Microbial-derived residues and their role in carbon stabilization and aggregate formation in southern Wisconsin cropping systems

by

Angela Y. Leon Tinoco

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The dissertation is approved by the following members of the Final Oral Committee:

Randal Jackson, Professor, Plant and Agroecosystem Sciences
Jean-Michel Ané, Professor, Plant and Agroecosystem Sciences
Matthew Ruark, Professor and Extension Specialist, Soil and Environment Sciences
Zachary Freedman, Assistant Professor, Soil Sciences

DEDICATION

To my dear family and friends who always support me.

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1. Abstract

Soil aggregation is a key process that enhances soil health by improving water and carbon (C) retention. It has been associated with two types of binding agents: glomalin-related soil proteins (GRSP), traditionally related to arbuscular mycorrhizal fungi (AMF), and extracellular polymeric substances (EPS), primarily composed of polysaccharides and proteins secreted by bacteria and archaea. This review explores the roles of GRSP and EPS in soil aggregation, their extraction and quantification methodologies, and the effects of agroecosystem management on their abundance.

GRSP, traditionally defined as a glycoprotein found in the cell walls of fungal hyphae, is extracted by autoclaving soil samples, extracting GRSP, and quantified using the Bradford protein assay. Despite ongoing debates about its precise origin (i.e., fungal or bacterial) GRSP is widely recognized for its role in promoting soil aggregation and C storage. AMF symbiosis with plants enhances plant biomass production, increasing C allocation to fungal hyphae and indirectly influencing GRSP abundance. Soil management practices such as reduced tillage and organic fertilizer additions promote AMF abundance and GRSP accumulation, whereas excessive inorganic fertilizer addition can weaken the symbiotic relationship between AMF and plants, consequently reducing GRSP concentrations in the soil.

EPS, considered a transient binding agent, has also been positively correlated with soil aggregation. Its amphiphilic nature allows it to become positively or negatively charged, influencing its ability to bind minerals. Initially, transient binding agents were defined as microbial exocellular mucilage, primarily composed of polysaccharides that interact with clay to

form soil aggregates. Early studies showed that periodate, a compound that oxidizes polysaccharides, reduced soil aggregation. Additionally, nuclear magnetic resonance spectroscopy revealed a higher proportion of alkyl C relative to O-alkyl C, supporting the microbial origin of these binding agents. Later, a method for extracting EPS from soil samples was developed allowing its quantification as EPS-polysaccharides or EPS-proteins. In vitro studies have demonstrated that EPS enhances soil aggregation, and that soil microbes store an excess of available C as EPS. Unlike AMF, soil microorganisms obtain C indirectly from root exudates and aboveground litter decomposition as dissolved organic C. Therefore, management practices that increase root biomass and litter inputs indirectly enhance C supply to microbial biomass, thereby promoting EPS production. While few field studies have analyzed EPS, evidence suggests that GRSP, AMF, and roots have a greater impact on soil aggregation than EPS.

2. Introduction

Soil aggregation plays a vital role in creating a favorable agricultural environment by promoting water infiltration and retention, facilitating root expansion, and reducing soil erosion (Jastrow et al., 1998). Aggregation also protects soil C from microbial degradation (Lehmann and Kleber, 2015) that may allow C to accumulate in soils (King et al., 2019). Here, I review the key binding agents involved in soil aggregation – glomalin related soil proteins (GRSP) and extracellular polymeric substances (EPS) – which are produced by arbuscular mycorrhizal fungi (AMF) and soil bacteria, respectively.

A conceptual model developed by Tisdall and Oades (1982) explains the mechanism of aggregate formation in soils where organic matter is considered as the main binding agent. It begins with the interaction of inorganic (e.g., Fe, Mn, Al, and Si) and organic (e.g.,

polysaccharides) elements and compounds driven by electrostatic surface forces, resulting in the formation of microaggregates (i.e., diameter <250 µm; Totsche et al., 2018). When microaggregates bind together, they form bigger structures called small (250 to 2000 µm) or large (>2000 µm) macroaggregates (Liu et al., 2020). Organic binding agents have been classified into 3 groups: transient (microbial exopolysaccharides); temporary (roots and fungal hyphae); and persistent (humic materials associated with polyvalent metal cations and polymers strongly sorbed to clay; Tisdall and Oades, 1982).

GRSP traditionally has been associated with fungal hyphae, whereas EPS more recently has been linked to microbial exopolysaccharides. Understanding the impact of agroecosystem management on these binding agents and soil aggregation helps us make better management decisions to improve soil health. Here, I review the literature to explore the role of GRSP and EPS in soil aggregation, development in their extraction and quantification methodologies, and the influence of agroecosystem management on their abundance.

3. Literature review

3.1 Glomalin Related Soil Proteins (GRSP) – a temporary binding agent putatively produced by arbuscular mycorrhizal fungi (AMF)

Glomalin initially was defined as a glycoprotein produced in the cell walls of AMF hyphae (Wright and Upadhyaya, 1998), but recent analyses indicate that much of what is called glomalin are polysaccharides, i.e., glomalose (Alptekin et al., 2025). Operationally, glomalin is obtained by autoclaving soil and extracting the soil solution with sodium citrate, resulting in an extract known as Glomalin Related Soil Protein (GRSP) because the fraction of this substance that is actually glomalin is uncertain (Rillig, 2004). Depending on the autoclave time, GRSP is divided into easily extractable (EE-GRSP) or total (T-GRSP) GRSP pools (Wright and

Upadhyaya, 1998). After autoclaving, the protein concentration of the extracted GRSP typically is analyzed using the Bradford assay (Wright et al., 1996). Using this methodology, Liu et al. (2020) reported that GRSP increased soil aggregate stability by binding microaggregates to form macroaggregates. However, studies have indicated that GRSP contains mostly proteins of non-mycorrhizal origin (Gillespie et al., 2011). The enzyme-linked immunosorbent method was developed to identify fungal protein levels after the autoclaving step. This method, known as immunoreactive glomalin (IR-GRSP), involves the use of an antibody (MAb32B11) that specifically targets proteins from various fungi in the genus *Glomus* (Wright et al., 1996). IR-GRSP had better correlation with aggregate stability than EE-GRSP (R^2=0.84 vs R^2=0.69) across 37 soil samples with different soil types and cropping systems (Wright and Upadhyaya, 1998). Some studies have focused on the effect of management (inorganic and organic fertilizers and tillage) on the abundance of GRSP (Wright et al., 2007; Xie et al., 2015; Zhang et al., 2023). While the precise origin of GRSP remains uncertain, a consensus exists regarding its beneficial effects in soil including promoting aggregation and C storage (Irving et al., 2021).

3.1.1 Symbiotic relationship between crops and AMF

AMF form symbiotic associations with more than 80% of terrestrial plants (Smith and Smith, 1996). In this association, AMF provides nutrients like N and P to the plant and receives close to 20% of the plant's fixed C (Basu et al., 2018). Plants tend to increase their biomass as a result of this symbiotic association; however, the development of new plant genotypes (domestication) can affect this relationship (Lehmann et al., 2012). In their meta-analysis, Lehmann et al. (2012) reported that new (from 1950 to 2010) and old annual cultivars (1950 to 1900) had less AMF root colonization (32 and 30 vs 41%, respectively) than ancestral annual cultivars developed before 1900. However, AMF responsiveness (increase of biomass mediated

by AMF) was higher in new and old cultivars than ancestor cultivars (0.48 and 0.63 vs 0.27, respectively; Lehmann et al., 2012). When Primieri et al. (2022) evaluated the effect of AMF and rhizobia on the biomass of annual and perennial legumes, inoculation with either AMF or rhizobia alone increased the biomass of both compared to non-inoculated plants. Furthermore, simultaneous inoculation with both AMF and rhizobia had an additive effect on the biomass of both plant types, but a synergistic effect was observed only for perennial legumes (Primieri et al., 2022). It is worth noting that the annual legumes studied were mostly domesticated crops, whereas perennial legumes were largely non-domesticated (Primieri et al., 2022). Management practices can also influence AMF colonization of crops. For instance, Bowles et al.'s (2017) meta-analysis revealed that winter cover crops led to a 28.5% increase in AMF colonization of summer cash crop roots compared to winter fallow conditions. Further, the authors reported that legumes used as cover crops exhibited a stronger colonization effect than graminoid or non-legume dicot cover crops. Interestingly, both AMF host and non-AMF host cover crops also increased cash crop root colonization compared with winter fallow (Bowles et al., 2017). Overall, the mutual benefits of the plant-AMF association seems to be reflected on the increase of plant biomass, which increases the provision of C photosynthate to the AMF and subsequently may enhance glomalin production (Treseder and Turner, 2007).

3.1.2 Agroecosystem management effects on AMF and GRSP

Application of inorganic N has been observed to reduce biomass of extraradical hyphae or AMF (Jach-Smith and Jackson, 2018; Zhang et al., 2023). However, nutrient addition may increase the concentration of GRSP and improve soil stability (Zhang et al., 2023). Jach-Smith and Jackson (2018) reported that high addition of ammonium nitrate (196 vs 56 or 0 N ha^{-1} yr^{-1}) decreased abundance of AMF extraradical hyphae biomass (15 vs 70 μg hyphae g $sand^{-1}$,

respectively) while no significant distinctions were found between the low or no N application on limited N soils for one year. These authors found soil N content was negatively correlated with AMF abundance, as assessed through root colonization measurements. Furthermore, Zhang et al. (2023) observed a decrease of AMF biomass quantified through phospholipids fatty acid analysis (PLFA; 1.1, 0.7, and 0.3 nmol g^{-1}) as the application rates of urea increased (0, 180, and 360 kg N ha^{-1}, respectively) after 16 years of application on soil with concentration of C and N of 8.79 and 0.96 g g^{-1}, respectively. However, these authors reported that application urea rate of 180 kg N ha^{-1} increased aggregate stability (measured as mean weight diameter; MWD) and C stock compared with the unfertilized control. Furthermore, these authors reported that MWD was significantly correlated with EE-GRSP (R^2=0.67) and SOC (R^2=0.95).

The concentration of nutrients in soil affects the impact of the application of organic N on AMF abundance, EE-GRSP concentration, soil stability, and C storage. A meta-analysis conducted by Jiang et al. (2021) showed that addition of organic fertilizer predominantly increased AMF abundance (as measured through PLFA) when applied to a host plant with strong mutualistic symbiosis, such as in nutrient-deficient soils. However, organic N addition mostly had a negative impact on AMF when a strong symbiosis was lacking (Jiang et al., 2021). When Xie et al. (2015) applied low or high levels of manure (13.5 and 27 t ha^{-1} y^{-1}, respectively), they observed that the concentration of EE-GRSP (0.38, 0.30, and 0.24 mg g^{-1}, respectively) and SOC (11.55, 10.40, and 7.95 mg g^{-1}, respectively) was higher with high manure, followed by low manure and control. However, aggregate stability (as MWD) only increased with low manure (1200 vs 940 μm, respectively) when applied to soils with C and N concentrations of 9.2 and 0.8 g g^{-1} (respectively; Xie et al., 2015).

The negative effect of tillage on C soil storage (Angers and Eriksen-Hamel, 2008), and aggregate stability (Liu et al., 2021) can be partly explained by its negative effect on AMF extraradical hyphae (Kabir, 2005). As was mentioned before, AMF hyphae itself is considered a binding agent but also is important to produce GRSP. Wright et al. (2007) reported no tillage systems had greater GRSP concentration than conventional tillage or organic systems (more intense tillage). Furthermore, these authors reported that the concentration of GRSP increased as aggregate size increases under no till systems, but this was not observed under till systems (Wright et al., 2007). Similarly, Caesar-TonThat et al. (2021) reported that systems under no-till have higher C storage across soil aggregates of 4750-2000 µm or 2000 to 1000 µm (14.5 vs 8.5 and 14.7 vs 8.8 g kg^{-1} aggregate, respectively) than under conventional tillage system. Furthermore, these authors reported that concentration of IR-GRSP across aggregates of 2000 to 1000 µm or 1000 to 500 µm (0.363 vs 0.051 and 0.322 vs 0.047 g kg^{-1}, respectively) were higher under no-till system than aggregates at the same size under conventional systems.

3.2 Extracellular polymeric substances (EPS) – a transient binding agent produced by bacteria and archaea

Extracellular polymeric substances (EPS) – comprised of proteins, polysaccharides, nucleic acids, and lipids – play a key role in cell aggregation, biofilm formation, and attachment to mineral surfaces (Flemming and Wingender, 2010). Their amphiphilic nature, with both hydrophobic and hydrophilic groups, allow EPS to become positively (protonated amine and amide) or negatively (dissociated carboxyl or hydroxyl groups) charged based on pH conditions (Martinez et al., 2002), which affects their binding capacity to minerals (Chen et al., 2021). Polysaccharides in the EPS of Gram-negative bacteria can be neutral or polyanionic, whereas the EPS of some Gram-positive bacteria is primarily cationic in nature (Donlan, 2002; Sutherland,

2001). For example, *Sinorhizobium meliloti* produces a negatively charged extracellular polysaccharide called succinoglycan, which interacts strongly with clay only under acidic conditions when the clay carries a positive charge (Labille et al., 2005). In contrast, the EPS of *Bacillus subtilis* binds to clay minerals such as kaolinite and montmorillonite, as well as the iron oxide goethite, primarily through interactions with peptides, phospholipids, and organic acid groups in the EPS (Chen et al., 2021). The binding of EPS to goethite occurs mainly via electrostatic interactions, where the positively charged surface of goethite is attracted to the negatively charged EPS groups of *B. subtilis*. Conversely, attachment to negatively charged clay minerals likely involves non-electrostatic mechanisms, such as hydrogen bonding and surface complexation (Chen et al., 2021).

3.2.1 Historical development of the extraction of EPS from soil samples

Transient binding agents were defined as microbial exocellular mucilage or gums produced by the rapid decomposition of plant or microbial substrates, which interact with clays to form transient soil aggregates (Tisdall and Oades, 1982). The rapid decomposition of these substrates contributes to the transient nature of the aggregates formed. Since these microbial mucilage or gums are mainly composed of polysaccharides, periodate was initially used for their analysis (Tisdall and Oades, 1982). Periodate, a compound that oxidizes polysaccharides, was observed to reduce soil aggregate size, particularly affecting aggregates <50 μm diameter (Stefanson, 1971).

Golchin et al. (1994) proposed a model for the formation and degradation of microaggregates (20 to 250 μm diameter) stating that soluble plant fractions are degraded by microorganisms, leading to the production of microbial mucilage, which interacts with mineral particles to form soil aggregates. These aggregates persist as long as labile plant particles remain

available to microorganisms; otherwise, soil aggregation begins to decline (Golchin et al., 1994). Occluded organic materials in microaggregates (aggregates resistant to ultrasonic destruction) were analyzed using nuclear magnetic resonance spectrometry (Golchin et al., 1994). The presence of a higher proportion of alkyl C compared to O-alkyl C indicates a greater extent of decomposition of plant-derived material and, mostly, the presence of microbial-derived compounds (Golchin et al., 1994).

Transient binding agents, assayed as hot-water soluble carbohydrates (hwsCHO), showed a significant correlation with the percentage of macroaggregates, though to a lesser extent than roots, external hyphae, or microbial biomass (Jastrow et al., 1998). However, hwsCHO exhibited a strong correlation with microbial biomass C, confirming their microbial origin, as a larger microbial biomass is likely to produce greater amounts of hwsCHO (Jastrow et al., 1998). Soil which was analyzed by mixing soil samples (<250 μm) with water at 80°C, followed by centrifugation and filtration through a 0.45-μm filter. The filtrate was then treated with a Ca-saturated cation exchange resin before sugar analysis (Jastrow et al., 1998).

