW1, CS < CSW1, CS < CSW2, CSW < CSW1, CSW < CSW2	<0.0001	Control < Low
Average Baseline Respiration	5.28	0.0037	MC < CSW1, C1 < CSW1, CS < CSW1, CS < CSW2, CSW < CSW1, CSW < CSW2	<0.0001	Control > Low, Control > High
Ammonium	0.58	0.7126		0.0116	Control > Med, Control > High
Nitrate	5.21	0.0057	MC < CSW1, MC < CSW2, C1 < CSW1, C1 < CSW2, CS < CSW1, CS < CSW2, CSW < CSW1, CSW < CSW2	<0.0001	Control < Med, Control < High
DOC	0.73	0.6128		<0.0001	Control < Low, Control < High

Table 3 (cont'd)

Parameter	ANOVA Results Crop Diversity		<i>Post hoc</i> Comparisons	Control vs. FTC	Dunnett's <i>Post-hoc</i> FTC
	F	P Value		P Value	
DON	0.82	0.559		0.0002	Control < Low, Control < Med, Control < High
MBC	7.61	0.0010	MC < CSW1, MC < CSW2, C1 < CSW2, CS < CSW1, CS < CSW2, CSW < CSW1, CSW < CSW2	0.0021	Control > High
MBN	0.32	0.8911		0.0005	Control < Low
C:N	0.5167	0.89		0.0239	Control > Low
BG	13.10	<0.0001	MC < C1, MC < CSW, MC < CSW1, MC < CSW2, C1 < CSW1, C1 < CSW2, CS < CSW1, CS < CSW2, CSW < CSW2	<0.0001	Control < Med, Control < High
CBH	11.7	<0.0001	MC < C1, MC < CSW, MC < CSW1, MC < CSW2, C1 < CSW1, C1 < CSW2, CS < CSW1, CS < CSW2, CSW < CSW1, CSW < CSW2	<0.0001	Control < Med, Control < High
NAG	5.37	0.005	MC < C1, MC < CSW1, MC < CSW2, CS < CSW1, CS < CSW2, CSW < CSW1	<0.0001	Control < Med
PHOS	5.34	0.0051	MC < C1, MC < CSW, MC < CSW1, MC < CSW2, CS < C1, CS < CSW1, CS < CSW2	<0.0001	Control < Med, Control < High
LAP	3.45	0.0284	MC < CSW1, MC < CSW2, C1 < CSW1, CS < CSW1	<0.0001	Control < Low, Control < Med, Control < High
Total Oxidase	2.40	0.0869		<0.0001	Control < Med, Control < High

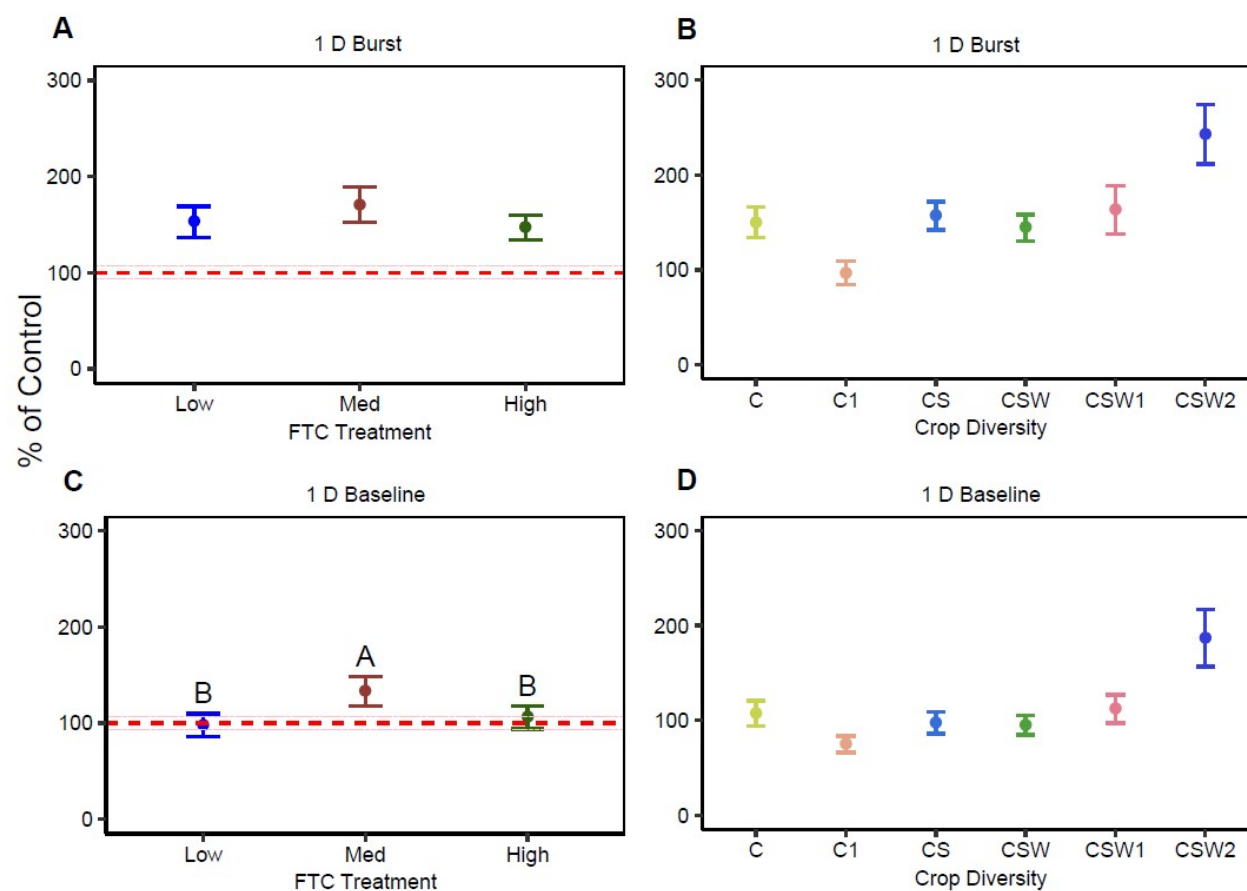


Figure 1. Soil respiration rates as a percentage of non-frozen control soils on the first day (1 d) of the incubation experiment. Data are presented as burst (thawing) respiration rates by freeze-thaw cycle (FTC) frequency (a) or crop diversity (b) and as baseline (post thawing) respiration rates by FTC frequency (c) or crop diversity (d). Crop rotational diversity abbreviations: monoculture corn (MC), corn + red clover cover crop (C1), corn-soy (CS), corn-soy-wheat (CSW), corn-soy-wheat + red clover (CSW1), and corn-soy-wheat with red clover and rye cover crops (CSW2). Data are means $\pm$ one standard error (n=4) and letters indicate significant differences between treatments. The red dotted line represents a 0% difference between treatment and control soils $\pm$ one standard error.

