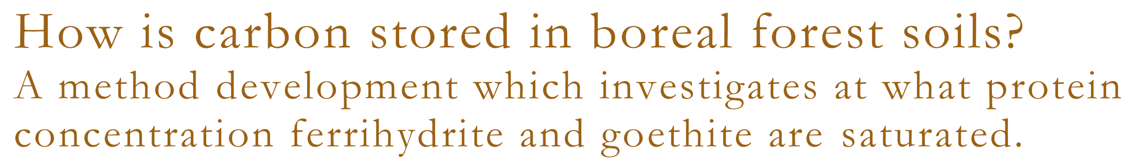

How is carbon stored in boreal forest soils?

A method development which investigates at what protein concentration ferrihydrite and goethite are saturated.

LOUISE BERILD 2023

MVEK12 EXAMENSARBETE FÖR KANDIDATEXAMEN 15 HP

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2023

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Abstract

Boreal forests store the majority of their carbon in the soil and play an important role in the global carbon cycle. However, the mechanisms that control carbon storage in boreal soils are not well understood. In recent studies, there has been a specific focus on how ectomycorrhizal (ECM) fungi influence carbon dynamics in boreal forests, particularly through their nitrogen acquisition. A major part of the nitrogen in boreal forests is present in organic form, as proteins, and adsorbed on soil minerals. However, it is generally unknown whether the ECM fungi can access this nitrogen. In this study, a laboratory method development was carried out. This provided useful information for future studies which can investigate if ECM fungi can acquire nitrogen from proteins adsorbed on two common iron oxide minerals in boreal forests. Concretely, the maximum adsorption of the protein Bovine serum albumin (BSA) on ferrihydrite and goethite was investigated. The result of this study showed that goethite is saturated at 0,58 mg BSA/ml goethite and ferrihydrite at approximately 1,25-1,3 mg BSA/ml ferrihydrite. This result was consistent with the hypothesis which stated that ferrihydrite, with its larger surface area compared to goethite, would have a greater capacity to adsorb proteins. These results can subsequently be used to create films with different protein loadings on minerals which then can be used to study if ECM fungi can acquire nitrogen from protein-mineral complexes. By studying this, we can increase our understanding of soil carbon storage and tree growth in boreal forests, as nitrogen is a limiting nutrient for tree growth.

Populärvetenskaplig sammanfattning

Marken är en viktig spelare när det kommer till att lagra kol, särskilt i boreala skogar. Dessa skogar lagrar nämligen största delen av sitt kol i jorden och fungerar som en viktig kolsänka. Men vi vet faktiskt inte helt vad det är som styr detta. Ny forskning har dock lyft ektomykorrhizasvampar som en viktig faktor. Mer konkret så föreslås nu ektomykorrhizasvampars förmåga att frigöra grundämnet kväve från organiskt material vara en viktig mekanism som påverkar kollagringen i marken. I denna studie genomfördes en metodutveckling som ska kunna hjälpa oss att förstå detta bättre.

Den boreala skogen sträcker sig över norra delen av jordklotet och är hem för en speciell typ av svamp som kallas ektomykorrhizasvamp. Denna sorts svamp har en unik förmåga att bilda ett nära samspel (symbios) med träden i skogen. Symbiosen innebär att svampen ger näring åt trädet i utbyte mot kol. Ett viktigt ämne som ektomykorrhizasvampen bidrar med, är kväve, som är nödvändigt för att träden ska växa. I den boreala skogen förekommer det mesta av kvävet i organisk form som protein. Dessutom är proteinet ofta adsorberat (fäst på ytan) på mineralerna i jorden. Dock vet vi inte om ektomykorrhizasvampen kan frigöra kvävet som är bundet till dessa mineral. I denna studie genomfördes en metodutveckling som bidrog med viktig information för framtida studier av ektomykorrhizasvampars förmåga att frigöra kväve från protein som adsorberats på två vanliga järnoxidsmineraler i boreala skogar. Mer konkret så undersöktes den maximala adsorptionen av protein på järnoxidsmineralerna ferrihydrit och goethit. Detta undersöktes genom att addera olika mängder utav proteinet BSA (Bovint serumalbumin) och sedan observera vid vilken koncentration som mineralerna var mättade och alltså inte kunde adsorbera mer protein. Studiens resultat visade att goethit var mättat vid 0,58 mg BSA/ml goethit och ferrihydrit var mättat vid 1,25-1,3 mg BSA/ml ferrihydrit. Detta resultat bekräftade studiens hypotes om att ferrihydrit, på grund av dess större ytarea, kan adsorbera mer protein än goethit. Dessa resultat är betydelsefulla då de kan ligga till grund för att skapa filmer där ferrihydrit och goethit har olika mycket protein adsorberat på sig. Filmerna kan sedan användas för att undersöka om ektomykorrhizasvampen kan frigöra kväve från protein som adsorberat på dessa mineral. Genom att undersöka detta kan vi få en djupare insikt om hur kol lagras i boreala skogar vilket kan vara viktigt för att förstå oss på hur klimatförändringar kan påverka denna kolsänka. Dessutom kan det ge oss mer kunskap om hur träd får tag i näringsämnet kväve vilket kan vara viktigt för skogsbruket.