The term extracellular polymeric substances (EPS) originally described microbial biofilms composed primarily of proteins, nucleic acids, and lipids (Geesey, 1982). Later, polysaccharides were also included as part of EPS (Flemming and Wingender, 2010). Extraction of EPS without intracellular compounds was one of the main problems when dealing with soil samples (Flemming and Wingender, 2010). However, a methodology for extracting EPS from soil was developed (Redmile-Gordon et al., 2014). Briefly, this process begins with a $CaCl_2$ pre-extraction to separate soluble microbial products, followed by EPS extraction using a cation exchange resin and a specific buffer solution. After shaking and centrifugation, the supernatant is quantified based on polysaccharide (EPS-polysaccharides) or protein (EPS-proteins)

concentrations (Redmile-Gordon et al., 2014). Cation exchange resin removes cations that are associated with the anionic groups of EPS carbohydrates and proteins (Fr¢lund et al., 1995).

3.2.2 Impact of extracellular polymeric substances on the formation of macroaggregates under *in vitro* conditions

Extracellular polymeric substances contribute to the formation of macroaggregates (Costa et al., 2018) and improve soil stability (Sher et al., 2020). For instance, the inoculation of EPS-producing bacteria *Pseudomonas chlororaphis* and *Bacillus proteolyticus* in soil increased water-stable macroaggregates from 8 to 17 and 20%, respectively, compared to the non-inoculated control (Cheng et al., 2020). Sher et al. (2020) reported a positive covariance between EPS-polysaccharides (EPS-CHO) and water-stable aggregates after cultivation of switchgrass (*Panicum virgatum*). These authors suggested that increased root biomass could have contributed to higher C availability (measured as dissolved organic C) for microorganisms, thereby enhancing the abundance of EPS-CHO (Sher et al., 2020). Consistently, a previous study hypothesized that soil microorganisms might store excess available C externally as EPS (Redmile-Gordon et al., 2015).

3.2.3 Agroecosystem management effects on EPS abundance

While AMF receives C directly from the roots, soil microorganisms assimilate C through the uptake of dissolved organic matter (DOM) coming from root exudates and aboveground litter (Zhang et al., 2021). Soluble litter contributes directly to the DOM pool, while structural litter (hydrolysable and unhydrolyzable) requires a prior depolymerization process (Zhang et al., 2021). Increasing root biomass enhances the abundance of DOM and provides a greater quantity of C compounds to microorganisms (Sher et al., 2020). Therefore, microorganisms produce more EPS, leading to improved soil aggregate stability (Sher et al., 2020).

Perennial roots have greater biomass and exudates than annual roots that enhance not only microbial biomass but also its EPS production. Root biomass is greater in cool-season grass, poplar tree, and switchgrass systems (~6 Mg ha^{-1}) than annual corn or soybean crop fields (~6 vs < 2.3 Mg ha^{-1}) (Sprunger et al., 2017). Root exudates provide C substrates to microbial biomass, however, when these C sources are limited, soil microbes like fungi and gram-positive bacteria can degrade old soil organic matter, and decrease soil C storage (Feng et al., 2022). When a corn field was transformed to prairie vegetation, microbial biomass increased from 0.09 to 0.3 μmol g^{-1} soil, respectively, in only 2 years (Herzberger et al., 2014). Long-term switchgrass cultivation has been shown to increase EPS-polysaccharide levels compared to paired tilled annual crop fields (10 vs 4 μg EPS g^{-1} soil, respectively) in the surface 30 cm of soil (Sher et al., 2020).

Cropping system management can influence microbial C use efficiency. The addition of legumes or manure lowers the ratio of C:N of inputs, which improves the microbial C use efficiency because microbial N demands are better satisfied (Rui et al., 2022). More microbial C efficiency leads to an increase not only microbial biomass but also microbial products like EPS. Redmile-Gordon et al. (2020) used a regression model to investigate the relationship between MWD with EPS-protein or EPS-CHO *in situ*, reporting a stronger association between MWD and EPS-protein (R^2 = 0.30) compared to EPS-CHO (R^2 = 0.15). However, the authors also observed that an increase in MWD was not always solely attributable to an increase in EPS content. They explained that root and hyphal contributions might have played a role in enhancing MWD, but it was not evaluated. Further, these authors reported that concentrations of EPS-protein (126, 185, and 213 μg g^{-1}, respectively) and SOC (0.84, 1.64, and 3.67%, respectively) increased across fallow, fertilized crops, and unfertilized grassland established for over 50 years. Similarly, Sher et al (2020) reported that switchgrass exhibited higher EPS-CHO levels than

paired plots under rye or wheat and sorghum (10 vs 3 and 4 μg g^{-1}, respectively). Compared to Redmile-Gordon et al. (2020), these authors explained that the significantly lower EPS concentration may be due to low C availability, and microbial biomass of the sites evaluated. Despite variations in EPS values, it is evident that cropping systems generally have lower EPS levels than perennial systems.

4. Conclusions

GRSP and EPS are important binding agents in soil aggregation. While GRSP, often linked to AMF, has consistently been associated with greater soil aggregation in field studies, EPS produced by bacteria and archaea has primarily been studied *in vitro*. Overall, soil health management practices – i.e., minimizing soil disturbance, incorporating cover crops, legumes, or manure, and reestablishing perennial systems – appear to enhance the abundance of both GRSP and EPS binding agents and soil aggregation. However, field studies suggest that GRSP, AMF, and roots have a greater impact on soil aggregation than EPS. Therefore, field studies simultaneously examining both binding agents are needed.

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Chapter 2: Carbon stabilization, aggregate stability, and microbial residues covary in cropping systems of Wisconsin

1. Abstract

Soil organic carbon (SOC) stabilization is critical to climate change mitigation and adaptation strategies, but the mechanisms promoting it are not fully understood. Significant SOC stabilization is thought to occur within macroaggregates, where particulate organic matter (POM) is converted into mineral-associated organic matter (MAOM) by soil microbes. We examined a) abundance of stable SOC (i.e., MAOM-C) within macroaggregates (i.e., >250 um), b) soil microbial binding agents abundance, and c) macroaggregate abundance across a range of cropping systems typical of the upper Midwest US to better understand the role of microbial residues believed to act as soil binding agents. We assayed soil microbial binding agents as easily extractable glomalin-related soil protein (EE-GRSP) and extracellular polymeric substances (EPS). We selected two cropping systems with no agricultural soil disturbance (rotationally grazed perennial pasture and restored tallgrass prairie) and two with periodic soil disturbance (continuous maize and organic maize-alfalfa forage rotation). Restored prairie had greater macroaggregate stocks than disturbed systems, while both perennial grassland systems had greater POM-C stocks within macroaggregates at the surface compared to systems with periodic soil disturbance. Perennial grassland showed greater MAOM-C and EE-GRSP stocks within macroaggregates at the surface indicating an important contribution of EE-GRSP to stabilized soil C. EE-GRSP, but not EPS, was affected by the cropping system or correlated with macroaggregates. Although the exact source of EE-GRSP remains uncertain, it is widely believed to originate from arbuscular mycorrhizal fungi residues, which appear to be more strongly associated with SOC accumulation than EPS produced by bacteria and archaea.

2. Introduction

Soil organic carbon (SOC) accumulation in soil is critical to climate change mitigation efforts to reduce atmospheric CO_2 (Amelung et al., 2020). Enhancing SOC also improves water holding capacity and nutrient availability, making it an important part of climate change adaptation in agroecosystems (Jordan et al., 2018). But significantly increasing SOC (Amundson and Biardeau, 2018) in agricultural soils is difficult (Raffeld et al., 2024). A long-term field experiment in southern Wisconsin showed that cover crops, legume inclusion, tillage reduction, and pasture establishment have not significantly increased SOC when evaluated rigorously (i.e., across full soil depth, corrected for equivalent soil mass, and monitored over time (Dietz et al., 2024). However, well-managed grazed pasture and restored tallgrass prairie maintained SOC stocks over a 30-yr period, with surface 30-cm gains offset by losses in deeper soil layers (Dietz et al., 2024). Accumulation of C in the surface of well-managed grazed pastures was mainly as ‘stabilized C’ or mineral-associated organic matter-C (MAOM-C) (Rui et al., 2022). When organic compounds are bound to minerals within aggregates, they can be protected from microbial degradation (Lehmann and Kleber, 2015) favoring organic matter accumulation. In fact, long turnover times of macroaggregates favor C stabilization by allowing the transformation of coarse particulate organic matter (POM) into fine POM (Six et al., 2000). This fine POM can be encrusted with clay particles to form occluded microaggregates (Six et al., 2000) helping to stabilize C within macroaggregates. Macroaggregate formation is affected by plant roots, fungal hyphae, and polysaccharides (Lützow et al., 2006; Six et al., 2002), including biotic residues known to function as binding agents in the soil. These binding agents are thought to arise from fungi, known as glomalin related soil protein (GRSP), and bacteria and archaea producing extracellular polymeric substances (EPS).

A glycoprotein bound to a monoclonal antibody (MAb32B11) raised against *Rhizophagus irregularis* spores, a species of arbuscular mycorrhizal fungi (AMF) in the Glomeraceae family, was dubbed “glomalin” in 1996 (Wright and Upadhyaya, 1996). Operationally, glomalin-related soil protein (GRSP) refers to the fraction extracted from soil after autoclaving with sodium citrate, followed by protein quantification. When extracted once, this fraction is defined as *easily extractable* GRSP (EE-GRSP)(Wright and Upadhyaya, 1996). While the exact origin of glomalin remains uncertain, its positive effects on soil stability and C sequestration were well documented in a review of 25 years of glomalin research (Irving et al., 2021).

Agroecosystem management practices affect the abundance of GRSP. For instance, inorganic nutrient addition reduces AMF biomass as plant roots become less dependent on AMF-provided N (Jach-Smith and Jackson, 2018). However, N addition may increase GRSP concentrations and enhance soil aggregate stability (Zhang et al., 2023). Conversely, organic fertilizers such as manure tend to increase AMF biomass and GRSP concentrations, with higher manure applications correlated with greater soil stability and C storage (Xie et al., 2015). Tillage has been reported to decrease GRSP concentrations, whereas GRSP abundance has been positively correlated with aggregate stability under no-till cropping systems (Wright et al., 2007).

Extracellular polymeric substances (EPS) – consisting of proteins, polysaccharides, and other organic compounds (Flemming and Wingender, 2010) – contribute to the formation of macroaggregates (Costa et al., 2018) and improve soil stability (Sher et al., 2020). Inoculating soil with EPS-producing bacteria such as *Pseudomonas chlororaphis* and *Bacillus proteolyticus* increased the proportion of water-stable macroaggregates compared with an uninoculated control

(Cheng et al., 2020). Further, EPS abundance in soil was positively correlated with increased aggregate stability (Redmile-Gordon et al., 2020; Sher et al., 2020).

As a symbiont, AMF receives C directly from plant roots, while most free-living soil microorganisms assimilate C through the uptake of dissolved organic matter (DOM) coming from root exudates and aboveground litter (Zhang et al., 2021). Soluble litter contributes directly to the DOM pool, while structural litter (hydrolysable and unhydrolyzable) requires a prior depolymerization process (Zhang et al., 2021). Increasing root biomass enhances the abundance of DOM and provides a greater quantity of C compounds to microorganisms (Sher et al., 2020). Greater activity of microbes results in more EPS, leading to improved soil aggregate stability (Sher et al., 2020).

We selected 3 cropping systems: continuous maize, organic maize-alfalfa rotation, and rotationally grazed pasture because they showed different patterns of C accumulation that appeared largely related to soil microbial dynamics (Rui et al., 2022), as well as restored tallgrass prairie known for AMF abundance (Herzberger et al., 2014; Jesus et al., 2016) and their capacity to accumulate persistent C (Sanford et al., 2022). Management differences among these systems include organic and inorganic nutrient addition and variation in soil disturbance ranging from chisel tillage to none, which are known to affect GRSP (Wright et al., 2007) and EPS (Redmile-Gordon et al., 2020) abundance in soil. We evaluated the abundance of these microbial residues across these four systems and explored relationships among them and aggregate stability, macroaggregate abundance, and soil C pools.

3. Materials and methods

3.1 Study site

The study was conducted at the Wisconsin Integrated Cropping Systems Trial (WICST) on Plano silt loam (fine-silty, mixed, superactive, mesic Typic Argiudoll). Mean annual temperature at Arlington was 7.9 °C with mean annual precipitation of 894 mm over a 30-yr period (1991–2020, NOAA, and 2021-2022, IEM). WICST was established in 1989 in a randomized complete block design with four blocks, except for the prairie system, which was established in 1999 nested within the bigger experimental setup and with 3 blocks instead of 4. Plots are 0.3 ha and typical farm equipment is used for all field work.

Continuous maize (*Zea mays* L.) receives commercial fertilizer at recommended rates. Organic forage is a 3-year rotation of maize, oats/alfalfa (*Medicago sativa* L.), and alfalfa. Dairy slurry is applied in the fall prior to maize and first-year alfalfa seeding in Organic forage. Continuous maize and Organic forage are chisel plowed in the fall before maize planting. A field cultivator is used in the spring prior to planting maize in continuous maize and Organic forage. A chisel plow is used prior to alfalfa seeding in Organic forage. Additional cultivation, including use of a tine weeder, a rotary hoe, and Danish tine and/or disk hiller row cultivator for weed control, is performed as needed in continuous maize and Organic forage. Perennial pasture is comprised of cool-season grasses – Timothy (*Phleum pratense* L.), Kentucky bluegrass (*Poa pratensis* L.), orchardgrass (*Dactylis glomerata* L.), ryegrass (*Lolium perenne* L.), and *Festulolium* (×*Festulolium* Asch. & Graebn.) – dandelion (*Taraxacum officinale* F.H. Wigg.), and clover (*Trifolium pratense* L. and *T. repens* L.). Pastures have received occasional fertilizer applications of 34 years and are rotationally grazed by six heifers each year between 1 May and 10 October for a stocking rate of ∼14 AU ha^{-1} y^{-1} (Rui et al., 2022). The restored prairie is burned in spring every 2 to 3 years to remove accumulating litter and is comprised of a mixture

of warm- and cool-season grasses and forbs (Liang et al., 2016) that do not receive agricultural fertilizers (Dietz et al., 2024).

3.2 Soil sampling & assays

Soil samples were taken October 2022 from each block of each system using a tractor mounted hydraulic soil sampler fitted with a 5-cm diameter soil probe. Each repetition was divided into three sections (north, center, and south). A total of two (for continuous maize and Organic forage) or one (for perennial pasture and restored prairie) soil cores were collected from the middle of each section. Two cores were taken from the systems that receive fertilizer or slurry, and sampling was conducted both within rows and between rows and subsequently combined. Soil cores were divided into 3 sections (0 to 15, 15 to 30, and 30 to 60 cm). Core soils from each repetition or block were mixed by depths. At least 1000 g of soil was sieved to 2000 μm and divided into two subsamples. One subsample of approximately 100 g of fresh sieved soil (<2000 μm) was stored at -20 °C for EE-GRSP, and EPS analysis in separated whirl-pak bags. The other group was air-dried and separated into three categories by size: macroaggregates (2000 to 250 μm), microaggregates (250 to 53 μm), and silt and clay (<53 μm) by wet sieving. At least 60 g of macroaggregates were collected for the analysis of EE-GRSP and POM-C/MAOM-C.

3.3. Extracellular polymeric substances (EPS) extraction

The extraction of EPS was done January 2024 following the methodology of Redmile-Gordon et al. (2014) with addition of a filter step after extraction (Sher et al., 2020) due to the present of tiny roots on the supernatant. Readily soluble organic material was removed from 2 g of moist soil (1.6 g dry weight equivalent) using 0.01-M $CaCl_2$ in centrifuge tubes. These tubes were shaken at 120 rpm and then centrifuged at 3750 rpm for 30 min. The supernatant was discarded, and EPS was extracted from the remaining pellet by re-suspending it in extraction

buffer. Cation exchange resin (Na ion exchange resin, strongly acidic, 20 to 50 mesh) was added to the pellet along with the buffer (2 mM $Na_3PO_4 \cdot 12H_2O$, 4 mM $NaH_2PO_4 \cdot H_2O$, 9 mM NaCl, 1 mM KCl), adjusted to pH 7. After shaking for 2 h and centrifuging at 3750 rpm for 30 min, the supernatant containing EPS was filtered through a 0.2-μm nylon filter, transferred into new tubes, and stored at -20 °C for carbohydrate analysis. The supernatant was utilized to measure EPS-polysaccharides using the Total Carbohydrate Assay Kit (Cell Biolabs, Inc., San Diego, CA, USA).