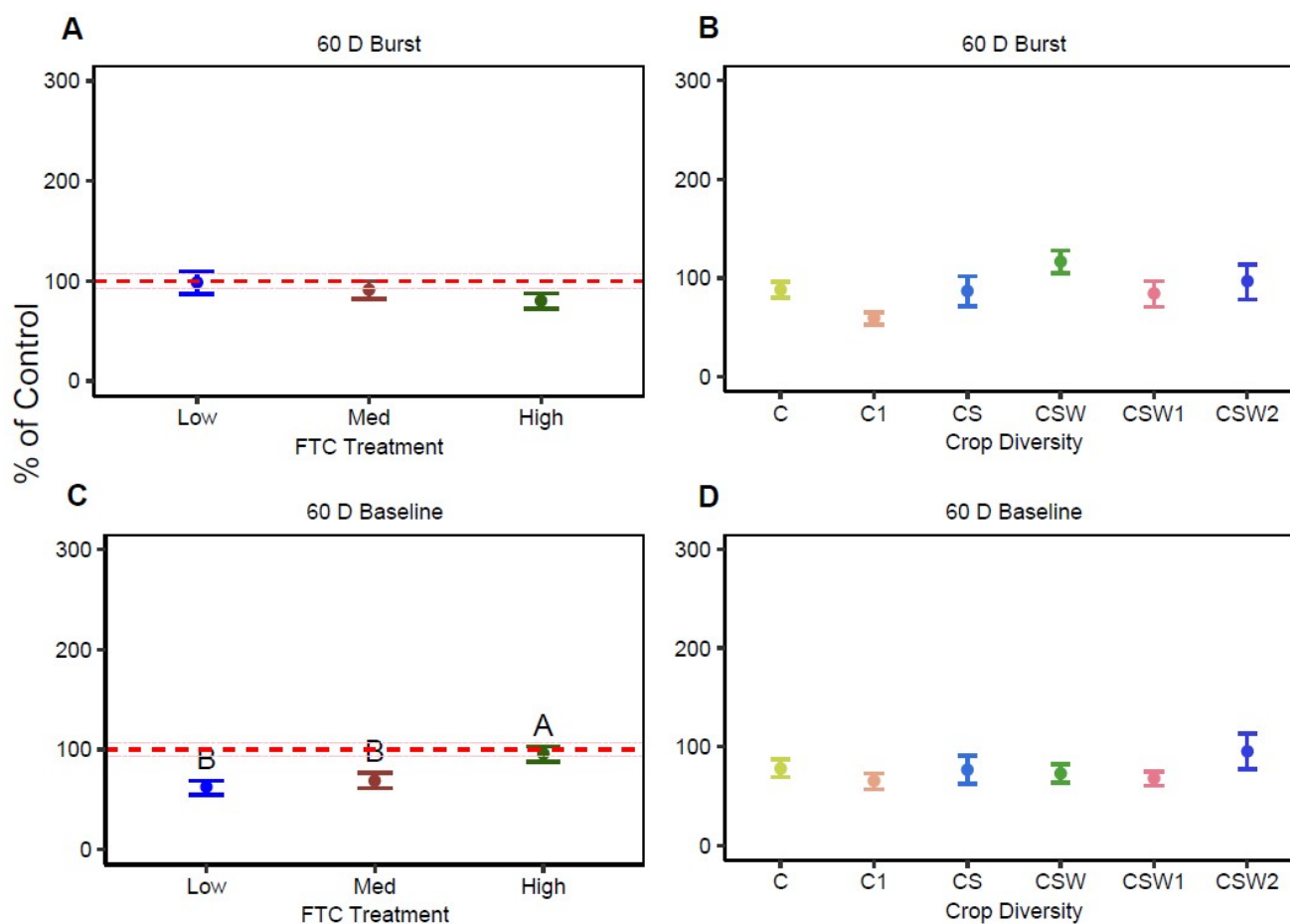


Figure 2. Soil respiration rates as a percentage of non-frozen control soils on the last day (60 d) of the incubation experiment. Data are presented as burst (thawing) respiration rates by FTC frequency (a) or crop diversity (b) and as baseline respiration rates by FTC frequency (c) or crop diversity (d). Data are means $\pm$ one standard error (n=4) and letters indicate significant differences between treatments. The red dotted line represents a 0% difference between treatment and control soils $\pm$ one standard error.

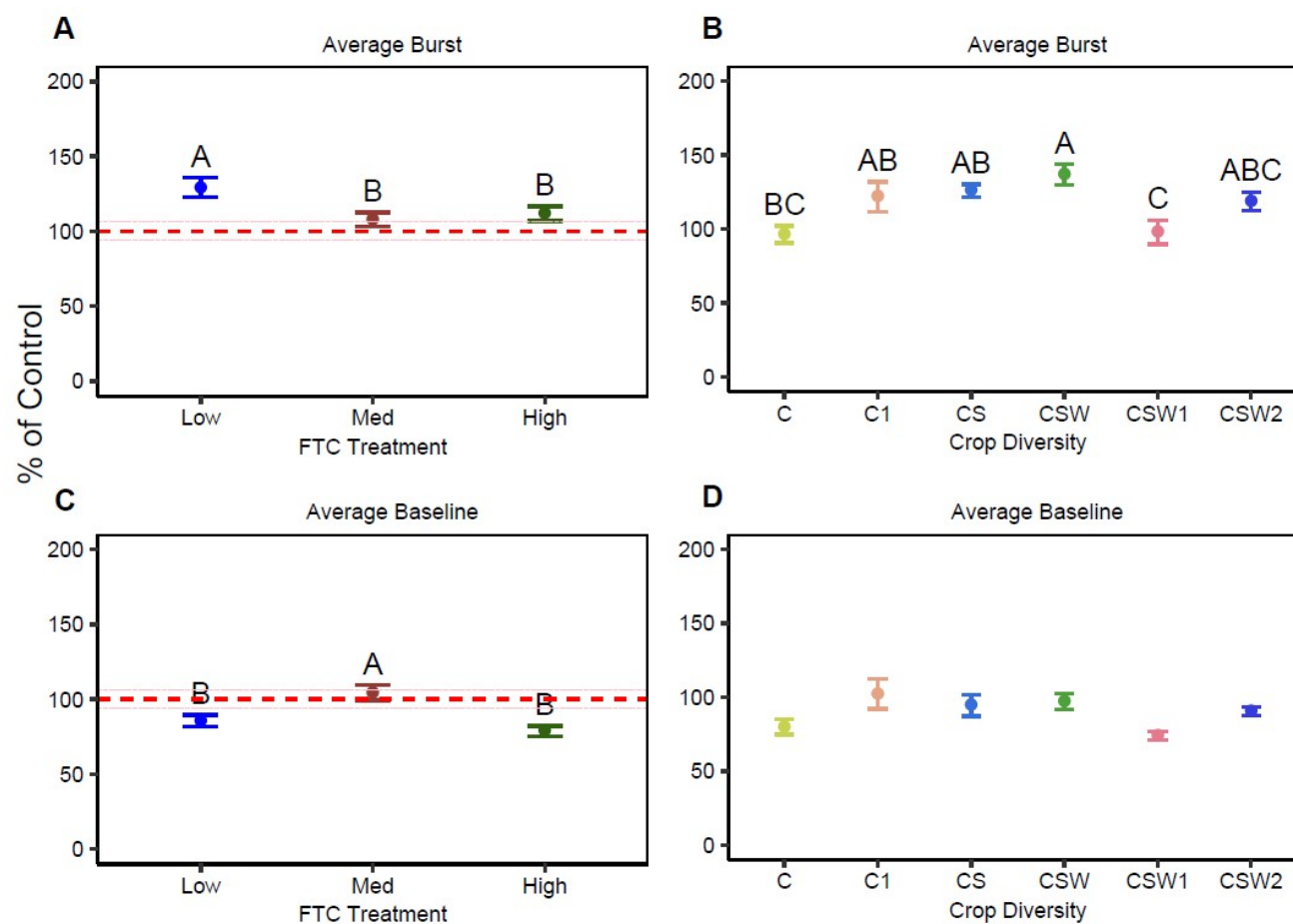


Figure 3. Soil respiration rates as a percentage of non-frozen control soils during the incubation experiment. Data are presented as the average burst (thawing) respiration rates by FTC frequency (a) or crop diversity (b) and as average baseline respiration rates by FTC frequency (c) or crop diversity (d). Data are means $\pm$ one standard error (n=4) and letters indicate significant differences between treatments. The red dotted line represents a 0% difference between treatment and control soils $\pm$ one standard error.