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1.Introduction

1.1 Carbon storage in boreal forests

The boreal forest, also known as taiga, stretches like a ring over the northern hemisphere of the earth and covers land across Canada, northern Europe, Russia, China, and Japan (Thiffault, 2019). In these forests, a major fraction of the carbon is stored in the soil as soil organic matter (SOM) (Pan et al., 2011). Even though boreal forests only cover 27 % of the global forest area (Sarre, 2020), they still store approximately one third of the entire global terrestrial carbon stock (Bradshaw & Warkentin, 2015; Pan et al., 2011). Thus, these forests play an important role in the global carbon cycle (Pan et al., 2011). However, the mechanisms that control the carbon storage in boreal soils are not well understood.

1.1.1 The role of ectomycorrhizal fungi in soil carbon dynamics

In boreal forests, ectomycorrhizal (ECM) fungi are abundant (Tedersoo et al., 2012). ECM fungi are a type of fungi that form symbiotic relationships with plants, i.e. the fungus provides the tree with nutrients in compensation for carbon (Smith & Read, 2008). One important nutrient that ECM fungi provide boreal trees with is nitrogen (Hobbie & Högberg, 2012). In fact, tree growth in boreal forests is limited by nitrogen availability (Högberg et al., 2017; Tamm, 1991). This is due to that the majority of the soil nitrogen occurs in organic form as protein (Friedel & Scheller, 2002; Nannipieri & Eldor, 2009) which boreal trees have a limited capacity to assimilate (Näsholm et al., 2009). Consequently, the boreal trees are dependent on their symbiosis with ECM fungi to obtain nitrogen (Hobbie & Högberg, 2012). However, a major part of the protein is often bound to or is complexed with different compounds in the soil and is consequently present as part of soil organic matter (SOM) (Nannipieri & Eldor, 2009). Thus, to access this nitrogen, the ECM fungi need to decompose the organic matter (Lindahl & Tunlid, 2015), but it is important to mention that the extent to which ECM fungi can access organic nitrogen bound in SOM is highly debated in the field of microbial research (Zak et al., 2019). The potential of ECM fungi to participate in SOM decomposition is, however, important for understanding plant growth (Terrer et al., 2016) and soil carbon storage which has been suggested to be affected by the activity of mycorrhizal fungi (Averill et al., 2014; Orwin et al., 2011). Yet, there are still

uncertainties regarding why soil carbon storage in boreal soils seems to be linked to ECM fungi (Zak et al., 2019) but their nitrogen acquisition has been proposed to be one important mechanism (Averill et al., 2014; Zak et al., 2019). There are several theories regarding how ECM fungi's nitrogen acquisition can affect the storage of soil carbon (Zak et al., 2019). One example is that it affects the amount of carbon stored as necromass. Due to that approximately 10% of the total host net primary production (Leake et al., 2004) is allocated to belowground symbionts (as a result of the symbiosis), ECM necromass could play a role in the soil carbon storage (Zak et al., 2019). In fact, a large amount of the stored carbon derives from root associated organisms (Clemmensen et al., 2013).