3.4 Easily extractable-glomalin related soil protein (EE-GRSP) assay

For EE-GRSP extraction from bulk soil and macroaggregates, samples were mixed with 20 Mm sodium citrate solution (pH 7.0) and autoclaved at 121 °C for 30 min following the procedure of Wright and Upadhyaya (1998). After centrifugation, the extract was analyzed for protein content using the Bradford assay (Bradford, 1976).

3.3.4 MAOM-C and POM-C determination

The method of Cotrufo et al. (2019) was used to determine MAOM-C and POM-C within fractionated macroaggregates and microaggregates. Sodium hexametaphosphate solution was added to the soil samples and shaken for 18 h and then sieved to 53 μm to finally obtain two fractions: POM (>53 μm) and MAOM (<53 μm). The C content in each fraction (MAOM-C and POM-C) was analyzed using an elemental analyzer (PDZ-Europa ANCA-GSL).

POM-C or MAOM-C within macroaggregates present in the bulk soil was calculated as follows:

$$\frac{Macroaggregates\ (\%) \times MAOM\ in\ macroaggregates\ (\%) \times C\ in\ MAOM\ (\%)}{10000}$$

$$= MAOM - C\ in\ macroaggregates\ present\ in\ bulk\ soil\ (\%)$$

3.3.5 Equivalent soil mass calculations

To avoid misleading conclusions driven by changes in bulk density (von Haden et al., 2020), we assess our response variables as stocks after ESM adjustment. The percentages of macroaggregates, EE-GRSP, and EPS in bulk soil, as well as EE-GRSP in macroaggregates, MAOM-C and POM-C in macro- and micro-aggregates across systems were analyzed using the equivalent soil mass (ESM) approach (von Haden et al., 2020) and converted into stocks. To calculate ESM, we used soil organic matter and bulk density data from Dietz et al. (2024).

3.4 Statistical analysis

Stocks of EE-GRSP from bulk soil and macroaggregates, EPS from bulk soil, and POM-C and MAOM-C in macroaggregates were analyzed using linear mixed-effects models (PROC GLIMMIX, SAS v9.4) to compare cropping systems and depths (fixed effects), while replicated blocks were included as a random effect. Means, standard errors, and p-values were calculated using the LSMEANS statement, with an alpha level of 0.1 used for all statistical comparisons of stocks. Statistical comparisons of indicators expressed as percentages are provided in the Appendix section, along with the correlations between macroaggregate stocks with stocks of EE-GRSP or EPS. These correlations were assessed using the Spearman method in RStudio (*ggpubr* package).

4. Results

Within macroaggregates, perennial grasslands with undisturbed soils had higher POM-C and MAOM-C stocks than the annual cropping systems (Table 2-1). POM-C was greater in the perennial systems in both the shallow and deep intervals, while MAOM-C was significantly higher only in the surface 15-cm. The significant interaction between cropping system and depth for POM-C within macroaggregates indicated that the differences in cropping system were not observed in the 15 to 30-cm depth.

Macroaggregate stocks were generally highest at the 30 to 60-cm depth than the surface 30 cm and across cropping systems, restored prairie had higher macroaggregate stocks than all others (Table 2-2). Microaggregate stocks had a similar pattern across depths but differences in cropping systems were only significant in the surface 15-cm and the 30 to 60-cm intervals indicating that restored prairie had lower microaggregate stocks than the other systems.

Restored prairie had higher bulk soil EE-GRSP stocks at the 30 to 60-cm depth, but EE-GRSP stocks within macroaggregates were greater in perennial pasture in the surface 15-cm. Restored prairie had significantly higher within-macroaggregate EE-GRSP at the deepest layer sampled. EPS-Polysaccharide stocks were much higher in the surface 30-cm than the lowest sampling depth, but not significantly different across cropping systems (Table 2-2).

Table 2-1. MAOM-C and POM-C stock within macroaggregates and microaggregates of bulk soil across cropping systems and depths at WICST

	Cropping Systems						P		
Depth (cm)	Continuous maize	Organic forage	Perennial pasture	Restored prairie	Mean	SEM	CS	Depth	CS*Depth
MAOM-C within macroaggregates (Mg ha^{-1})									
0-15	17.82	19.64	31.53	33.05	25.51^{A}	5.92	0.08	0.02	0.49
15-30	15.92	17.10	20.21	19.53	18.19^{B}				
30-60	14.76	12.10	18.22	28.42	18.37^{B}				
Mean	16.17	16.28	23.32	27.00					
MAOM-C within microaggregates (Mg ha^{-1})									
0-15	17.05	17.30	12.29	8.11	13.68^{B}	5.00	0.33	0.0005	0.89
15-30	19.16	18.26	17.08	11.91	16.60^{B}				
30-60	25.26	27.44	31.50	23.72	26.98^{A}				
Mean	20.49	21.00	20.29	14.58					
POM-C within macroaggregates (Mg ha^{-1})									
0-15	4.60^{b}	4.43^{b}	7.80^{a}	7.79^{a}	6.15^{A}	0.54	0.0002	<0.0001	0.003
15-30	1.24	1.63	1.62	1.16	1.41^{B}				
30-60	0.53^{b}	0.63^{b}	2.10^{a}	1.52^{ab}	1.19^{B}				
Mean	2.12^{B}	2.23^{B}	3.84^{A}	3.49^{A}					
POM-C within microaggregates (Mg ha^{-1})									
0-15	2.02	2.28	2.94	1.26	2.12^{A}	0.80	0.21	0.04	0.48
15-30	0.90	1.10	0.86	0.58	0.86^{B}				
30-60	1.53	0.84	3.42	2.06	1.97^{A}				
Mean	1.48^{AB}	1.41^{B}	2.41^{A}	1.30^{B}					

Different lowercase letters indicate significant differences (P<0.1) within the same depth among cropping systems, capital letters indicate differences across cropping systems (CS) or depths (P<0.1).

Table 2-2. Macroaggregates, microaggregates, EE-GRSP, EE-GRSP in macroaggregates, and EPS-Polysaccharide in bulk soil across cropping systems and depths at WICST

Depth (cm)	Cropping Systems				Mean	SEM	P		
	Continuous maize	Organic forage	Perennial pasture	Restored prairie			CS	Depth	CS*Depth
Macroaggregates (Mg ha^{-1})									
0-15	771.48	846.40	1114.72	1371.02	1025.91^{B}	142.94	0.005	0.0008	0.11
15-30	745.92	765.05	896.18	1170.94	902.02^{B}				
30-60	1183.83	988.68	971.10	1781.92	1231.38^{A}				
Mean	900.41^{B}	876.71^{B}	994.00^{B}	1441.29^{A}					
Microaggregates (Mg ha^{-1})									
0-15	757.50^{a}	725.89^{a}	526.75^{ab}	301.98^{b}	578.03^{C}	115.34	0.001	<0.0001	0.008
15-30	981.09	947.41	882.88	661.55	868.23^{B}				
30-60	2283.23^{a}	2520.53^{a}	2467.39^{a}	1741.64^{b}	2253.20^{A}				
Mean	1340.60^{A}	1397.94^{A}	1292.34^{A}	901.72^{B}					
EE-GRSP within bulk soil (g m^{-2})									
0-15	2.35	1.92	2.36	2.19	2.20^{B}	0.20	0.19	<0.0001	0.0001
15-30	1.83	1.60	1.63	1.41	1.62^{C}				
30-60	2.07^{b}	2.30^{b}	2.34^{b}	3.52^{a}	2.56^{A}				
Mean	2.09^{AB}	1.94^{B}	2.11^{AB}	2.37^{A}					
EE-GRSP within macroaggregates (g m^{-2})									
0-15	0.79^{c}	0.87^{bc}	1.31^{a}	1.23^{ab}	1.05^{A}	0.14	0.02	<0.0001	0.003
15-30	0.62	0.52	0.65	0.75	0.63^{B}				
30-60	0.57^{b}	0.52^{b}	0.50^{b}	1.34^{a}	0.73^{B}				
Mean	0.66^{C}	0.64^{C}	0.82^{B}	1.11^{A}					
EPS-Polysaccharide within bulk soil (g m^{-2})									
0-15	3.37	4.62	3.32	4.14	3.86^{A}	1.41	0.51	<.0001	0.89
15-30	3.92	5.65	3.67	5.05	4.57^{A}				
30-60	0.96	0.77	0.81	0.34	0.72^{B}				
Mean	2.75	3.68	2.60	3.18					

Different lowercase letters indicate significant difference (P<0.1) within the same depth among cropping systems, capital letters indicate differences across cropping systems (CS) or depths (P<0.1).

5. Discussion

As others have demonstrated at WICST, the perennial grasslands with undisturbed soils had greater soil C (Dietz et al., 2024; Rui et al., 2022; Sanford et al., 2012), but differences in so-called ‘stabilized’ C (i.e., MAOM-C) were only observed in surface soil macroaggregates, indicating the importance of aggregate formation and stability in stabilizing C flowing into the agroecosystem. The C inputs to the system, characterized as the ‘less stable’ POM-C pool, were also higher within macroaggregates of the perennial grasslands overall, demonstrating their importance as the initial source of C processed by microbiota to become MAOM-C. Perennial grassland systems also tended to have more macroaggregate stocks than annual cropping systems, which is partly why there is more soil C in these systems overall.

At WICST, continuous maize is chisel-tilled annually, while Organic forage undergoes chisel-tillage every 3 years. In contrast, perennial pasture and restored prairie remain undisturbed. Cates et al. (2016) found no significant differences in macroaggregate mass between undisturbed (pasture) and disturbed systems (organic forage or continuous maize) in the surface 25-cm. They concluded that the high biomass input in tilled systems (Cates et al., 2016) was sufficient to maintain aggregates between disturbed and undisturbed systems. However, recent work has shown over double the amount of belowground biomass in perennial pasture and restored prairie compared to an annual cropping system of corn and soybeans (Moore et al., 2025), similar to findings by Sprunger et al. (2020). The greater macroaggregate stock in the restored prairie (dominated by C4 plants) may be partly explained by its higher belowground biomass compared to perennial pasture (dominated by C3 plants). Saunders et al. (2006) reported that systems dominated by C4 plants have greater belowground biomass than those dominated by

C3 plants (4119.1 vs. 3256.9 g m^{-2}, respectively). Moreover, the higher diversity of the restored prairie may give it an advantage over the perennial pasture (Sprunger et al., 2017)

Although perennial pasture did not have significantly greater macroaggregate stocks, it showed greater POM-C in macroaggregates at the surface, along with the restored prairie – and was the only system to show greater POM-C at the deepest layer. Rui et al. (2022) reported that, in bulk soil, pasture had the highest POM-C stock, followed by organic forage and continuous maize at the 0 to 30-cm depth (3.48, 3.31, and 2.62 mg g^{-1} soil, respectively). They explained that this accumulation stemmed from lower C:N ratio of the POM fractions of pasture and Organic forage, compared to the continuous maize system (14.7, 15.0, and 17.4, respectively) allowing for higher microbial C use efficiency (0.45, 0.27, and 0.20 respectively). Although these authors did not evaluate POM-C within macroaggregates, their results help to explain our findings. In contrast to our results, Cates et al. (2016) reported that the stock of occluded microaggregate-C or coarse POM-C in macroaggregates was similar between undisturbed (pasture) and disturbed (continuous maize or organic forage) systems. In our study, we did not fractionate macroaggregates into three components as Cates et al. (2016) did (occluded coarse POM, >250 μm; occluded microaggregates, 250–53 μm; and occluded silt and clay, <53 μm). Instead, we fractionated macroaggregates into POM (>53 μm) and MAOM (<53 μm) pools. The longer period after perennial pasture, continuous maize, and organic forage reached equilibrium in this study compared to Cates et al. (2016) may help explain the different results (13 vs. 4 years, respectively). According to Dietz et al. (2024), these systems reached soil organic C equilibrium—where soil C inputs equal soil C outputs—around 2009. After this stage, a slow rate of C accumulation is expected (Dietz et al., 2024). Although restored prairie was expected to reach equilibrium by 2019 (Dietz et al., 2024), its greater belowground biomass may have

positively influenced the accumulation of POM-C observed in macroaggregates. Cates et al. (2016) reported a positive correlation between belowground biomass C and POM-C. Although this correlation was observed in bulk soil, it may also help explain POM accumulation within macroaggregates.

In undisturbed systems, POM-C in macroaggregates may gradually transform into MAOM-C, potentially leading to significantly greater MAOM-C stocks in these aggregates over time. According to Six et al. (1999), macroaggregate formation occurs similarly in both no-till and tilled systems. However, undisturbed systems have lower macroaggregate turnover rates, allowing macroaggregates to persist longer (Six et al., 2000). This extended stability provides more time for the decomposition of coarse POM into finer or more stable C within macroaggregates (like occluded microaggregates) compared to disturbed systems (Six et al., 2000). When Fulton and Cotrufo (2019) tracked the incorporation of isotopes of C from leaves or roots to soil aggregates, they observed that C was incorporated mostly within occluded microaggregates as MAOM. These authors concluded that the role of aggregates is not only to protect organic matter from microbial degradation but also to promote the formation of persistent organo-mineral structures. We expect that our undisturbed perennial systems will accumulate more stable MAOM-C within macroaggregates than disturbed annual systems in the future.

The lack of significant differences in EE-GRSP stocks between disturbed and undisturbed systems at 0 to 30-cm might be attributed to recalcitrant residues causing overestimated EE-GRSP values in disturbed systems. This overestimation may occur because GRSP extraction also retrieves polyphenolic compounds that react with the dye, thus inflating protein measurements (Whiffen et al., 2007). Whiffen et al. (2007) further noted that the Bradford assay may capture both protein and recalcitrant organic material from the soil. In

perennial systems like pasture and prairie, 80 to 90% of belowground biomass is found in the surface 30-cm of soil (Moore et al., 2025). Although annual systems have less belowground biomass than perennial systems, their C:N ratio is higher and therefore more recalcitrant than perennial systems.

Perennial pasture had greater EE-GRSP stock in bulk soil than continuous maize or organic forage, though only restored prairie showed a significant difference at 30 to 60 cm. When Oehl et al. (2005) compared the vertical distribution of AMF spore abundance in perennial vs annual systems they reported that spore abundance decreased with soil depth in both systems. However, perennial systems showed higher spore counts than annual systems (20 vs. 4 spores g^{-1} soil, respectively) even at greater depths (35 to 50 cm). Using lipid markers, Liang et al. (2016) reported that a prairie system on Plano silt loam had lower AMF biomass at 50-cm than the surface (5 vs. 24 μg g^{-1} soil, respectively). Although AMF biomass and spore abundance decrease with soil depth, annual systems are expected to have significantly lower AMF abundance than perennial systems at greater depths.

Perennial pasture showed a significant greater stock of EE-GRSP in macroaggregates compared to the disturbed systems at the surface. This finding may help explain why only perennial pasture had significantly greater POM-C stock in macroaggregates than continuous maize or organic forage. As mentioned, macroaggregates protect organic matter from microbial degradation, which may be enhanced by lower macroaggregate turnover rates in undisturbed compared to disturbed systems (Six et al., 2000). Further, greater EE-GRSP concentration has been reported under no-till systems when compared with chisel-plow tillage (Wright et al., 2007). Under *in vitro* conditions, Morris et al. (2019) reported that AMF inoculation not only enhanced macroaggregate formation but also increased their stability by extending its turnover

time compared to those formed without AMF inoculation (139 vs. 76 days, respectively). Therefore, we suggest that a greater stock of EE-GRSP in macroaggregates helps slow their turnover rates, potentially enhancing POM-C and MAOM-C stabilization inside this structure. It is important to highlight that in addition to EE-GRSP and AMF hypha, plant roots can promote aggregation (Morris et al., 2019).