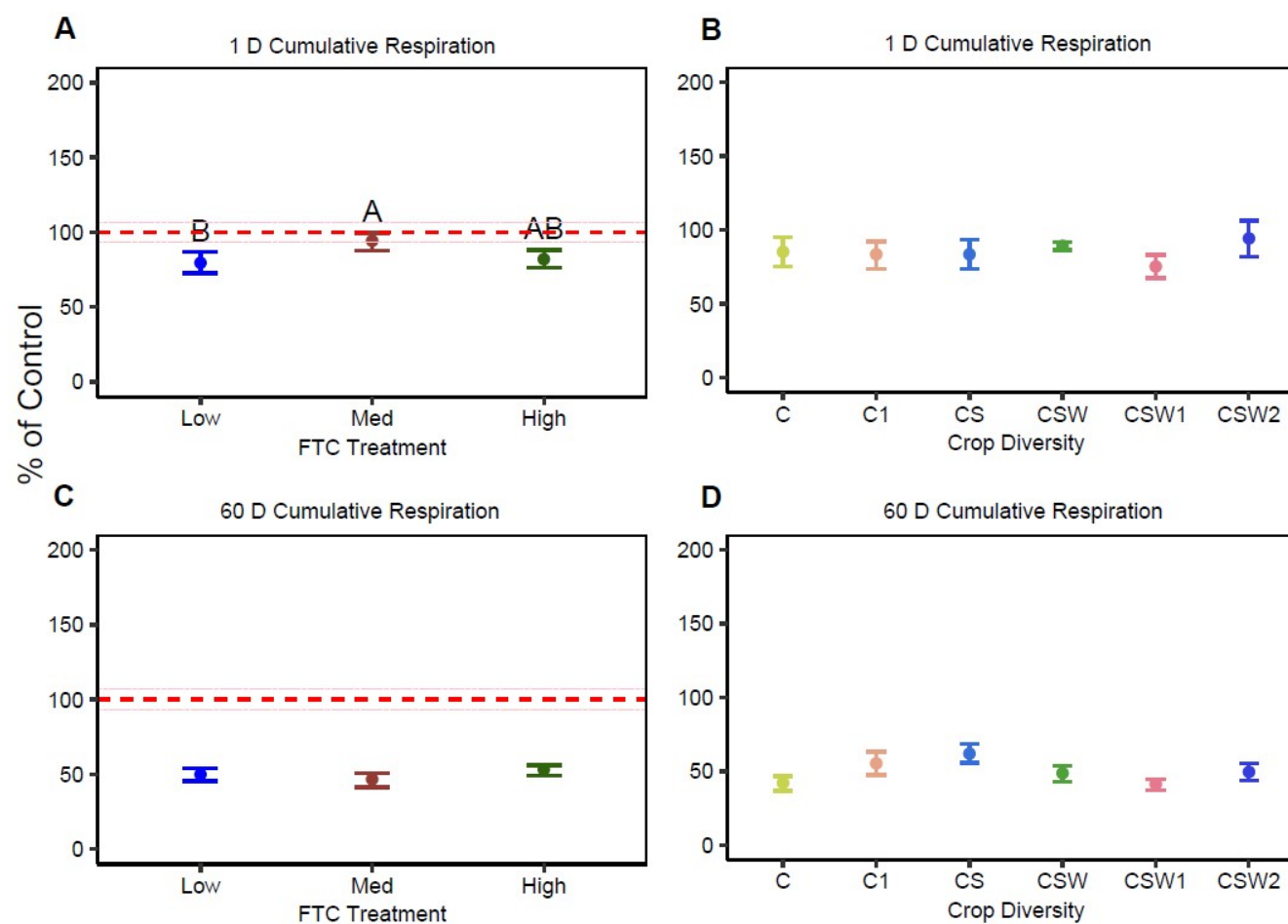


Figure 4. Cumulative CO₂ respiration as a percentage of non-frozen control soils on 1 d and 60 d of the incubation experiment. Data are presented as the cumulative respiration on 1 d by FTC frequency (a) or crop diversity (b) and as cumulative respiration on 60 d by FTC frequency (c) or crop diversity (d). Data are means $\pm$ one standard error (n=4) and letters indicate significant differences between treatments. The red dotted line represents a 0% difference between treatment and control soils $\pm$ one standard error.