1.2 Decomposition by ectomycorrhizal fungi

As previously mentioned, the extent of ECM fungi's ability to acquire nitrogen bound to SOM is a controversial topic in the field of microbial research (Zak et al., 2019). In general, there are two ways fungi can decompose organic matter: by enzymatic processes and non-enzymatic processes. Some research argues that since ECM fungi have lost many genes encoding for degrading enzymes, they have a reduced capacity to decompose SOM (Kohler et al., 2015) and furthermore a reduced capacity to liberate nitrogen (Pellitier & Zak, 2018). However, recent studies have challenged the hypothesis that ECM fungi can not decompose SOM (Rineau et al., 2012; Shah et al., 2016) and acquire nitrogen through decomposition (Op De Beeck et al., 2018). Rineau et al. (2018) and Op De Beeck (2018) suggest that certain ECM fungi may use a non-enzymatic mechanism to degrade SOM. For instance, Rineau et al. (2012) have demonstrated that the ECM fungus *Paxillus involutus* uses hydroxyl radicals (•OH) produced by the Fenton reaction (R1) to decompose SOM. In addition, Op De Beeck et.al (2018) investigated whether hydroxyl radicals are involved in the ECM fungus *P. involutus* process of acquiring nitrogen. Their result suggests that this type of fungus uses hydroxyl radicals to oxidize SOM which then makes SOM more vulnerable to enzyme degradation.

$$Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + {}^{\bullet}OH + H_2O \text{ (R1)}$$

1.3 Adsorption on minerals

There are different mechanisms that are thought to protect SOM from decomposition in boreal forests (Adamczyk, 2021). One of these mechanisms is adsorption onto minerals (Baldock & Skjemstad, 2000; Mikutta et al., 2006; Torn et al., 1997). Some research suggests that strong adsorption of SOM onto minerals physically protects organic matter from decomposition (Chevallier et al., 2003). However, there is overall little evidence for this. Proteins are one organic compound that adsorbs strongly onto minerals (Chevallier et al., 2003). In fact, a large fraction of the soil nitrogen (proteins), is associated with minerals (Jilling et al., 2018). Nonetheless, it is generally unknown whether ECM fungi can acquire this nitrogen.

1.3.1 Ferrihydrite and goethite

Iron oxides are common minerals in the soil (Cornell & Schwertmann, 2003). There are 16 different types of iron oxides (Cornell & Schwertmann, 2003) but this study will focus on ferrihydrite and goethite, which are two common iron oxide minerals in boreal forests. Goethite is one of the most thermodynamically stable iron oxides (Cornell & Schwertmann, 2003). This iron oxide is shaped like needles that have a surface area that can range from 8-200 m^2/g (Cornell & Schwertmann, 2003) (figure 1). Ferrihydrite on the other hand, is less thermodynamically stable, has a surface area that can vary between 100-700 m^2/g and is shaped like spherical particles (Cornell & Schwertmann, 2003) (figure 1). Due to that ferrihydrite has a larger average surface area than goethite (Cornell & Schwertmann, 2003), this mineral should be able to adsorb more protein and thus also offer more physical protection against ECM fungal degradation. But as mentioned above, there is little empirical evidence that these minerals provide physical protection against degradation and thus this needs to be investigated further.

Figure 1: Simplified and illustrative figure of the shape of ferrihydrite (left) and goethite (right). Note that the minerals are not drawn in true relative size to each other.

It is also worth considering the presence of iron in the iron oxide minerals. As mentioned above, Op De Beeck et al. (2018) found results suggesting that the ECM fungus *P. involutus* uses hydroxyl radicals produced by the Fenton reaction to liberate nitrogen. These results raise the question as to whether the iron in the iron oxide minerals may result in a production of hydroxyl radicals which can speed up decomposition. This in turn raises the following question: Do protein-mineral complexes protect the protein from ECM decomposition or accelerate it? In order to study if mineral associations protect proteins from decomposition or accelerate it, and if there is any difference between goethite and ferrihydrite, it must be possible to detect which processes take place. Thus, there must be a method that enables this to be studied. The development of such a method is the main focus of this work.