EPS-polysaccharide stocks were not affected by cropping systems, but they decreased with increasing soil depth. Similarly, Sher et al. (2020) found that EPS-polysaccharide decreased with depth (10 μg g soil^{-1} at 0 to 30 cm to 4.5 μg g soil^{-1} at 60 to 90 cm) in a long-term switchgrass field. They further explained that plant roots may stimulate EPS-polysaccharide production both directly – through the production of root exudates – and indirectly by reducing water potential. The decomposition of aboveground biomass along with root exudates contributes to the abundance of dissolved organic C in soil (Zhang et al., 2021). Consequently, the abundance of soluble C substrates was expected to decline with soil depth resulting in reduced C input for microbial EPS production. Although undisturbed systems have greater belowground biomass than disturbed systems and may thus provide more C substrates to support microbial EPS production, disturbed systems also have greater aboveground biomass, which may have compensated for C substrate availability to the microbial community. Overall, these results show that stock of macroaggregates are correlated with stock of EE-GRSP in bulk soil (Appendix Figure 2-1) or with EE-GRSP within macroaggregates (Appendix Figure 2-2) but not with EPS-polysaccharides (Appendix Figure 2-3).

5. Conclusions

Within-macroaggregate POM-C stocks were higher in perennial grasslands in surface and deepest soils, pointing to the importance of high C inputs from dense, fibrous, productive roots in

perennial grasslands. Within these same macroaggregates, MAOM-C stocks and EE-GRSP stocks were higher in surface soils of perennial grasslands even though macroaggregate stocks were not significantly different across systems indicating a more important contribution of AMF to more stabilized soil C via hyphal biomass inputs compared to its ability bind and stabilize macroaggregates. However, AMF's ability to act as a binding agent may have driven greater overall macroaggregate stocks across all soil depths in restored prairie, especially in the deepest soil increment sampled (30 to 60-cm), which had almost 3x the amount of EE-GRSP stocks as other systems at this depth. EE-GRSP, but not EPS, was affected by the cropping system or correlated with macroaggregates. These findings are important for cropping system management to fight climate change because they help us better understand which factors contribute to soil C accrual and stabilization.

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7. Appendices

Appendix Table 2-1. Bulk density and macroaggregates as concentrations across cropping systems and depths at WICST.

Depth (cm)	Cropping Systems				Mean	SEM	P		
	Continuous maize	Organic forage	Grazed pasture	Restored prairie			CS	Depth	CS*Depth
Bulk density (g cm^{-3})						0.06	0.005	<0.0001	0.09
0-15	1.25	1.21	1.13	1.02	1.16^{B}				
15-30	1.37	1.31	1.21	1.26	1.29^{A}				
30-60	1.36	1.34	1.25	1.36	1.33^{A}				
Mean	1.33^{A}	1.29^{A}	1.20^{B}	1.22^{AB}					
Macroaggregates (%)									
0-15	43.69^{b}	48.66^{b}	63.72^{a}	79.86^{a}	58.98^{A}	6.04	0.005	<0.0001	0.03
15-30	37.66^{b}	40.85^{b}	47.21^{ab}	62.86^{a}	47.15^{B}				
30-60	33.15	24.64	25.17	44.07	31.76^{C}				
Mean	38.17^{C}	38.05^{C}	45.37^{B}	62.26^{A}					

Different lowercase letters indicate significant difference within the same depth among cropping systems. Capital letters indicate differences across cropping systems (CS) or depths.

Appendix Table 2-2. EE-GRSP within bulk soil, EE-GRSP within macroaggregates, and EPS-polysaccharides as concentrations across cropping systems and depths at WICST.

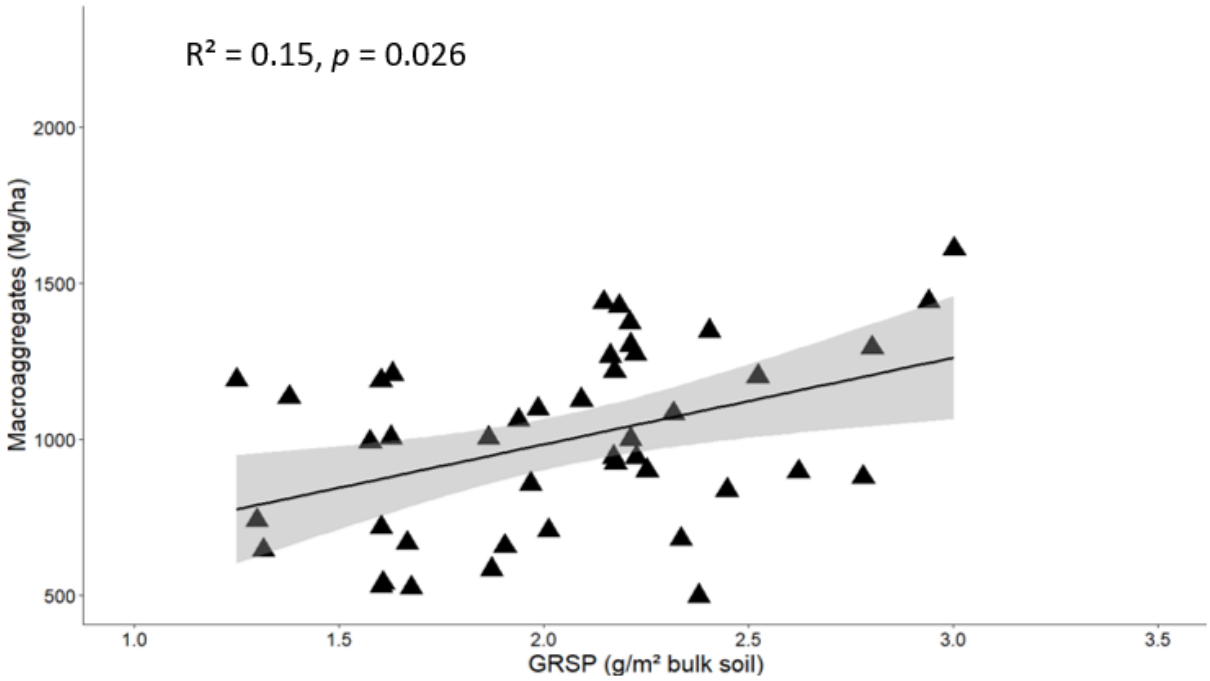
Depth (cm)	Cropping Systems				Mean	SEM	P		
	Continuous maize	Organic forage	Grazed pasture	Restored prairie			CS	Depth	CS*Depth
EE-GRSP (mg g^{-1} bulk soil)									
0-15	1.34^{a}	1.10^{b}	1.35^{a}	1.29^{ab}	1.27^{A}	0.07	0.05	<0.0001	0.001
15-30	0.92	0.82	0.86	0.77	0.84^{B}				
30-60	0.53^{b}	0.58^{b}	0.60^{ab}	0.85^{a}	0.64^{C}				
Mean	0.93^{A}	0.83^{A}	0.94^{A}	0.97^{A}					
EPS-polysaccharides (mg g^{-1} bulk soil)									
0-15	1.98	2.67	1.89	2.29	1.27^{A}	0.74	0.54	<0.0001	0.89
15-30	1.92	2.74	1.95	2.91	0.84^{B}				
30-60	0.13	0.16	0.27	0.11	0.64^{C}				
Mean	1.34	1.88	1.37	1.77					
EE-GRSP (mg g^{-1} macroaggregate)									
0-15	1.02^{ab}	1.04^{ab}	1.17^{a}	0.91^{b}	1.04^{A}	0.06	0.46	<0.0001	0.0004
15-30	0.82^{a}	0.62^{b}	0.74^{ab}	0.66^{ab}	0.71^{B}				
30-60	0.46^{b}	0.54^{ab}	0.49^{b}	0.72^{a}	0.55^{C}				
Mean	0.77	0.74	0.80	0.77					

Different lowercase letters indicate significant difference within the same depth among cropping systems. Capital letters indicate differences across cropping systems (CS) or depths.

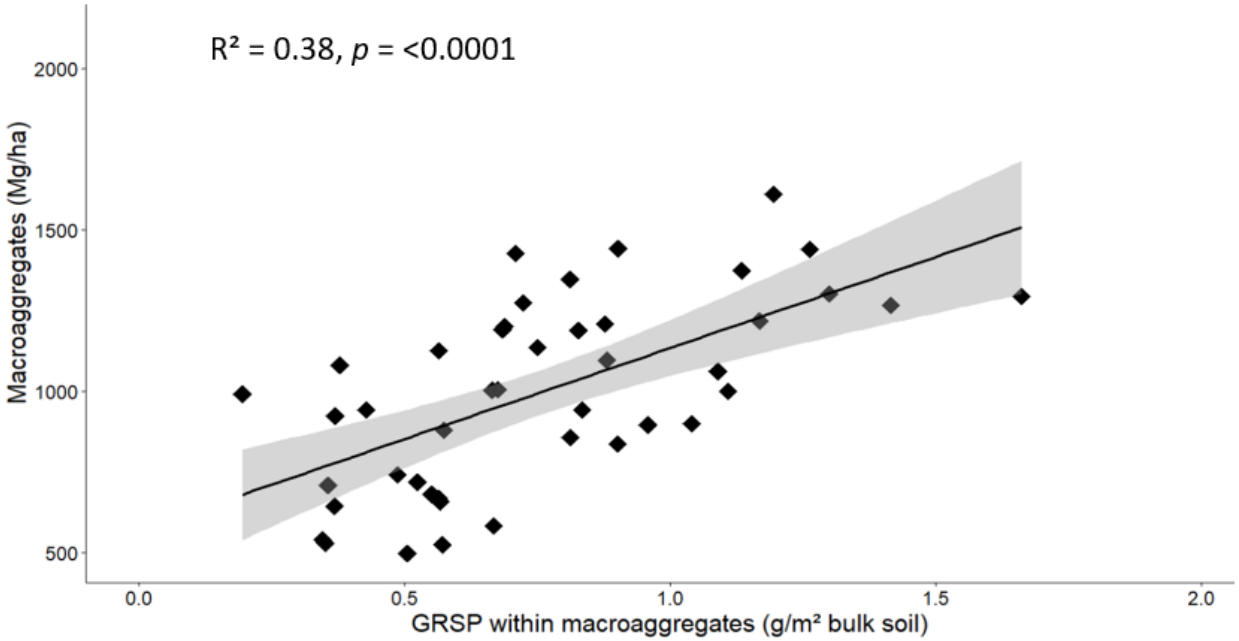
Appendix Table 2-3. MAOM-C and POM-C concentrations within macroaggregates and microaggregates across cropping systems and depth at WICST.

Depth (cm)	Cropping Systems				Mean	SEM	P		
	Continuous maize	Organic forage	Grazed pasture	Restored prairie			CS	Depth	CS*Depth
MAOM-C in macroaggregates (%)									
0-15	2.58	2.50	3.23	2.77	2.77^{A}	0.37	0.03	<0.001	0.67
15-30	2.25	2.29	2.48	1.93	2.24^{B}				
30-60	1.22	1.31	1.97	1.53	1.51^{C}				
Mean	2.02^{B}	2.03^{B}	2.56^{A}	2.08^{AB}					
MAOM-C in microaggregates (%)									
0-15	2.40	2.64	2.88	3.07	2.75^{A}	0.38	0.46	<0.001	0.82
15-30	2.08	2.07	2.15	2.03	2.08^{B}				
30-60	1.13	1.07	1.47	1.70	1.35^{C}				
Mean	1.87	1.93	2.17	2.27					
POM-C in macroaggregates (%)									
0-15	7.25	6.49	6.79	5.88	6.60^{A}	0.88	0.15	<0.001	0.47
15-30	2.24	3.13	3.75	1.77	2.72^{B}				
30-60	1.04	1.11	3.08	1.23	1.61^{C}				
Mean	3.51	3.58	4.54	2.96					
POM-C in microaggregates (%)									
0-15	3.58	3.36	3.42	3.59	3.49^{A}	0.40	0.51	<0.001	0.52
15-30	1.20	1.48	1.41	1.08	1.29^{B}				
30-60	0.71	0.77	1.81	0.98	1.07^{B}				
Mean	1.83	1.87	2.21	1.88					

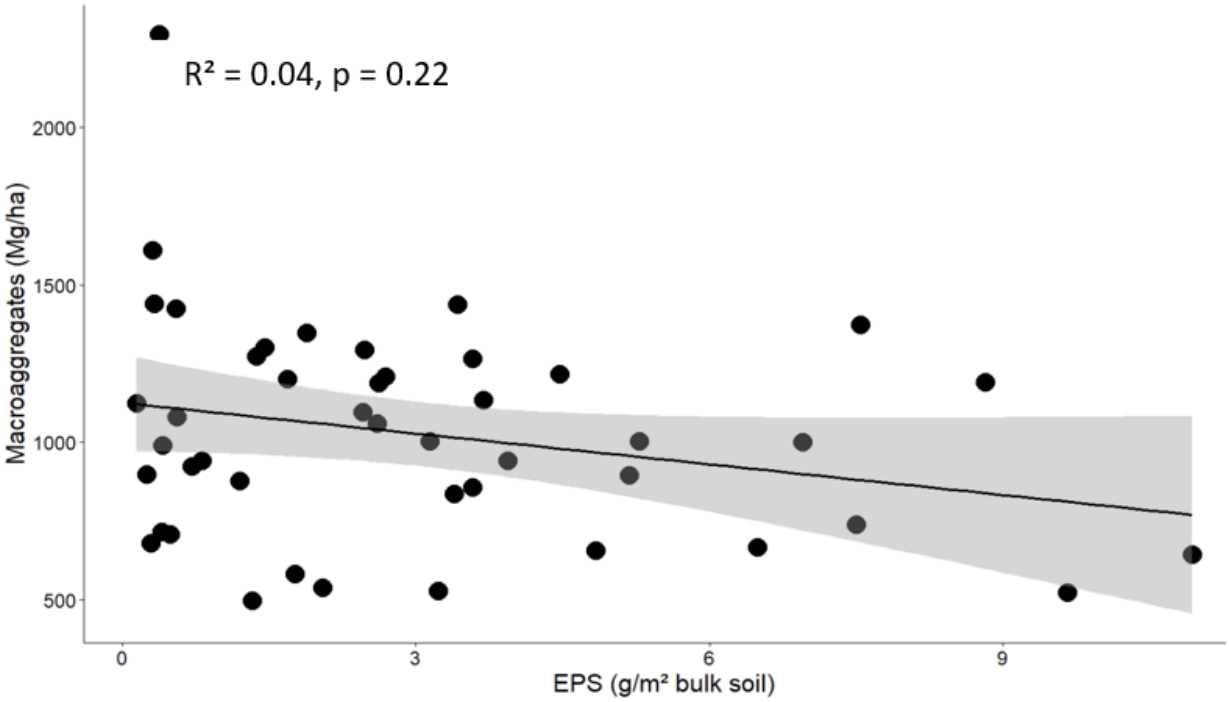
Different lowercase letters indicate significant difference within the same depth among cropping systems. Capital letters indicate differences across cropping systems (CS) or depths.



Appendix Figure 2-1. Correlation between macroaggregates and EE-GRSP in bulk soil stocks across 4 cropping systems at 3 depths



Appendix Figure 2-2. Correlation between macroaggregates and EE-GRSP within macroaggregates stocks across 4 cropping systems at 3 depths



Appendix Figure 2-3. Correlation between macroaggregates and EPS-polysaccharide stocks across 4 cropping systems at 3 depths

Chapter 3: Storage duration, but not temperature, affected EPS-polysaccharide extraction

1. Abstract

Extracellular polymeric substances (EPS) can be important binding agents in soil aggregation, particularly for macroaggregate formation. EPS extraction typically is performed on soil samples that have been refrigerated for some time before the extraction, but logistical constraints may affect pre-extraction handling and storage. Because soil samples commonly are stored at 4°C or -20°C for microbial analyses, we evaluated the effects of these storage temperatures on EPS abundance. We collected 32 soil samples from a cool-season pasture rotationally grazed by dairy heifers to 12-cm depth, sieved these samples to <2 mm, and stored soils in plastic bags. A 2×4 factorial design was used to compare two storage temperatures (4°C and -20°C) and four storage durations (1, 9, 19, and 43 d), with four replicates per combination. As storage duration increased, EPS-polysaccharide abundance and microbial biomass C (MBC) increased. Initially higher DOC concentrations at -20°C compared to 4°C may stem from increased microbial lysis under freezing conditions, along with greater access to organic matter from disrupted aggregates. However, the lack of significant difference of temperature on MBC may be due to the release of nutrients from freezing could offset the lower microbial viability when compared to cold storage. Storage duration is an important consideration for studies extracting EPS from soil. Future work should explore a range of soil types with differing organic C content to provide a more comprehensive understanding of storage and handling effects on EPS assays.