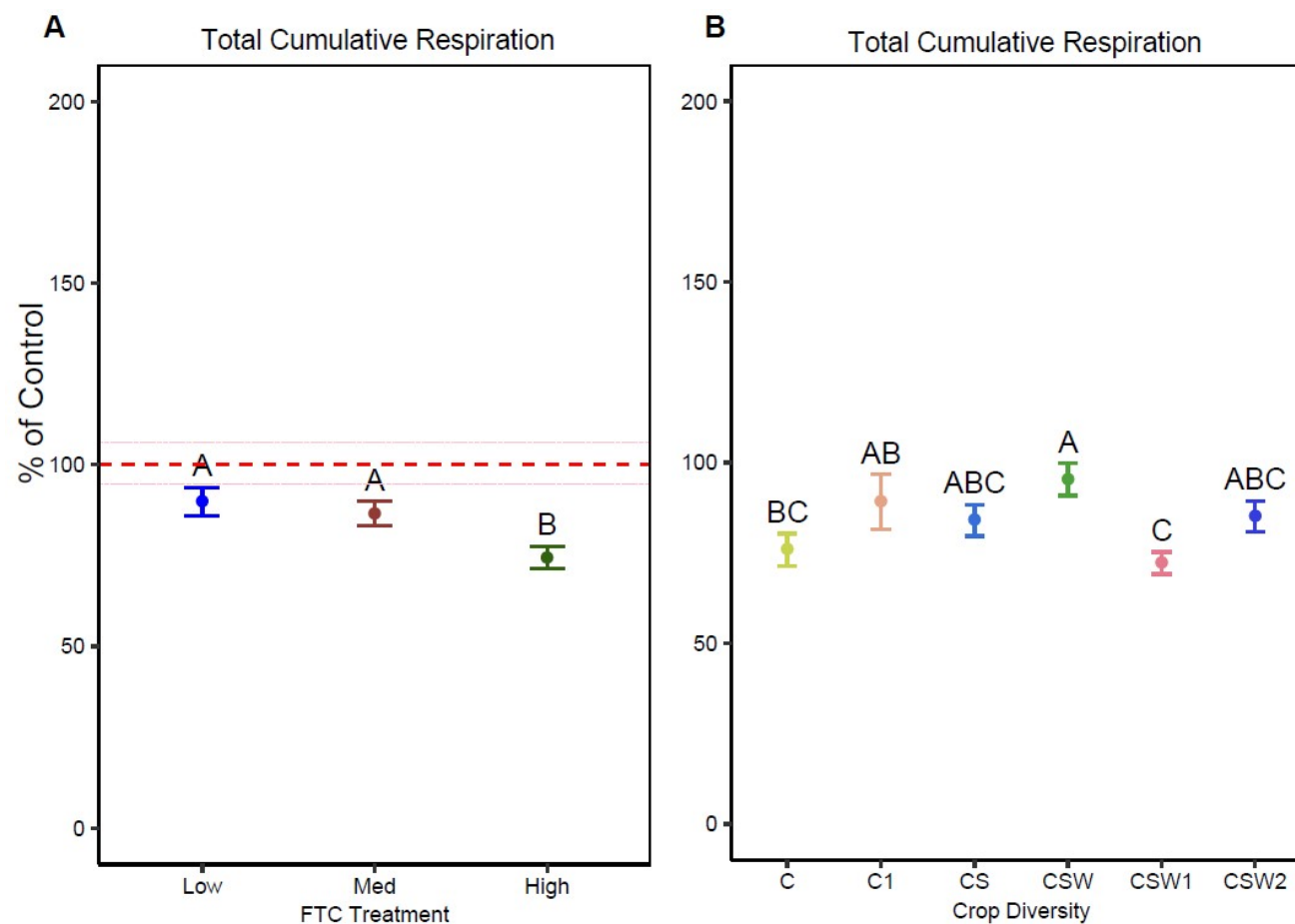


Figure 5. Total Cumulative CO₂ respiration as a percentage of non-frozen control for the incubation experiment. Data are presented as the cumulative respiration by FTC frequency (a) or crop diversity (b). Data are means $\pm$ one standard error (n=4) and letters indicate significant differences between treatments. The red dotted line represents a 0% difference between treatment and control soils $\pm$ one standard error.

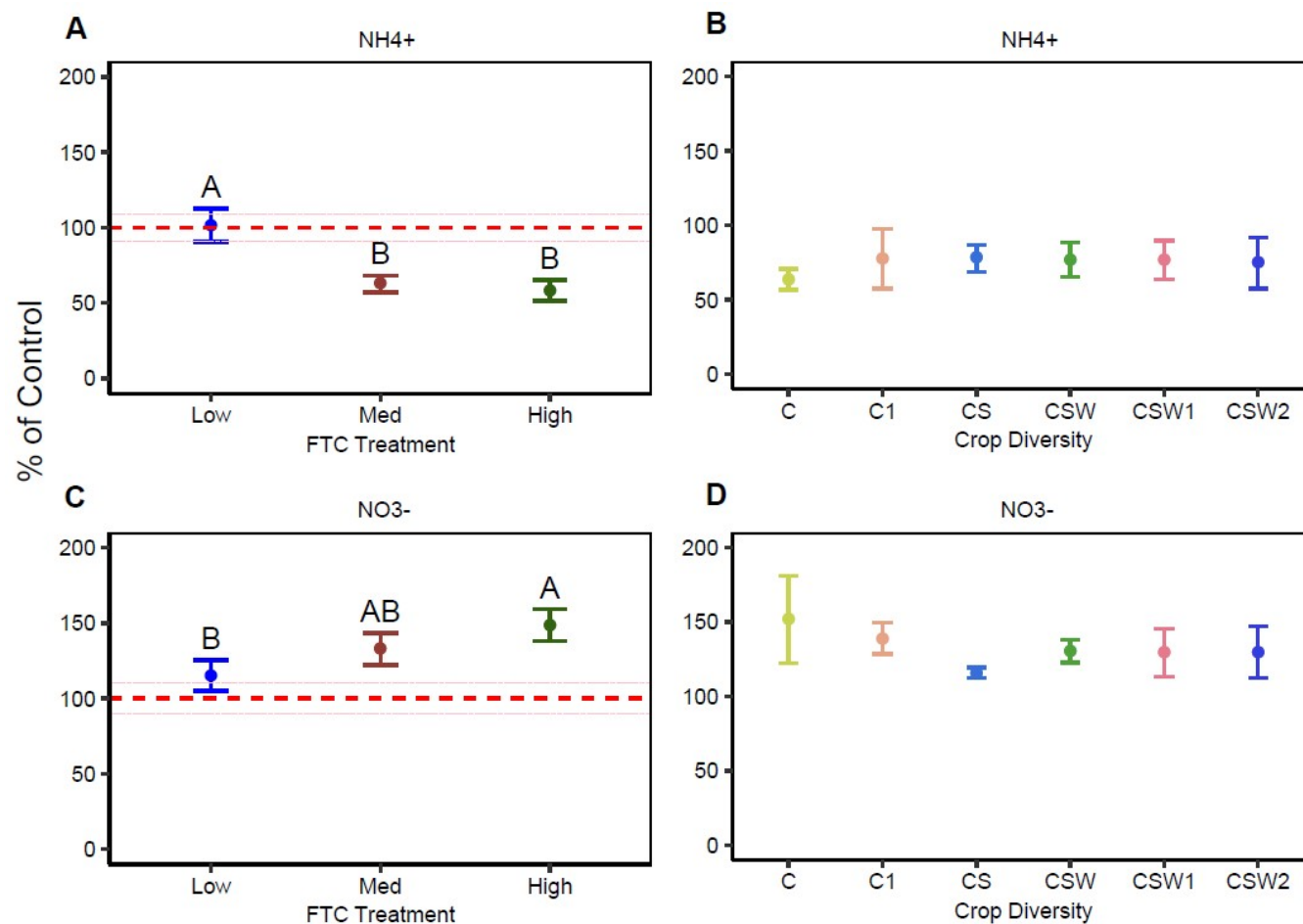


Figure 6. Ammonium (NH_4^+) and nitrate (NO_3^-) content as a percentage of non-frozen control after the incubation experiment. Data are presented as the NH_4^+ content by FTC frequency (a) or crop diversity (b) and as NO_3^- content by FTC frequency (c) or crop diversity (d). Data are means $\pm$ one standard error ($n=4$) and letters indicate significant differences between treatments. The red dotted line represents a 0% difference between treatment and control soils $\pm$ one standard error.