2. Introduction

Extracellular polymeric substances (EPS) are produced by bacteria and archaea in soil ecosystems improving microbial activity and soil functions such as aggregation and organic matter accrual (Poli et al., 2018). EPS are comprised of proteins, polysaccharides, nucleic acids, and lipids, which serve to retain extracellular enzymes and sequester particulate and dissolved nutrients for further microbial use as an energy source (Flemming and Wingender, 2010). Under *in vitro* conditions, Redmile-Gordon et al. (2015) reported that addition of available C (glycerol) to the soil increased the abundance of EPS carbohydrates and proteins compared to an unamended control. However, when only inorganic N was added, EPS abundance decreased, as microbes broke down EPS for use as a C source. In the field, root exudates (Sher et al., 2020) and plant litter decomposition (Zhang et al., 2021) may increase EPS abundance by enhancing the pool of dissolved organic C available to soil microorganisms. However, these microorganisms may also use EPS as a C source. Under storage conditions, therefore, microbes are likely to use dissolved organic nutrients and/or EPS to meet their basal energy requirements.

Stenberg et al. (1998) examined the effects of cold and frozen storage on microbial parameters in 12 agricultural soils observing fluctuations in microbial biomass during 13 months of storage. Freezing had less impact on microbial biomass than cold storage (Stenberg et al., 1998) so these authors recommended freezing soil samples for microbial analysis, particularly in environments naturally exposed to freeze-thaw cycles where microbes may be adapted to slower nutrient availability (Stenberg et al., 1998). Other studies indicated that freezing has little or no impact on selected soil parameters. For instance, Sun et al. (2015) found that freezing did not affect the concentration of labile organic C in soil samples stored for one month compared to fresh samples. However, Kim et al. (2023) observed that freeze-thaw cycles decreased the

concentration of labile dissolved organic C in Arctic soils, as microbes decomposed available C during thawing. Interestingly, extracellular enzymes necessary for breaking down recalcitrant organic matter were unaffected by freeze-thaw cycles, indicating that microbes preferentially consume labile over recalcitrant organic matter (Kim et al., 2023).

Given the common use of cold and frozen storage in microbial analysis (Tian et al., 2025), we analyzed the effects of storage temperature (4°C and -20°C) and storage duration (1, 9, 19, and 43 d) on the abundance of EPS. We hypothesized that frozen storage has less impact on EPS abundance than cold storage because microbes primarily use dissolved organic nutrients to meet their basal metabolic needs, but when dissolved organic nutrients are relatively unavailable, microbes likely utilize EPS as an alternative energy source.

3. Methods

3.1 Soil sampling

Soil samples were collected from a rotationally grazed pasture (PAS) established in 1990 as part of the Wisconsin Integrated Cropping System Trial (WICST) at the University of Wisconsin-Madison's Arlington Agricultural Research Station (43.30 N, -89.33 W). WICST is a randomized complete block experiment with four blocks, each containing 0.3-ha plots. All fieldwork is conducted using large farm-scale equipment. The PAS system consisted of a mix of cool-season grasses Timothy (*Phleum pratense* L.), Kentucky bluegrass (*Poa pratensis* L.), orchardgrass (*Dactylis glomerata* L., ryegrass (*Lolium perenne* L.), and festulolium (xFestulolium Asch. & Graebn.), dandelion (*Taraxacum officinale* F.H. Wigg.), and clover (*Trifolium pratense* L. and *T. repens* L.). Rotational grazing by six heifers takes place annually from May 1 to October 10, maintaining a stocking rate of ~14 AU ha^{-1} yr^{-1}.

Soil samples from the PAS system were taken on 14 Oct 2024. One block of the PAS was divided into four transects, and a total of four cores were taken from each transect. Soil samples were collected from the surface 12 cm and composited by mixing them very well by hand, resulting in a total of one composite soil sample per experimental unit. In the lab, the field-moist samples were sieved to 2 mm and all visible plant material was removed. Each composite soil sample was then divided into two storage temperature groups (4°C and -20°C), and each group was further divided into four subgroups for the analysis of storage duration (1, 9, 19, and 43 d). Each storage duration was subdivided into 4 bags or repetitions. Each soil sample stored at 4°C or -20°C in plastic bags and discarded after analysis to avoid interference caused by freezing-thawing. Direct thawing at room temperature of samples stored at -20°C was done for 2 h. Soil moisture was analyzed on soil samples by determination of loss of weight after 12 h at 60°C.

3.2 Extracellular polymeric substances (EPS) extraction

We followed the methodology modified by Redmile-Gordon et al. (2014) with addition of a filter step after extraction (Sher et al., 2020) to remove tiny roots from the supernatant. Readily soluble organic material was removed from 2.5 g of moist soil (2 g dry weight equivalent) using 0.01 M $CaCl_2$ in centrifuge tubes. These tubes were shaken and then centrifuged at 3750 rpm for 30 min. The supernatant was discarded, and EPS was extracted from the remaining pellet by re-suspending it in extraction buffer. Cation exchange resin (Na ion exchange resin, strongly acidic, 20-50 mesh) was added to the pellet along with the buffer (2 mM $Na_3PO_4 \cdot 12H_2O$, 4 mM $NaH_2PO_4 \cdot H_2O$, 9 mM NaCl, 1 mM KCl), adjusted to pH 7. After shaking for 2 h and centrifuging at 3750 rpm for 30 min, the supernatant containing EPS was filtered through a 2.5-μm nylon filter, transferred into new tubes, and stored at -20°C for carbohydrate

analysis. The supernatant was used to measure EPS-polysaccharides with a Total Carbohydrate Assay Kit (Cell Biolabs, Inc., San Diego, CA, USA).

3.3 Microbial biomass C and dissolved organic C

Microbial biomass C (MBC) was determined using the direct-chloroform extraction method, following Gregorich et al. (1990) with minor modifications. This method involves ethanol-free chloroform, which disrupts microbial cell membranes and kills microbes. The surviving microbes metabolize the available C, leading to a flush of CO_2, which is captured by K_2SO_4 (Kandeler, 2015). The quantification of this flush of C is performed in the liquid fraction, and extracted C is converted to MBC using a conversion factor of 0.45 (Vance et al., 1987) or 0.17 (Gregorich et al., 1990).

Soil samples were divided into two subsamples of 10 g (dry weight equivalent), each treated with 40 mL of K_2SO_4. One subsample was fumigated with 0.5 mL of ethanol-free chloroform, while the other remained unfumigated. All subsamples, including blanks, were mixed on a rotary shaker for 10 min, centrifuged at 3750 RPM for 10 min, and the supernatant was filtered through a 2.5-μm pore filter. Fumigated samples were sparged with air to remove residual chloroform. Unfumigated samples and blanks were processed in the same manner for consistency. The C content from the unfumigated subsamples (Jones and Willett, 2006) was used to determine dissolved organic carbon (DOC) using a Shimadzu TOC-L analyzer (Shimadzu Corp., Kyoto, Japan). MBC was calculated as the difference in C between the fumigated and unfumigated subsamples, divided by a conversion factor of 0.17 (Gregorich et al., 1990). To ensure comparability with previous MBC studies, we converted MBC to flush C using their reported factors and applied the 0.17 factor when necessary. To minimize the effects of sampling before microbial C analysis, a pre-incubation or conditioning step is often recommended

(Franzluebbers, 1999). However, in this study, soil samples were collected from the same plot and homogenized by thorough hand mixing, without a pre-incubation step, as the focus was on evaluating storage conditions' effects on EPS.

3.4 Data analysis

The experiment followed a 2×4 factorial design where levels of each factor were randomly assigned to PAS soil samples. In this setup, a factorial combination of two storage temperatures (4°C and -20°C) and four storage durations (1, 9, 19, and 43 d) were assigned to the PAS soil samples with 4 repetitions of each factorial combination. ANOVA was performed using R (LME4 package) and means were separated using the Tukey HSD procedure. Residuals were analyzed for normality and homogeneity of variance.

4. Results

Storage duration, but not storage temperature, affected EPS abundance and MBC, while both factors influenced DOC and soil moisture (Table 3-1). EPS concentrations increased from day 1 to day 9 and then remained stable for the rest of the storage period (Figure 3-1). MBC followed a similar trend but showed a significant decline between days 19 and 43 (Figure 3-1). Although the initial DOC level was higher under freezing than cold temperatures, levels converged by the end of the storage period (Figure 3-2). Similarly, despite fluctuations, soil moisture decreased to the same level under both temperatures by the end of the storage period (Figure 3-2).

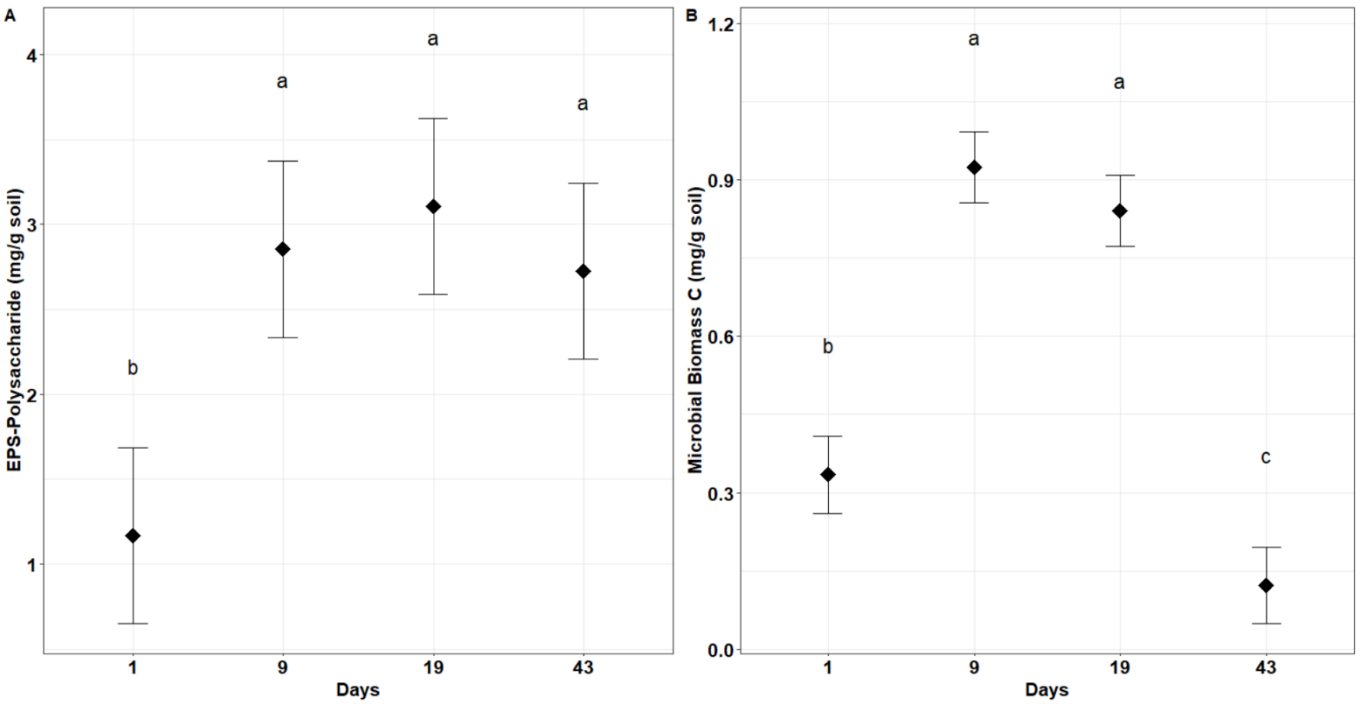


Figure 3-1. (A) Mean (±SE) EPS-polysaccharide and (B) Mean (±SE) MBC over storage time.

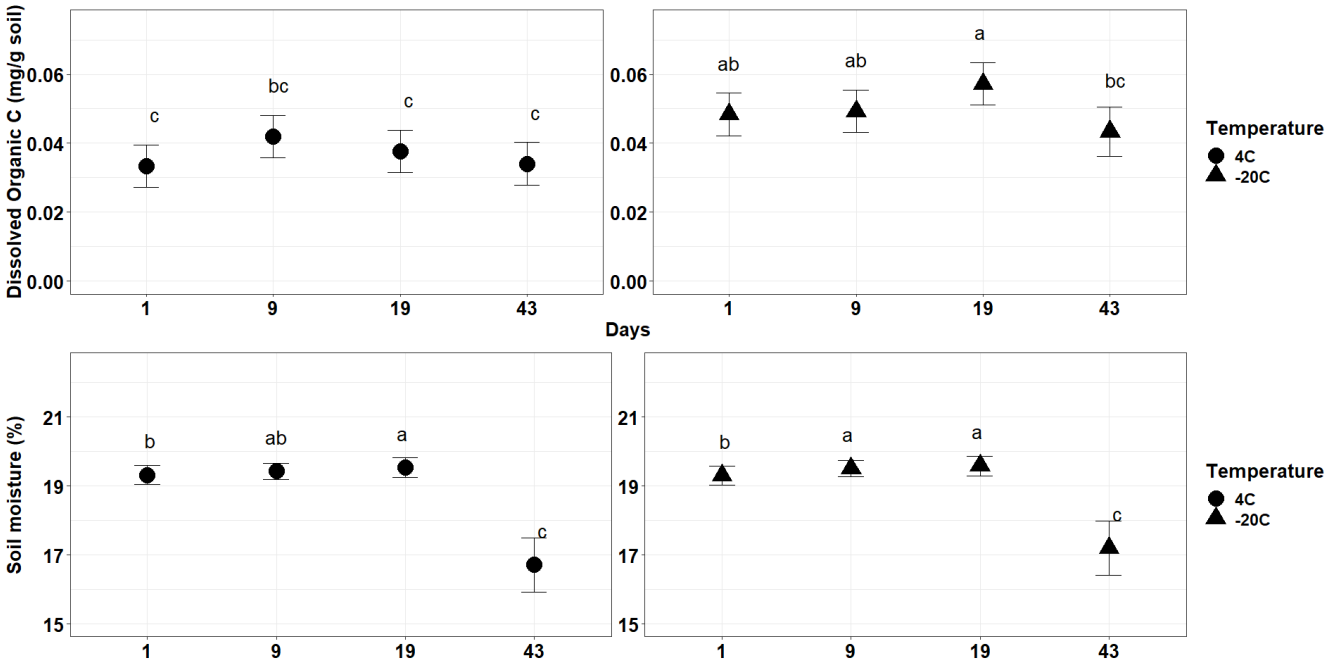


Figure 3- 2. Mean (±SE) DOC and soil moisture over storage time.

Table 3-1. Effect of storage conditions on microbial biomass C, dissolved organic C, soil moisture, and abundance of EPS-polysaccharides

Response	Day				SEM	*P*		
	1	9	19	43		Day	Tem	Day*Tem
Temperature								
Microbial biomass C (mg g^{-1} soil)								
-20°C	0.417	0.939	0.819	0.123	0.027	<0.0001	0.12	0.06
4°C	0.251	0.909	0.862	0.121				
Mean	0.334^{a}	0.924^{b}	0.840^{b}	0.122^{c}				
Dissolved organic C (mg g^{-1} soil)								
-20°C	0.0484^{ab}	0.0493^{ab}	0.0572^{a}	0.0434^{bc}	0.002	<0.0001	<0.0001	0.03
4°C	0.0334^{c}	0.0419^{bc}	0.0377^{c}	0.0341^{c}				
Moisture (%)								
-20°C	19.31^{b}	19.51^{a}	19.59^{a}	17.19^{c}	0.12	<0.0001	0.06	0.05
4°C	19.32^{b}	19.43^{ab}	19.53^{a}	16.71^{c}				
EPS-Polysaccharides (mg g^{-1} soil)								
-20°C	1.017	3.472	3.159	3.187	0.25	<0.0001	0.06	0.14
4°C	1.313	2.234	3.052	2.260				
Mean	1.16^{b}	2.85^{a}	3.11^{a}	2.72^{a}				

Different lowercase letters meant significant differences between storage temperature or storage duration.

5. Discussion

Storage duration increased EPS abundance and microbial biomass C at the beginning of the incubation; however, while EPS abundance was maintained, MBC decreased by the end of the storage period. On day 1, EPS-polysaccharide abundance was higher than the value reported by Redmile-Gordon et al. (2020) for a grassland system using CER extraction (0.35 mg g^{-1}) but remained within the range (0.05 to 1.4 mg g^{-1}) reported by Chenu (1995). Although there was no direct relationship between EPS-polysaccharide abundance and soil organic carbon (SOC), previous studies suggest that systems with higher SOC tend to have increased EPS levels. For instance, Redmile-Gordon et al. (2020) reported that grassland soils with 3.67% SOC had 0.35 mg g^{-1} of EPS-polysaccharide, whereas arable or fallow systems with lower SOC levels (1.64% and 0.87%, respectively) exhibited lower EPS abundance (0.32 and 0.29 mg g^{-1}, respectively). In our study, SOC levels were approximately 3.30 ± 0.62% (data from 2019, as reported in Dietz et

al., 2024). Sher et al. (2020) suggested that, in addition to SOC, microbial biomass and DOC availability may influence EPS-polysaccharide abundance. The relatively high EPS-polysaccharide values observed in our study may be attributed to differences in microbial biomass and DOC availability compared to previous studies.

Microbial biomass on day 1 (fresh samples) was affected by soil sample pretreatments. Our MBC result for day 1 (0.33 mg g^{-1} of soil) was lower than those reported by Bailey et al. (2002) (1.06–2.65 mg g^{-1} of soil) for agricultural soils converted to prairie for at least five years. Notably, Bailey et al. (2002) oven-dried their samples, rewetted them, and conducted a 10-d pre-incubation at 21°C in the dark before analysis. In contrast, we analyzed moist soil without pre-incubation or pretreatment; fresh-moist samples were only sieved through a 2-mm mesh. Our results fall within the range (0.26 to 0.79 mg g^{-1} of soil) reported by Schroeder et al. (2021) for cropland samples evaluated after 1 d of pre-incubation at 15°C in the dark. When Schroeder et al. (2021) compared pre-incubation periods of 1 d versus 14 d, they found that the extended pre-incubation reduced microbial biomass, likely because microorganisms had consumed a significant portion of the readily available C. However, a short pre-incubation period may help restore microbial activity after the disturbance caused by sampling (Franzluebbers, 1999). The lack of pre-incubation period after the disturbance of samples in this study may cause a decrease in microbial biomass when compared to Bailey et al. (2002) due to the lack of time for recovering after sampling and sieving (Stenberg et al., 1998).

Longer storage likely increased microbial mortality, with dead microbes serving as a nutrient source during sample thawing, increasing measured microbial biomass C. MBC increased on days 9 and 19 compared to day 1. These results agree Lee et al. (2007), who reported a 20% increase in MBC after storing soil at 4°C for four weeks compared to fresh

samples. These authors attributed this increase to the high initial C concentration and nutrient availability, which supported microbial growth. Similarly, Winter et al. (1994) observed higher MBC in soil samples frozen at -15°C for seven days compared to day 1 or nonfrozen samples. Both studies used the chloroform extraction method, but Winter et al. (1994) further suggested that freezing disrupted or dispersed soil aggregates, making organic matter more accessible to microbes upon thawing. The presence of liquid water during thawing softens aggregates and reduces their stability (Dagesse, 2013), which may facilitate microbial access to previously protected C sources. While freezing can lead to higher microbial mortality due to increased salt concentrations and membrane damage caused by intra- and extracellular ice crystal formation (Ragoonanan et al., 2010) compared with cold storage, the enhanced C extraction efficiency from reduced aggregate stability may offset these losses (Winter et al., 1994). The higher microbial mortality under freezing than cold storage may be reflected in the higher DOC observed on day 1.

As storage duration increased, microbial mortality rose and cell viability declined, reducing the amount of microbial biomass able to survive chloroform fumigation. This likely resulted in the observed reduction of MBC by day 43. The effect of storage temperature on microbial biomass is time-dependent (Tian et al., 2025). The drop of soil moisture at day 43 also contributed to the increase of microbial mortality as a consequence of microbial water stress (Manzanera, 2021). Cold storage allows for continued microbial activity, leading to a gradual depletion of available nutrients over time (Coxson and Parkinson, 1987). Lee et al. (2007) observed increased β-glucosidase activity—a key enzyme that hydrolyzes cellobiose and releases glucose for microbes—during storage at 4°C compared to fresh soil samples and suggested this increase as evidence of the low C availability. Limited C availability reduces microbial viability

and increases microbial mortality. As Tian et al. (2025) reported that microbial cell viability was 77.0%, 71.2%, and 47.3% when stored at 4°C, and 69.1%, 55.6%, and 38.1% when stored at -20°C, for storage durations of 5, 40, and 210 d, respectively, compared to fresh samples from semiarid grasslands. Their findings suggest that microbial cell viability is generally higher at 4°C than at -20°C. However, Stenberg et al. (1998) found that after 3 months, MBC and basal respiration rate decreased by 27% and 30%, respectively in refrigerated soils while a reduction of 13% and 16%, respectively, were observed in frozen soils compared to fresh samples. These authors suggested that freezing is better than cold temperatures for microbial analysis of soil samples. Freezing can increase available C due to microbial lysis caused by ice crystal formation or osmotic stress, as well as aggregate dispersion, potentially resulting in higher MBC than cold storage (Winter et al., 1994). Lack of difference between storage temperatures may stem from release of nutrients during freezing, which could offset the lower microbial viability when compared to cold storage. In addition, soil samples were collected from areas subject to annual freeze-thaw cycles, potentially making the microbial community more resilient to low temperatures (Stenberg et al., 1998). However, as storage duration increases, declining microbial cell viability leads to less biomass surviving chloroform fumigation, resulting in lower measured microbial biomass.

The increase in EPS-polysaccharide followed the same pattern observed for MBC. To our knowledge, this is the first study to examine the effect of storage conditions on EPS abundance. However, previous research provides insights that may help explain our findings. As noted earlier, the rise in MBC was likely due to increased access to organic matter resulting from decreased aggregate stability and from the lysis of microbes during storage. Redmile-Gordon et al. (2015) found that adding available C (glycerol) to incubated soil increased EPS-

polysaccharide concentrations compared to a control without glycerol. They hypothesized that microbes may store an excess of available C as EPS. Based on this, we suggest that the observed increase in EPS-polysaccharide resulted from microbes storing an excess of available C in this form. Additionally, microorganisms secrete EPS as a protection from environmental conditions, in particular, to retain moisture under drought conditions which make possible microbial activity when the diffusion of substrate are limited (Malik and Bouskill, 2022). The decrease in soil moisture observed on day 43 may have stimulated the production of EPS, although MBC was low.

6. Conclusions

EPS-polysaccharide abundance was higher in pasture soil stored for >9 d compared to fresh soil samples. Pre-incubation should be taken into consideration for EPS abundance comparisons in fresh soil. EPS appeared to be associated not only with organic C, but also with microbial biomass. Further research is needed to assess storage effects across a range of soil textures and C contents over longer storage periods to better understand EPS dynamics under storage conditions.

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Chapter 4: Arbuscular mycorrhizal fungal necromass promotes macroagregate formation in a loamy sand

1. Abstract

Soil is an important C reservoir, but microbial degradation of organic matter releases C to the atmosphere. Although soil aggregation plays a key role in protecting C, the mechanisms of organic matter formation and stabilization remain uncertain. Microbial residues are known to bind mineral particles and promote aggregate formation that can occlude organic matter C. In the lab, we experimentally added residues of bacteria (extracellular polymeric substances, EPS) and fungi to soils with no growing plants and monitored soil aggregate, microbial biomass (phospholipid fatty acids) and microbial residues (glomalin related soil protein or GRSP and EPS) over time. We extracted EPS from gram positive (*Bacillus spizizenii*) and gram negative (*Sinorhizobium meliloti*) bacteria (hereafter EPSP and EPSN, respectively) and germinated and dried arbuscular mycorrhizal fungal (AMF) spores and collected the AMF necromass (AMFN). We added these cultivated residues to a loamy sand soil and also monitored a control (CON) with no residue added. Only AMFN addition promoted macroaggregate formation, with a peak observed at 14 d. AMFN addition did not increase GRSP, but stimulated overall fungal biomass, which was mainly AMF, a surprising result given the absence of plant growth. AMF residues promoted soil aggregation, and perhaps subsequent AMF growth, in a low-organic matter loamy sand.

2. Introduction

Soils store a significant pool of C (3500-4800 Pg C), containing more than aboveground biomass (420 Pg C) and the atmosphere (829 Pg C) combined (Lehmann and Kleber, 2015). Agricultural management that increases C inputs and reduces C outputs from soil is key to

helping mitigate climate change and adapt to its effects (Krupek et al., 2022). Soil aggregation is a key protective mechanism against microbial degradation of organic matter belowground (Lehmann and Kleber, 2015) that can help stabilize soil C (King et al., 2019).

Tisdall and Oades (1982) describe organic matter as the main binding agent of soil aggregates. Their model begins with the interaction of inorganic elements (e.g., Fe, Mn, Al, and Si) and organic molecules (e.g., polysaccharides) driven by electrostatic surface forces resulting in the formation of microaggregates (<250 µm diameter; Totsche et al., 2018). When microaggregates bind together, they form bigger structures called small (250 to 2000 µm) or large (>2000 µm) macroaggregates (Liu et al., 2020) by temporary and transient binding agents. Tisdall and Oades (1982) classified organic binding agents into 3 groups: transient (polysaccharides); temporary (roots, and fungal hyphae); and persistent (humic materials associated with polyvalent metal cations and polymers strongly sorbed to clay).

Arbuscular mycorrhizal fungi (AMF) and extracellular polymeric substances (EPS) play important roles in aggregation. AMF and EPS are consistently associated with both aggregate stability and C storage in soil (Sher et al., 2020, Redmile-Gordon et al., 2020, Irving et al., 2020). AMF hyphae transport C from plants to mineral surfaces, contributing to soil C accumulation (Averill et al., 2014). AMF hyphae also intertwine with soil particles, playing a key role in physical stabilization of macroaggregates (Tisdall and Oades, 1982), while microaggregates are primarily stabilized by bacteria, polysaccharides, and organo-mineral complexes (Tisdall, 1994). Extraradical AMF hyphae and spores secrete a special glycoprotein called *glomalin*, which is referred to as glomalin-related soil protein (GRSP) based on assay methods (Rillig, 2004). When mycorrhizal mycelia slough, GRSP is released during decomposition coating soil organic matter

and aggregate surfaces. This layer acts as a barrier that reduces loss of water, nutrients, and C from soil aggregates (Zou et al., 2014).

Extracellular polymeric substances (EPS) produced by bacteria and archaea are thought to be important soil binding agents (Poli et al., 2018). Composed of proteins, polysaccharides, nucleic acids, and lipids (Flemming and Wingender, 2010) these substances are externally deposited by microbes. EPS play a key role in cell aggregation, biofilm formation, and attachment to mineral surfaces (Flemming and Wingender, 2010). Their amphiphilic nature, with both hydrophobic and hydrophilic groups, allow EPS to become positively (protonated amine and amide) or negatively (dissociated carboxyl or hydroxyl groups) charged depending on pH (Martinez et al., 2002), which affects their binding capacity to minerals (Chen et al., 2021). For example, EPS from *Bacillus subtilis* binds to clay minerals (kaolinite and montmorillonite) and the oxide goethite, particularly through interactions with peptide, lipophospholipids, and organic acids (Chen et al., 2021). The binding of EPS to goethite occurs primarily through electrostatic interactions, where the positively charged surface of goethite is attracted to the negatively charged EPS groups of *B. subtilis*. In contrast, attachment to negatively charged clay minerals likely involves non-electrostatic mechanisms such as hydrogen bonding and surface complexation (Chen et al., 2021). Similarly, *Sinorhizobium meliloti,* an important alpha-proteobacterium that fixes N and forms a symbiotic relationship with certain legumes (Primo et al., 2020), produces a negatively charged extracellular polysaccharide called *succinoglycan*. This polysaccharide has been reported to interact strongly with clay only under acidic pH conditions, when the clay carries a positive charge (Labille et al., 2005).

Like GRSP, EPS can contribute to formation of macroaggregates (Costa et al., 2018) and improve soil aggregation (Sher et al., 2020). Cheng et al (2020) showed that separate addition of

EPS produced by *Bacillus proteolyticus* and *Pseudomonas chlorophis* to soil increased the percentage of water-stable macroaggregates compared with the control (no addition of EPS) after 30 d of incubation. Sher et al. (2020) reported a positive covariance between EPS-polysaccharides (EPS-CHO) and water-stable aggregates in the soil. The composition analysis of EPS-CHO revealed a predominantly microbial origin (Sher et al., 2020), but did not evaluate fungal biomass, which could also impact soil aggregation. In their study, Redmile-Gordon et al. (2020) investigated the relationship between mean weight diameter (MWD) with EPS-protein and EPS-CHO, reporting a stronger association between MWD and EPS-protein compared to EPS-CHO. However, the authors also observed that an increase in MWD was not always solely attributable to an increase in EPS. They explained that root and hyphal contributions might have played a role in enhancing MWD, but it was not evaluated. Further, these authors reported that concentrations of EPS-protein and SOC increased across fallow, fertilized crop, and unfertilized grassland established for over 50 yr.

At the Wisconsin Integrated Cropping Systems Trial (WICST), 30-yr monitoring of typical cash-grain and dairy-forage cropping systems on a silt loam Molllisol has shown perennial grasslands (rotationally grazed perennial pasture and restored tallgrass prairie) to have a higher abundance of SOC (Dietz et al. 2024, Sanford et al. 2012), but the mechanisms underlying these findings are not fully understood. Lack of soil disturbance in perennial pastures pointed to greater POM-C occluded in soil aggregates after ~20 yr (Cates et al. 2016), while microbial abundance and AMF taxonomic richness were higher under perennial pasture (Potter et al. 2022). Rui et al. (2022) showed higher C use efficiency and SOC stabilization in the grazed pastures indicating microbial composition and residues as likely drivers of SOC differences. Recent work demonstrated higher mineral-associated organic C and AMF residue in perennial

pastures, with a significantly correlation between GRSP and soil aggregates (Leon et al., Ch. 2 this volume). Here, we tested the independent contributions of AMF necromass and EPS on macroaggregates by adding them to a plant-free loamy sand as a sensitive substrate and monitoring aggregates, microbial biomass, and microbial residues over time.

3. Methods

3.1 Soil collection and characterization

Soil samples were collected from the Hancock Agriculture Research Station (HARS) in southern Wisconsin during June and July 2024 from a corn field that previously was planted to soybeans (2022 and 2023) and corn (2021). Soil samples (Table 3-1) were placed in aluminum containers and oven-dried at 60°C for 24 h. Once dried, they were sieved to <250 μm to remove macroaggregates and stored at 4°C until further analysis. For texture determination, the aggregates in the <250-μm soil fraction were dispersed using a sodium hexametaphosphate (SHMP) solution. Texture analysis followed the hydrometer method (Bouyoucos, 1962) with slight modifications from other protocols (Gee and Bauder, 2018; Moorberg and Crouse, 2021). A total of 40 g of soil was mixed with SHMP in a 1-L graduated cylinder. Liquid density measurements were recorded at 40 sec to estimate silt and clay concentrations, and again after 7 h to estimate clay concentration. Density readings were corrected for temperature and a blank sample. Clay mass proportion was calculated as the difference between the 40-sec and 7-h readings. Finally, sand mass proportion was determined by subtracting the sum of clay and silt proportions from 100%. Soil sieved <250 μm was classified as loamy sand (Table 4-1).