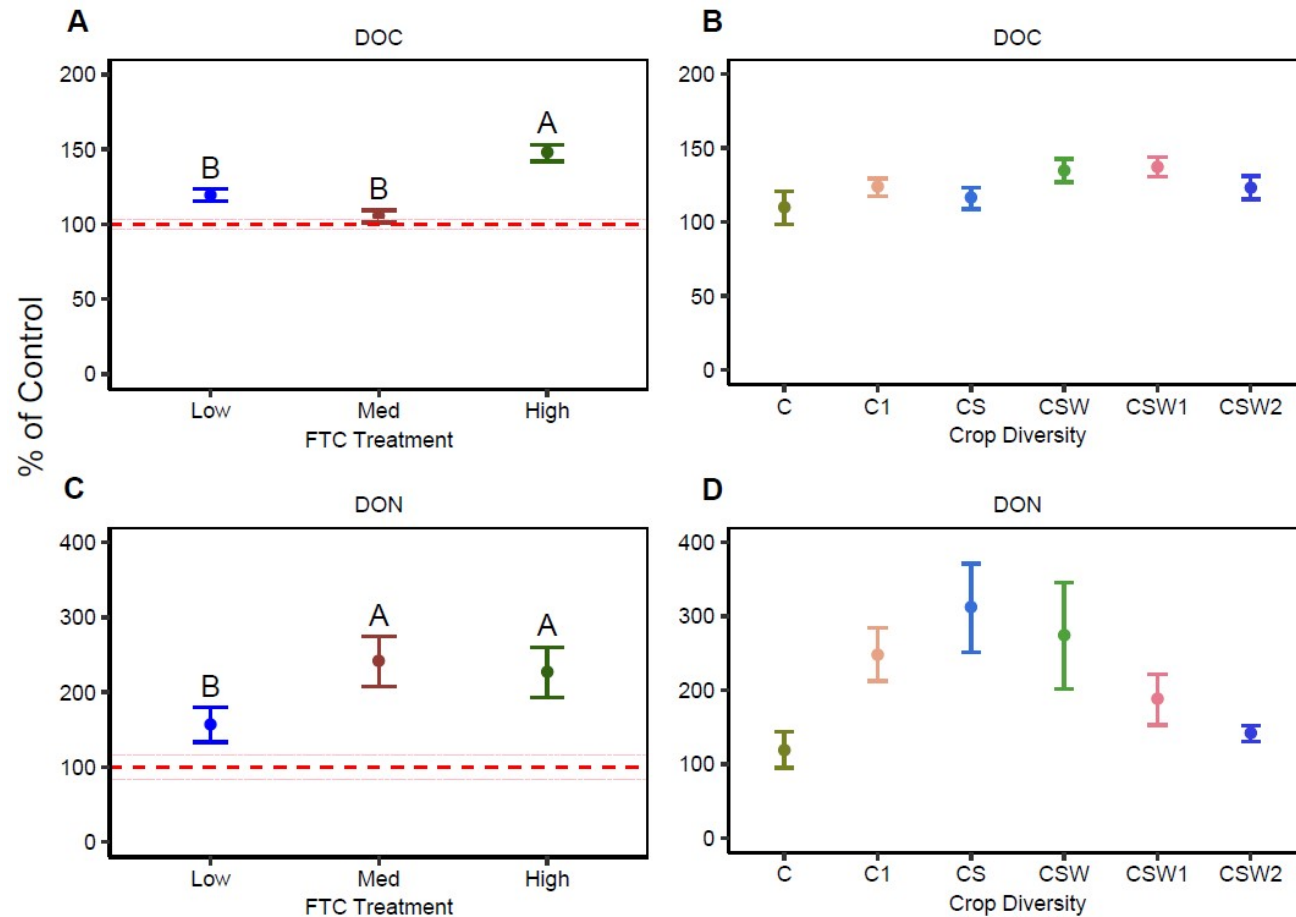


Figure 7. Dissolved organic carbon (DOC) and dissolved organic nitrogen (DON) as a percentage of non-frozen control after the incubation experiment. Data are presented as the DOC content by FTC frequency (a) or crop diversity (b) and DON content by FTC frequency (c) or crop diversity (d). Data are means $\pm$ one standard error (n=4) and letters indicate significant differences between treatments. The red dotted line represents a 0% difference between treatment and control soils $\pm$ one standard error.

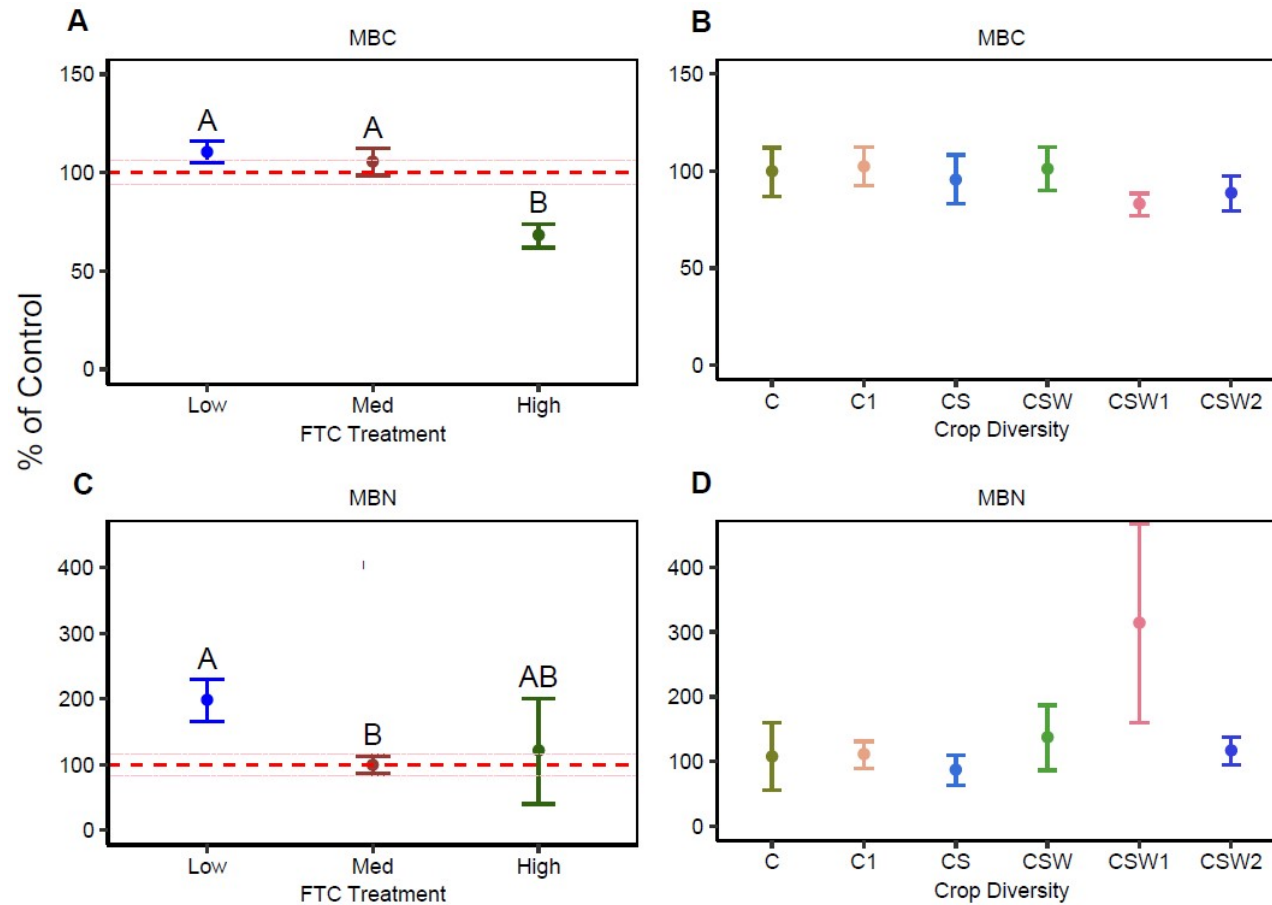


Figure 8. Microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) content as a percentage of non-frozen control after the incubation experiment. Data are presented as the MBC content by FTC frequency (a) or crop diversity (b) and MBN content by FTC frequency (c) or crop diversity (d). Data are means $\pm$ one standard error ($n=4$) and letters indicate significant differences between treatments. The red dotted line represents a 0% difference between treatment and control soils $\pm$ one standard error.