Table 4-1. Characteristics of HARS sandy soil in Fall 2022.

Parameter	Quantity
pH	6.8
CEC (meq 100 g^{-1} soil)	2
Organic matter (%)	1
Total P (mg kg^{-1})	55
Total K (mg kg^{-1})	169
Sand (%)	90
Silt (%)	5
Clay (%)	5
Sand (%) of sieved soil	78
Silt (%) of sieved soil	15
Clay (%) of sieved soil	7

3.2 Soil binding agent production and characterization

3.2.1 EPS production and extraction

Two bacterial strains were used to produce EPS – *Bacillus spizizenii* and *Sinorhizobium meliloti*. Cultivation of *B. spizizenii* (ATCC 6633) followed the protocol outlined by Chen et al. (2021). The pre-cultivation of *B. spizizenii* was done in 1 mL of LB-Miller medium (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl) at 30°C for 12 h. Then, it was transferred into 500 mL of fresh LB-Miller medium and cultured with shaking at 30°C for an additional 48 h. *B. spizizenii* and *S. meliloti* were cultivated in the Ané Lab, University of Wisconsin–Madison (details on the cultivation of *S. meliloti* in Appendix 4-1). After incubation, cells were separated from the culture medium by centrifugation at 3750 rpm – *B. subtilis* for 15 min at 4 °C, and *S. meliloti* for 45 min. After centrifugation, the solution was precipitated with three volumes of cold ethanol, and EPS was obtained by double centrifugation (3750 rpm for 15 min). The resulting EPS pellets were oven-dried at 55°C for 48 h. Once dried, EPS pellets were placed in 2-mL

microcentrifuge tubes with sterilized metal beads and pulverized using a shaker. Pulverized dried EPS were stored at 4°C. Many batches were cultivated to obtain >700 mg of EPS per bacterium.

3.2.2 Germination of AMF spores and collection of hyphal necromass

The germination of AMF spores was conducted according to the protocol outlined by Mukherjee and Ané (2011). Sterile *Glomus intradices* spores (Mycorise ASP; Premier Tech Biotechnologies, Rivière-du-Loup, Quebec, Canada) were washed three times with sterile distilled water and centrifuged at 3750 rpm for 10 min. After washing, spores were incubated in sterile distilled water at 25°C in the dark, where they germinated after 7 d. Once germinated, AMF hyphae were dried in an oven at 55°C for 72 h. Dried AMF necromass (AMFN) was collected in a microcentrifuge tube, and a sterilized metal bead was added to pulverize it by shaking. Pulverized AMFN was stored in a 2-ml microcentrifuge tube at 4°C.

3.3 EPS and fungal necromass effect on macroaggregate formation

Thirty-five grams of dry, sieved soil were placed into 50-ml centrifuge tubes. Four treatments were applied, each with five replicates: (1) CON (control; soil without the addition of EPS or fungal necromass), (2) EPSP (soil + EPS from *B. spizizenii*), (3) EPSN (soil + EPS from *S. meliloti*), and (4) AMFN (soil + AM fungal necromass). EPS or fungal necromass was added at a rate of 2 mg/g dry soil. After addition of EPS or AMF necromass, the treated soil was mixed by vortexing. Nanopore water was then added to each tube to achieve 50% of the soil's water holding capacity (WHC), resulting in a final bulk density of 1.3 g/cm^3. Tubes were incubated in the dark for 28 d at 25 °C, with sampling on days 14 and 28 resulting in the following treatments: CON-14, EPSP-14, EPSN-14, AMFN-14, and CON-28, EPSP-28, EPSN-28, AMFN-28. All tubes were destructively harvested at each sampling date. Additionally, five tubes were set aside for the CON treatment to measure initial soil characteristics on day 1 (CON-1). Throughout the

incubation period, nanopore water was added as needed to maintain 50% water holding capacity. The amount of water added was equivalent to the difference in tube weights compared to the initial weights recorded at the start of the experiment.

The analysis of water-stable macroaggregates followed the method of Elliot (1986), with slight modifications. Ten grams of fresh soil were dried for 24 h in an oven at 60°C. Then the dried soil was soaked in distilled water for 5 min to allow slaking. The mixture was then poured onto a 250-μm sieve and agitated by moving the sieve up and down 50x for 2 min. Aggregates >250 μm were collected, dried, and weighed for quantification of macroaggregates.

3.4 Microbial composition assay

The phospholipid fatty acid (PLFA) extraction and analysis protocol was adapted from methods used in the Jackson Lab (based on procedures by M. Miller, Argonne National Laboratory) and follows standard approaches described by Frostegård et al. (1991), Bligh and Dyer (1959), and Zelles (1999). Fresh soil samples were stored at –20 °C prior to freeze-drying. After freeze-drying and grinding, lipids were extracted from 3 g of soil using a chloroform:methanol:phosphate buffer solution (50 mM, pH 7.4) in a 1:2:0.8 ratio. After phase separation and isolation of the chloroform phase, phospholipids were isolated by silicic acid chromatography, using solid phase extraction columns containing 0.5 g of silicic acid (Thermo). The phospholipids were converted into methyl esters by mild base methanolysis, then concentrated and resuspended in hexane with a quantification standard (19:0 ethyl ester). The extracts were analyzed by gas chromatography with flame ionization detection (GC-FID) using an Agilent 6890 GC, J&W Ultra-2 column (25 m, 0.2 mm ID and 0.33 um film thickness) using hydrogen as carrier gas. Lipids were identified using the MIDI Sherlock software (MIDI, Inc., Newark, DE, now a division of Biolog, Inc.). Lipid biomarkers were used to infer microbial

groups such as Gram-positive and Gram-negative bacteria, fungi, and arbuscular mycorrhizal fungi (Zelles, 1999).

3.5 Microbial binding agent assay

EPS assessment was done following Redmile-Gordon et al. (2014) with addition of a filter step after extraction (Sher et al., 2020). Readily soluble organic material was removed from 2.5 g of moist soil (2 g dry weight equivalent) using 0.01 M $CaCl_2$ in centrifuge tubes. These tubes were shaken and then centrifuged at 3750 rpm for 30 min. The supernatant was discarded, and EPS was extracted from the remaining pellet by re-suspending it in extraction buffer. Cation exchange resin (Na ion exchange resin, strongly acidic, 20 to 50 mesh) was added to the pellet along with the buffer (2 mM $Na_3PO_4 \cdot 12H_2O$, 4 mM $NaH_2PO_4 \cdot H_2O$, 9 mM NaCl, 1 mM KCl), adjusted to pH 7. After shaking for 2 h and centrifuging at 3750 rpm for 30 min, the supernatant containing EPS was filtered through a 2.5-μm nylon filter, transferred into new tubes, and stored at −20°C for carbohydrate analysis. The supernatant was utilized to measure EPS-polysaccharides using the Total Carbohydrate Assay Kit (Cell Biolabs, Inc., San Diego, CA, USA).

For the extraction of GRSP from bulk soil, soil samples were mixed with 20 Mm sodium citrate solution and autoclaved one time at 121°C for 30 min following the procedure of Wright and Upadhyaya (1998). After centrifugation, the extracted sample was analyzed for protein content using the Bradford assay (Bradford, 1976). The content of C and N in EPSP, EPSN, and AMFN were analyzed using an elemental analyzer (PDZ-Europa ANCA-GSL).

3.6 Statistical analyses

Five replicate blocks of 9 treatments were randomly assigned to soils in a randomized complete block design where racks holding the centrifuge tubes were blocks. Statistical analyses

were conducted in R using the LME4 package with treatments as fixed and blocks as random effects. Residuals were checked for normality and homogeneity of variance. AMF abundance was log-transformed to normalize residuals, so P-values were calculated for models using the transformed response variable, but the original values are reported. Mean separation for these models was performed with the Tukey HSD test. Concentration of GRSP and the ratio of fungi to bacteria were analyzed using the Friedman test for non-parametric data, and the Nemenyi test was used for post-hoc comparisons of means (Demšar, 2006).

4. Results

Addition of AMF necromass increased water-stable macroaggregates at 14 d compared to the controls, but by 28 d macroaggregates were not significantly different between AMFN and EPSP at 14 d. Further, EPSP14 was not significantly different than controls (Figure 4-1). After overnight incubation, some macroaggregate formation was already observed in the control group, but the increase in macroaggregates was not reflected in changes in EPS or GRSP concentrations (Table 4-2) or total microbial biomass (Table 4-3). However, AMFN addition led to increased fungal biomass, primarily due to a rise in the AMF group (Table 4-3). EPSP addition increased gram positive biomass, but this was only observed on day 14 compared to the control. Compositional analysis of EPSP (Table 4-4) revealed a higher C concentration compared to EPSN, whereas AMFN had the highest C concentration and C:N ratio.

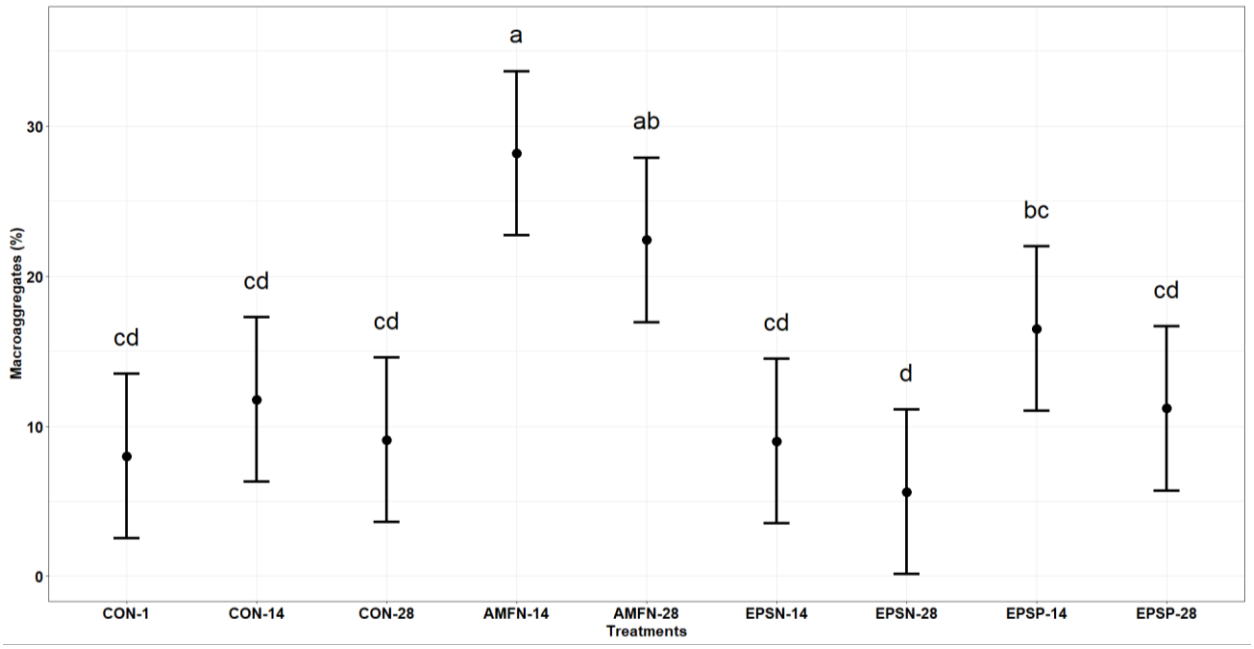

Figure 4-1. Mean (+/- SE) macroaggregate formation by bacterial and fungal binding agent treatment observed 1, 14 and 28 days after incubation.

Table 4-2. GRSP and EPS concentration observed 1, 14 and 28 days after incubation

Treatment	GRSP[1] (mg g^{-1} soil)	EPS (mg g^{-1} soil)
CON-1	1.18^{ab}	3.72^{a}
CON-14	1.14^{abc}	3.65^{a}
AMFN-14	1.16^{ab}	3.61^{a}
EPSN-14	1.19^{ab}	3.02^{ab}
EPSP-14	1.31^{a}	2.58^{abc}
CON-28	0.85^{c}	1.79^{bcd}
AMFN-28	0.93^{bc}	0.75^{d}
EPSN-28	0.92^{bc}	1.63^{cd}
EPSP-28	0.98^{bc}	0.79^{d}
SEM	0.0583	0.269
P-value		
Treatment		<0.001

[1]Friedman test was used for comparison of treatments, and Nemenyi test was used for post-hoc comparisons of means

Table 4-3. Biomass of functional microbial groups analyzed using PLFA expressed as nmol/ g soil

Treatment	Total Biomass	Fungal groups		Bacterial groups			Total fungal biomass	Total bacterial biomass
		AMF[1]	Fungi	Actinomycete	Gram Negative	Gram Positive		
CON-1	62.5	1.88^{c}	4.98	5.38^{a}	11.9	12.48^{b}	6.86^{c}	36.4^{ab}
CON-14	62.3	1.77^{c}	4.47	4.93^{a}	15.2	12.74^{b}	6.24^{c}	37.5^{ab}
AMFN-14	76.1	15.0^{a}	4.73	4.16^{ab}	15.3	11.38^{b}	19.74^{a}	37.5^{ab}
EPSN-14	65.4	1.85^{c}	4.56	5.05^{a}	16.1	13.42^{ab}	6.40^{c}	39.6^{ab}
EPSP-14	68.9	1.75^{c}	4.54	4.64^{ab}	16.4	17.68^{a}	6.29^{c}	44.1^{a}
CON-28	55.9	1.57^{c}	3.72	4.33^{ab}	13.4	11.58^{b}	5.29^{c}	33.8^{ab}
AMFN-28	57.6	9.56^{b}	3.83	3.24^{b}	11.9	9.05^{b}	13.38^{b}	29.6^{b}
EPSN-28	65.8	1.80^{c}	4.24	4.72^{ab}	15.8	12.92^{b}	6.04^{c}	39.6^{ab}
EPSP-28	55.3	1.46^{c}	3.70	3.80^{ab}	14.0	12.27^{b}	5.16^{c}	35.0^{ab}
SEM	4.55	0.428	0.356	0.338	1.12	0.955^{b}	0.7	2.65
P-value								
Treatment	0.06	<0.001	0.14	0.003	0.15	<0.001	<0.001	0.04

[1]*P*-values were obtained from log-transformed data; however, the original (untransformed) data are presented.

Table 4-4. Carbon and nitrogen analyzed in bacterial or fungal treatments

Sample	C (%)	N (%)	C/N
EPS from *Bacillus spizizenii* (EPSP)	40.2	8.5	5
EPS from *Sinorhizobium meliloti* (EPSN)	2.0	0.2	10
Arbuscular mycorrhizal fungi necromass (AMFN)	59.1	1.7	35

5. Discussion

5.1 Addition of AMF necromass increased macroaggregates

AMF necromass addition increased the formation of new macroaggregates compared to the control. These results align with previous studies by Helfrich et al. (2008), Bossuyt et al. (2001), and Martens (2000) who reported peak macroaggregate formation at 14, 12, and 9 days, respectively, following soil amendment with plant residues. Helfrich et al. (2008) and Bossuyt et al. (2001) also observed that fungal activity played a crucial role in macroaggregate formation, as the use of fungicide delayed and reduced aggregate formation, whereas bactericide had no such effect (Bossuyt et al., 2001). A meta-analysis by Leifheit et al. (2013) found that AMF inoculation had a more pronounced effect on soil aggregation in coarse-textured (sand-rich) soils and under *in vitro* conditions. Similarly, Morris et al. (2019) reported that AMF inoculation enhanced the formation of macroaggregates and increased its turnover time compared to those formed without AMF inoculation. These studies focused on the effects of living AMF forming a symbiotic relationship with plants, where macroaggregate formation resulted from AMF's ability to enmesh soil particles physically through its filamentous hyphae (Rillig and Mummey, 2006) and by altering biochemical properties of soil or root of host plants (Rillig et al., 2015).

Unlike previous research that focused on living AMF, we aimed to determine whether AMF necromass alone (without plants) contributed to macroaggregate formation. To our knowledge, no prior studies have specifically tested this, but the literature suggests potential mechanisms that may explain its role in soil aggregation. Ji et al. (2019) reported the direct effect of EE-GRSP, spore density, and hyphal length on mean weight diameter (MWD) reporting that the required energy to break down small macroaggregates was influenced by hyphal length and spore density, while large macroaggregates were also affected by EE-GRSP concentration. These

findings highlight the importance of hyphae, spores, and EE-GRSP not only in the enmeshment of soil particles to form macroaggregates but also in increasing their stability by enhancing their resistance to disintegration. At the microscopic level and using C isotopes, Jeewani et al. (2021) reported that plant-derived C compounds were transferred from roots to soil via AMF hyphae. They visualized these C compounds, such as polysaccharides, forming new small and large aggregates along with goethite (Fe-O mineral). The release of sticky compounds like polysaccharides or glycoproteins from AMF help to bind together soil particles forming aggregates (Jeewani et al., 2021).

Although glomalin has been traditionally described as the AMF soil glue (Wright et al., 1996), a new study indicates that the fungal soil glue -GRSP- is not a protein, but a polysaccharide dubbed "glomalose" (Alptekin et al., 2025). When AMF necromass was analyzed using an enzyme-linked immunosorbent (ELISA) method based on carbohydrates and using antibody MAb32B11, AMF necromass showed the same signal response to the antibody as AMF spores (Appendix Figure 4-1), however, EPS from *B. spizizenii* or *S. meliloti* didn't show any signal (Appendix Figure 4-2). We observed the sticky nature of AMFN during its manipulation.

5.2 EPS addition did not affect macroaggregates

Low clay content (7%) of our soils may be responsible for the lack of aggregation with EPS addition. EPS has been described as binding to clay minerals through electrostatic interactions (Chen et al., 2021). Cheng et al. (2020) reported that adding 2.8 mg of EPS-CHO g^{-1} soil resulted in a higher percentage of water-stable aggregates compared to the control after 30 d of incubation. However, their study used silt clay loam soil with a significantly higher clay and organic matter content than the loamy sand we used. Considering that soil organic matter is also related with higher aggregation (Chaney and Swift, 1984), we chose a low-organic-matter soil to

increase the likelihood of detecting the effects of EPS addition. Furthermore, *S. meliloti* produces an EPS negatively charged that binds more with clay positively charged and this interaction is stronger under acidic conditions (Labille et al., 2005). In this study, however, we used soil with an initial pH close to neutral. Lack of clay notwithstanding, we did observe aggregation of soils in the absence of binding agent addition, but with addition of water to maintain soil moisture. Helfrich et al. (2008) incubated at 15°C and rewetted soil samples (sieved <250 μm) reporting the formation of new macroaggregates between 1 and 5 g kg^{-1} soil during 84 d of incubation. According to Kaiser et al. (2015), the drying process enhances the organic matter-mineral interactions resulting in an increase of 8 to 41% of macroaggregates compared with moist samples.

5.3 Addition of AMF necromass did not increase GRSP, but increased AMF biomass

Traditionally, GRSP has been considered a fungal heat resistant glycoprotein located in the cell walls of AMF hyphae (Rillig, 2004). However, proteomic analyses suggest that GRSP contains primarily bacterial, rather than fungal, proteins (Gillespie et al., 2011). A recent study suggested that GRSP is a fungal polysaccharide rather than a fungal protein (Alptekin et al., 2025). Under field conditions, AMF forms symbiotic relationships with plant roots but can also support certain bacterial communities that consume AMF spores or hyphal exudates (Bonfante and Anca, 2009). Using ^{13}C labeling, plant-derived C was first detected in AMF, and later predominantly in Proteobacteria biomass (Drigo et al., 2010). Therefore, it is possible that AMF hyphae may have encapsulated some bacteria in the field. However, in our study, plant growth was excluded to isolate the effect of AMF hyphal structures on macroaggregate formation. The absence of GRSP response to AMFN addition appears to support findings suggesting that GRSP is not a fungal protein. Surprisingly, GRSP levels were higher in EPSP-14 than in EPSP-28. This

may be related to the greater Gram-positive bacterial biomass in EPSP-14, as these bacteria can form heat-resistant spores that survive autoclaving (Setlow, 2005; West et al., 1985), potentially leading to an overestimation of GRSP due to the persistence of these heat resistant spores.

Only the addition of AMFN increased fungal biomass, mainly due to a rise in AMF biomass. PLFA is a sensitive method for detecting the effects of treatments on active microorganisms (Blagodatskaya and Kuzyakov, 2013), as these phospholipids are found only in living cells (Zelles, 1999). As mentioned earlier, the AMF spore biomass was oven-dried after germination; however, we hypothesize that some ungerminated spores may have survived the heating process and germinated during incubation. It is likely that the PLFA analysis detected these newly germinated AMF structures.

5.4 Drying conditions may have stimulated production of EPS observed at the beginning of the incubation period

High EPS concentration on day 1 probably was a consequence of the previous pretreatment process. Our results showed higher EPS levels compared to those reported by Redmile-Gordon et al. (2014) for bare fallow soil (sandy loamy soil with 7.9% clay and 0.299% C), where EPS levels reached only 0.169 mg g^{-1} soil after 10 d of incubation. A key difference is that Redmile-Gordon et al. (2014) pre-incubated moist, sieved soil (<2000 μm) for 14 d at 40% WHC to stabilize the initial C flush caused by sieving, whereas in our study, we dried and sieved soil samples (<250 μm) as a pretreatment. Microbial EPS production has been shown to increase under drought stress. For example, Vardharajula (2014) reported that EPS levels increased from 100 to 300 mg mg^{-1} protein as water potential decreased from -0.05 to -0.73 MPa. EPS plays a protective role, helping microorganisms survive drought and other adverse conditions (Flemming and Wingender, 2010). We speculate that the drying process of our samples may have stimulated

EPS production, potentially explaining the high EPS concentrations observed on day 1. Additionally, the lower EPS abundance in Redmile-Gordon et al. (2014) study is expected, as bare fallow soil generally contains fewer nutrients compared to agricultural soils.

5.5 Lack of EPS increase after EPSP or EPSN addition suggests rapid microbial use, with the later decrease indicating continued consumption

Addition of EPSN or EPSP did not show an observable increase of EPS-CHO on day 14. We hypothesize that bacteria may have used EPSP as C-source along with other soluble nutrients available considering that EPSP-14 had higher gram positive biomass than CON-14. The lack of effect of EPSN may be due to its low C concentration when compared to EPSP. During incubation, EPS levels declined from day 14 to 28, likely due to continue microbial use as a C source once soluble nutrients were depleted. We hypothesize that during the early stages of incubation, microbes depleted readily available C sources, such as dissolved organic nutrients, and subsequently began using EPS as a nutrient source. Helfrich et al. (2008) investigated the impact of maize leaves and roots on macroaggregate formation in silty loam soils (<250 μm) incubated at 15°C for 3 months finding that macroaggregate formation peaked at day 14 before declining, likely due to the depletion of readily available C and the subsequent reduction in microbial activity. This suggests that continuous organic matter input is necessary to maintain macroaggregate stability. Further, the higher C:N ratio in AMFN compared to EPSN or EPSP may have limited bacteria's ability to use AMFN as a nutrient source (Bossuyt et al., 2001). In our study, we observed a similar trend of macroaggregate decline from day 14 to 28 across all treatments, although this decrease was not statistically significant.

6. Conclusions

Addition of AMF necromass, but not bacterial extracellular polymeric substances, significantly increased macroaggregate formation in a loamy sand. While many studies have examined the effect of AMF inoculation on soil aggregation, ours is the first show that the isolated effect of AMF necromass is positive for macroaggregates. We showed that addition AMF necromass did not increase GRSP, supporting the other findings that GRSP is not a fungal protein but likely a sticky fungal polysaccharide capable of forming aggregates by binding soil particles.

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8. Appendices

Appendix 4-1: Cultivation of *Sinorhizobium meliloti*

Preparation of modified Vincent Minimal Medium (mVMM)

Stock solutions

a. Basal Salt Mixture (2X): dissolve 2 g K_2HPO_4 and 2 g KH_2PO_4 in 1 liter of MilliQ water. Autoclave.
b. $MgSO_4$ (100X): 25g/l of $MgSO_4$ $7H_2O$. Autoclave
c. $FeCl_3$ (100X): 0.1g of $FeCl_3$, 6H2O in 100ml (1g/L). Filter 0.45 um.
d. $CaCl_2$ (100X): 6.71 g/l of $CaCl_2$, 6H2O. Autoclave.
e. 20% (20 g / 100 ml) Sodium succinate (100X). (final concentration 0.2%) Autoclave.
f. 10% (10 g / 100 ml) Sodium glutamate (100X): (final concentration 0.1%). Autoclave.
g. Biotin (5000X): 500 ug/ml. Filter 0.45 um.
h. Murashige Skoog Vitamin Mix (1000X) (M7150 SIGMA). Filter sterilize.
i. 60 g/l KNO_3 (100X) (final concentration 0.6 g/l). Autoclave.
j. 200 g/l sucrose (100X) (final concentration 2g/l). Autoclave

Procedure

First Pre-culture of *S. meliloti*

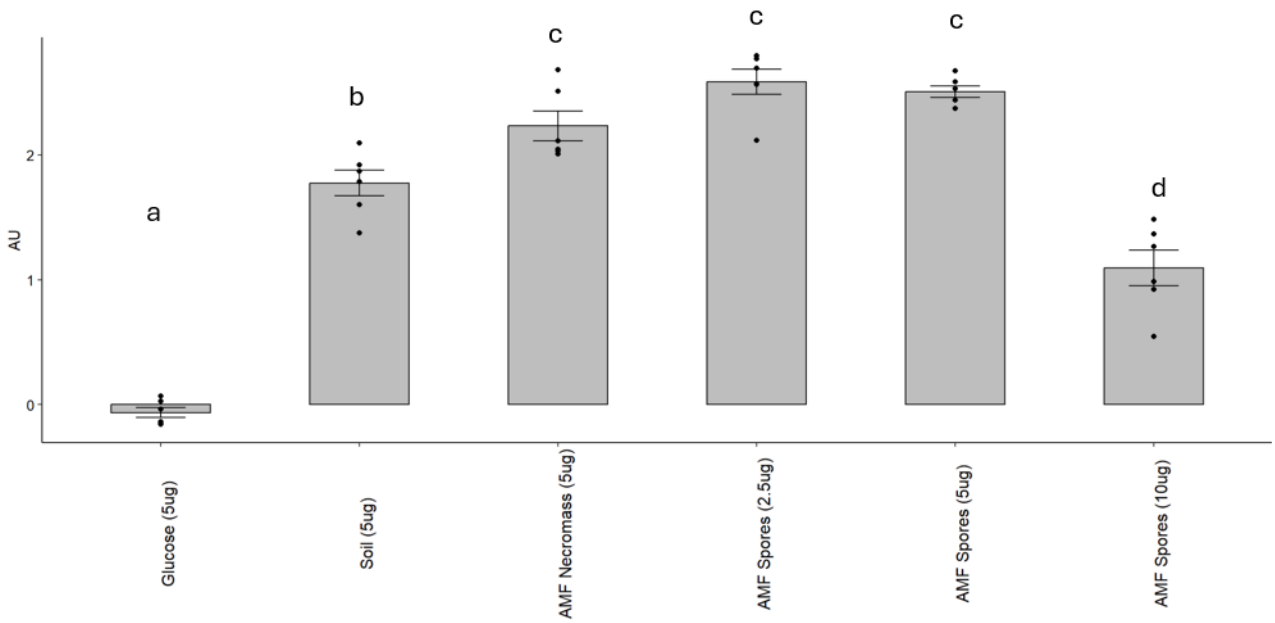
a. Inoculate 3 ml of Tryptone Yeast extract (TY) + tetracycline (tc) 5 µg/ml with *S. meliloti* from TY + Tc 10 µg/ml plate.
b. Grow under agitation 1 day at 30°C.

Second Pre-culture of *S. meliloti*

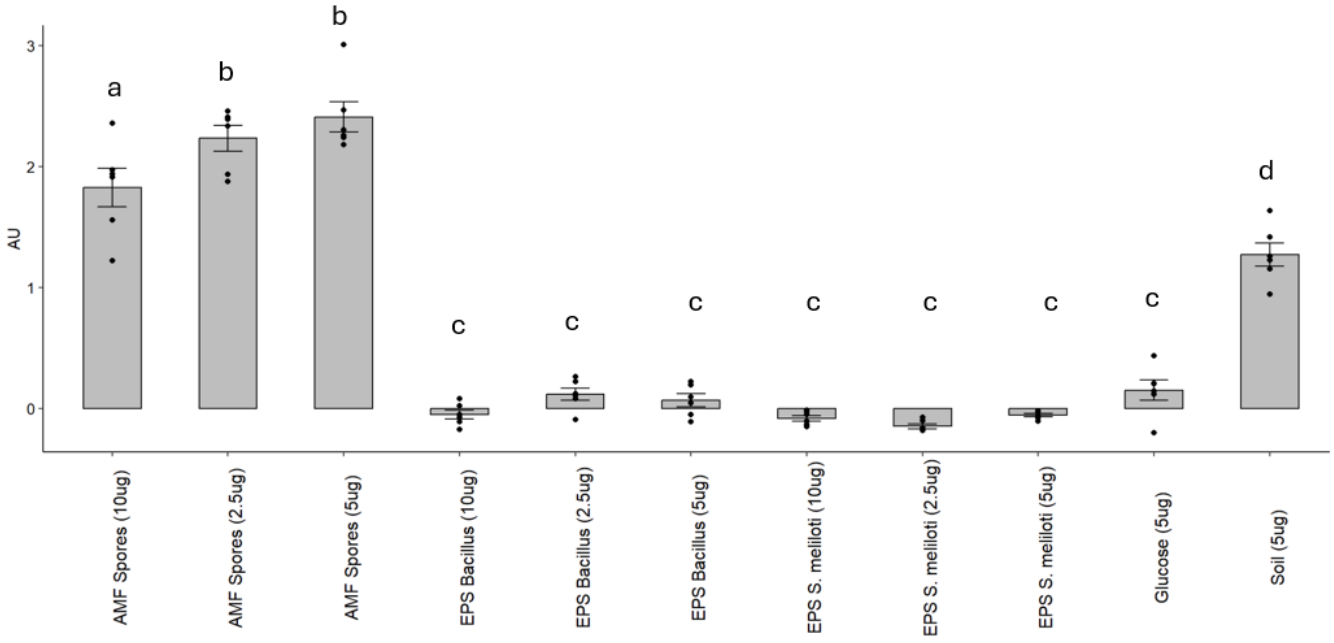
a. Inoculate 300 ml of Vincent medium + Tc 5 µg/ml with 3 ml of the first pre-culture.
b. Grow under agitation 2 days (or over the weekend) at 30°C.

Induced culture of *S. meliloti*

a. Prepare 3 liters of modified Vincent minimal medium.
b. Add 2 ml of luteolin stock (1,500X dilution) to 3 liters of Vincent medium (final concentration of 10 μM).
c. Inoculate 3 liters of modified Vincent minimal medium + luteolin with the 300ml *S. meliloti* second pre-culture.
d. Grow 2 days at 30°C under agitation (rpm 200) until ODR_{650nm} R=1.5-2.0 (with foam)



Appendix Figure 4-1. Reactivity of AMF necromass and AMF spores to antibody MAb32B11 based on carbohydrates measured as arbitrary units (AU) data provided by Burcu Alptekin from Ané Lab UW-Madison.



Appendix Figure 4-2. Reactivity of EPS from *Bacillus spizizenii,* EPS from *Sinorhizobium meliloti*, and AMF spores to antibody MAb32B11 based on carbohydrates measured as arbitrary units (AU) data provided by Burcu Alptekin from Ané Lab UW-Madison.