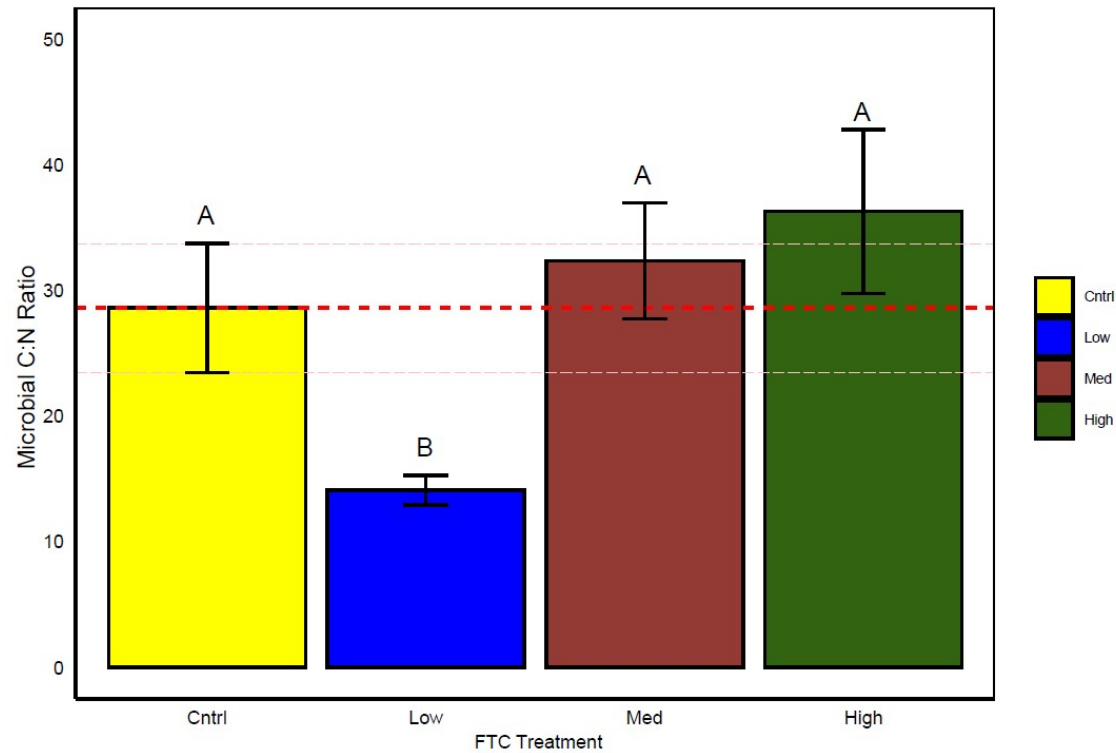


Figure 9. Microbial C:N ratio after the incubation experiment by FTC frequency. Data are means $\pm$ one standard error (n=4) and letters indicate significant differences between treatments. The red dotted line represents the average microbial C:N ratio of control soils $\pm$ one standard error.

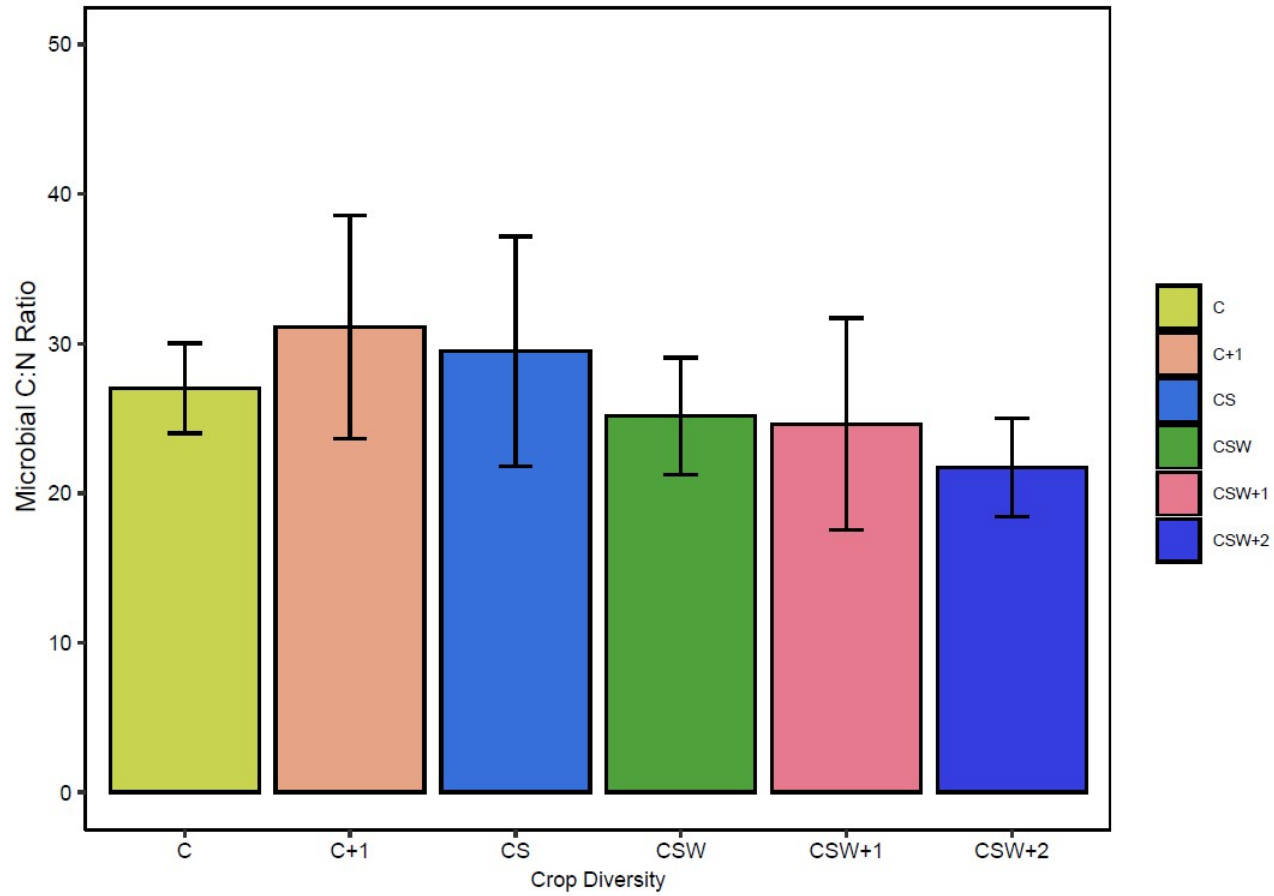


Figure 10. Microbial C:N ratio after the incubation experiment by crop diversity. Data are means $\pm$ one standard error (n=4) and letters indicate significant differences between treatments.

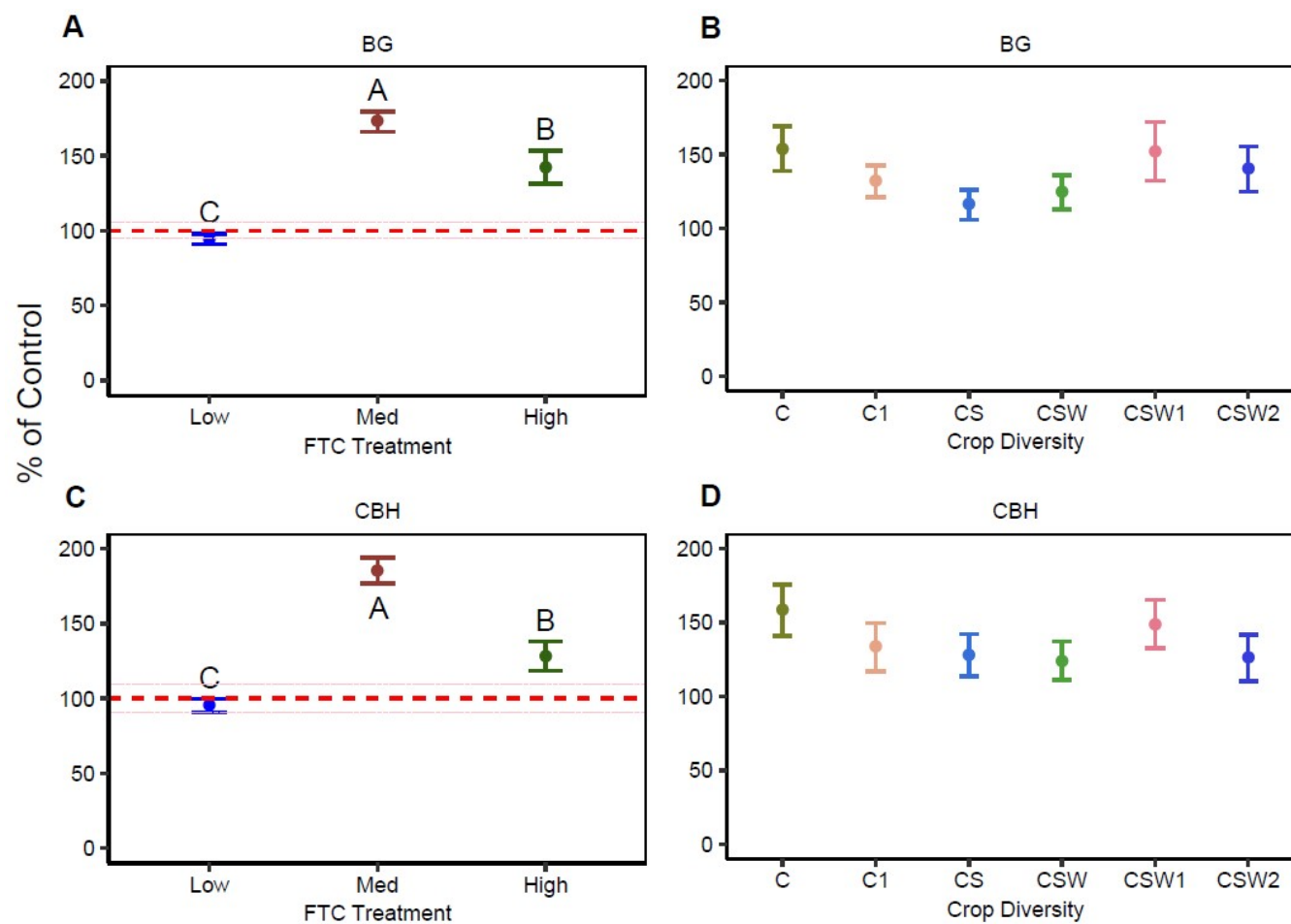


Figure 11. β -1,4-glucosidase (BG) and β -D-1,4-cellobiohydrolase (CBH) activity as a percentage of non-frozen control after the incubation experiment. Data are presented as BG activity by FTC frequency (a) or crop diversity (b) and CBH activity by FTC frequency (c) or crop diversity (d). Data are means $\pm$ one standard error (n=4) and letters indicate significant differences between treatments. The red dotted line represents a 0% difference between treatment and control soils $\pm$ one standard error.

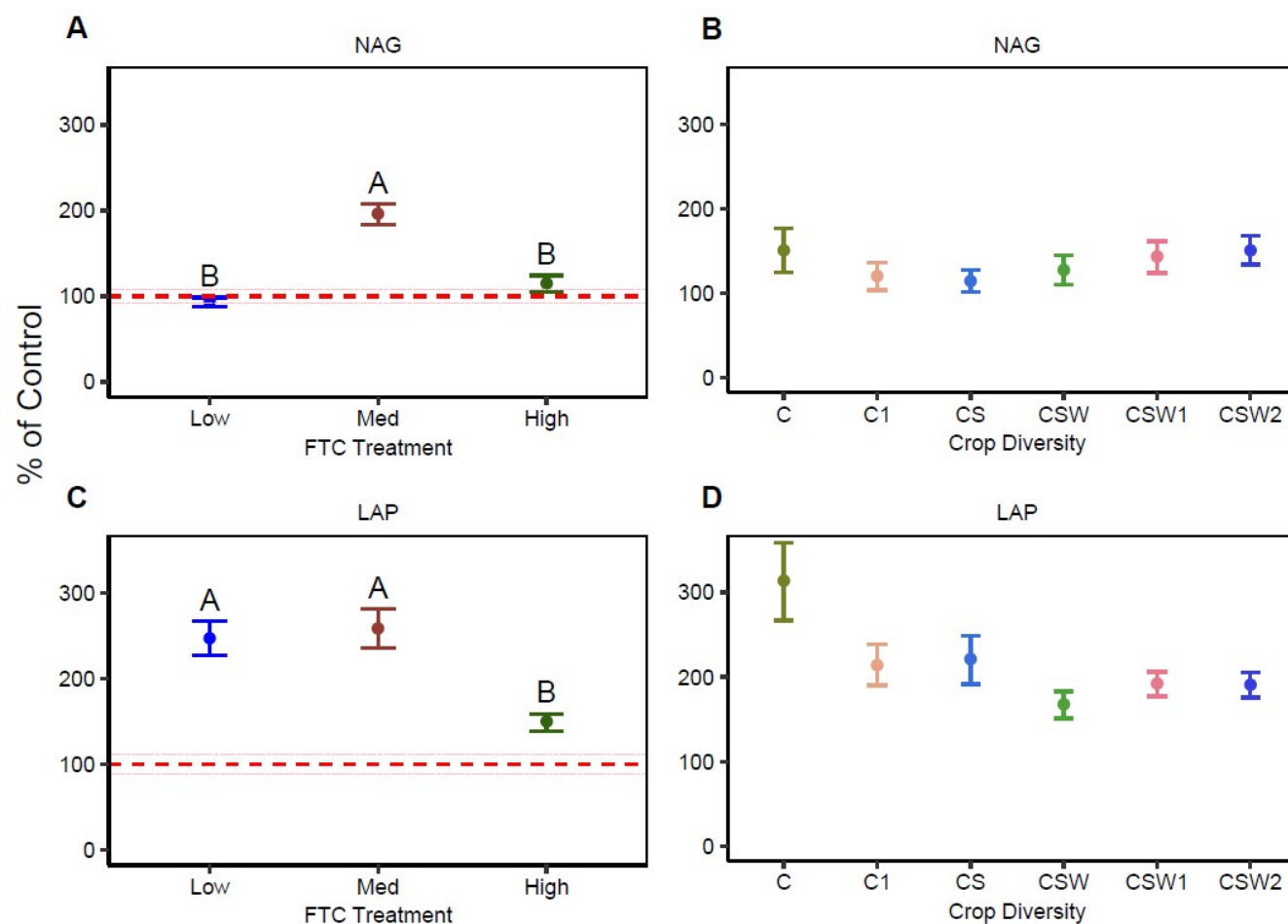


Figure 12. N-acetyl-glucosaminidase (NAG) and leucine aminopeptidase (LAP) activity as a percentage of non-frozen control after the incubation experiment. Data are presented as NAG activity by FTC frequency (a) or crop diversity (b) and LAP activity by FTC frequency (c) or crop diversity (d). Data are means $\pm$ one standard error (n=4) and letters indicate significant differences between treatments. The red dotted line represents a 0% difference between treatment and control soils $\pm$ one standard error.

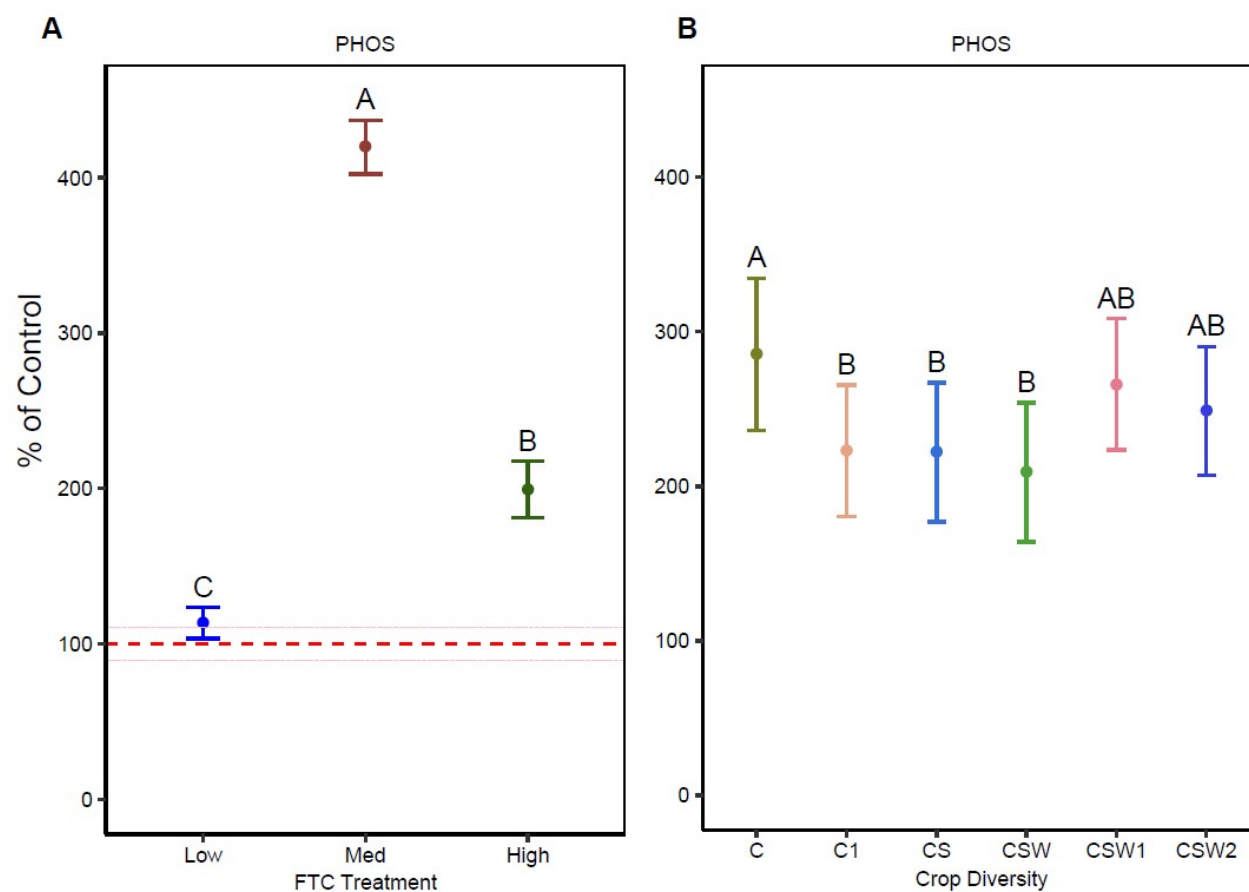


Figure 13. Acid phosphatase (PHOS) activity as a percentage of non-frozen control after the incubation experiment. Data are presented as PHOS activity by FTC frequency (a) or crop diversity (b). Data are means $\pm$ one standard error (n=4) and letters indicate significant differences between treatments. The red dotted line represents a 0% difference between treatment and control soils $\pm$ one standard error.

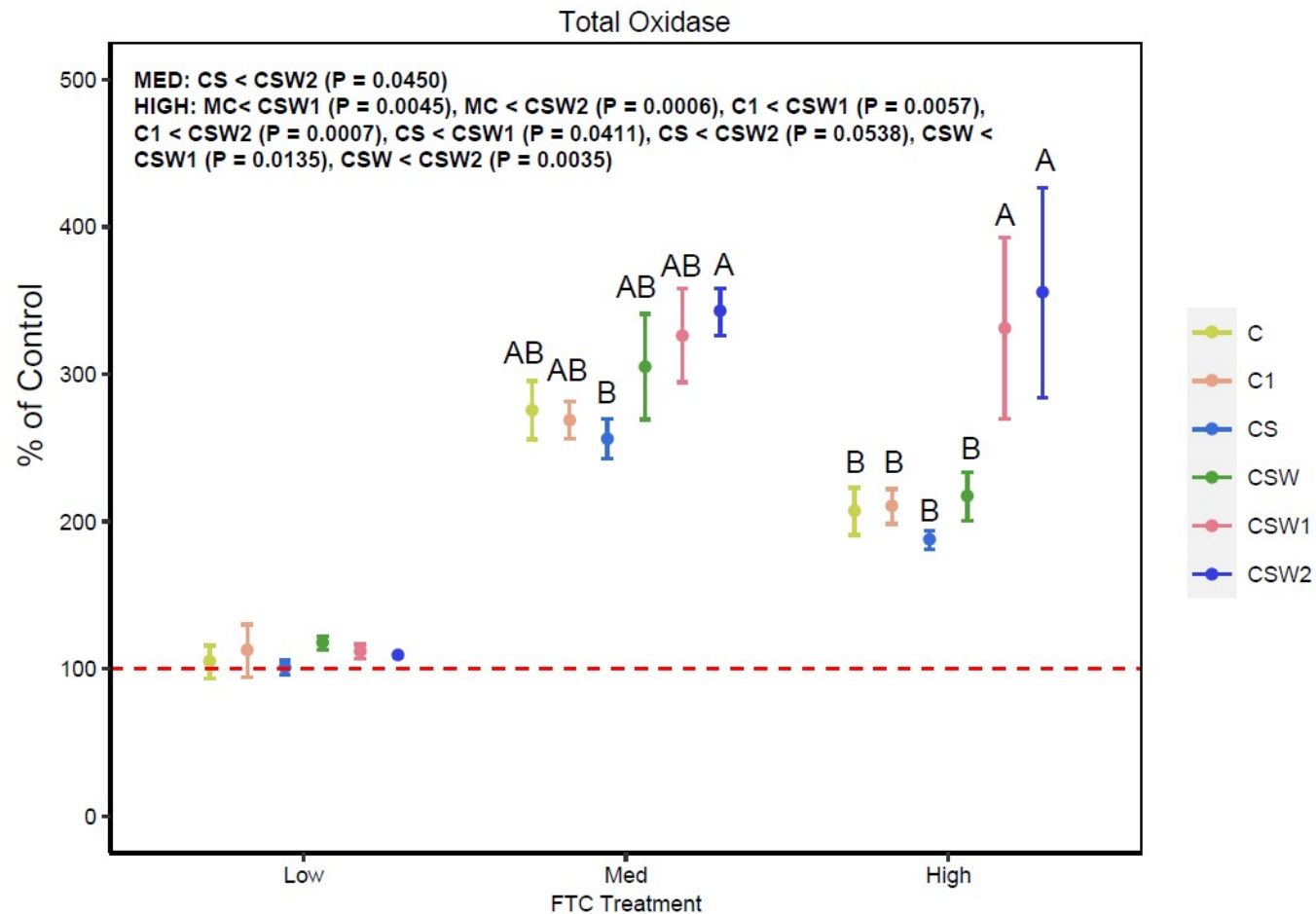


Figure 14. Phenol oxidase and peroxidase activity summed together as total oxidase activity presented as a percentage of non-frozen control after the incubation experiment. Data are presented as total oxidase activity by crop diversity within FTC frequency. Data are means $\pm$ one standard error ($n=4$) and letters indicate significant differences between treatments. The red dotted line represents a 0% difference between treatment and control soils $\pm$ one standard error.

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