1.4 Film method

One way of investigating whether protein adsorption on minerals provides protection from decomposition or accelerates it is to create films with protein adsorbed on ferrihydrite and goethite (Michiel Op De Beeck, personal communication, April 20, 2023). These films can be used to detect decomposition by an ECM fungus (hydrolysis and oxidation), with infrared spectroscopy. However, in order to investigate this, it is necessary to create various films with different protein loadings on the minerals. By studying several scenarios, more substantiated conclusions can be drawn about the ability of ECM fungi to liberate nitrogen from protein adsorbed to iron oxide minerals. This is because the ratio of oxidized/non-oxidized protein will be different depending on the BSA concentration (Michiel Op De Beeck, personal communication, April 20, 2023). For example, a film with a high loading of protein, might yield a low oxidation value and consequently a low ratio of oxidized/non oxidized protein. However, this result does not necessarily indicate that the fungus lacks the ability to oxidize the BSA. Instead, it could indicate that the fungus does not have the ability to "reach" all the protein. Furthermore, different protein loadings can also provide information regarding if a higher concentration of iron oxide minerals could accelerate decomposition through ECM fungal production of hydroxyl radicals. In addition, it could also provide information regarding if the iron oxide minerals in fact provides the proteins physical protection from decomposition (Michiel Op De Beeck, personal communication, April 20, 2023).

1.5 Aim and research question

As previously discussed, understanding the saturation of protein on ferrihydrite and goethite, is crucial for designing films that can examine whether mineral associations with protein provides protection from decomposition or accelerates it. Therefore, this study aims to find *at what BSA concentration ferrihydrite and goethite are saturated.* To accomplish this, the following questions have been formulated:

- At what protein concentration is ferrihydrite saturated?
- At what protein concentration is goethite saturated?

The hypothesis of this study is that the amount of protein that can get adsorbed on ferrihydrite is greater than for goethite due to that ferrihydrite has a bigger surface area (Cornell & Schwertmann, 2003).

1.5.1 Delimitations and relevance for the field of environmental science

Even though boreal forests store a significant amount of carbon in their soil (Pan et al., 2011), we still lack a lot of knowledge regarding the mechanisms that control the soil carbon storage. This study will provide useful information for future studies which can investigate if ECM fungi can acquire nitrogen from proteins adsorbed on two common iron oxide minerals in boreal forests. Subsequently, this will increase our understanding of soil carbon storage and tree growth in boreal forests (as nitrogen is a limiting nutrient for tree growth (Högberg et al., 2017; Tamm, 1991)).

This study has several limitations. One limitation of this study is its exclusive focus on protein-mineral complexes. Other mechanisms involved in soil organic nitrogen (SON) stabilization also require investigation to gain a deep understanding of how ECM fungi acquire nitrogen from SOM. Another limitation is that only two types of minerals will be studied even though there are other types of minerals that also need to be investigated in order to be able to fully understand how ectomycorrhizal fungi can acquire nitrogen from proteins adsorbed on soil minerals.

1.5.2 Ethical reflection

This project does not involve living organisms, which means that no ethical consideration needs to be taken to how these organisms are handled. Training for laboratory work has been carried out to make sure that Lund university's guidelines for laboratory work are met, ensuring that the project does not pose any danger to people or the environment. In addition, the protein and iron oxides that will be used

are common in nature (Cornell & Schwertmann, 2003) and are not hazardous to humans or plants.

2. Method

In this project, the protein Bovine Serum Albumin (BSA) (purity ≥98%, pH 7) was used. It is a protein that has occurred in many other studies within the same field of work (Op De Beeck et al., 2018; Wang et al., 2020). Furthermore, suspensions of ferrihydrite and goethite were used during laboratory experiments. The goethite suspension was prepared through the hydrolysis of ferrihydrite under alkaline conditions and the ferrihydrite suspension through the hydrolysis of $Fe(N0_3)_3*9H_20$ (Schwertmann & Cornell, 2000).

2.1 BSA standard curve

A BSA standard curve was created to enable the determination of the BSA concentration in samples with an unknown concentration. By creating a linear regression with the BSA concentration as a function of visible light absorbance, it was possible to determine the BSA concentration in a sample by measuring its absorbance and using the equation of the standard curve.

The Micro BCA Protein Assay Kit and a UV- visible spectrophotometer (Ultrospec 3000, Pharmacia Biotech, Uppsala, Sweden) were used to create the standard curve. The Micro BCA Protein Assay Kit was chosen since it allowed for a sensitive colorimetric protein assay at low concentrations of BSA (0,5 μg/ml – 20 μg/ml). This kit used a method in which BSA reacted with a working reagent, resulting in a colour change in the sample which consequently affected the absorbance. In other words, a high concentration of BSA resulted in a high absorbance, and vice versa. Something worth noting however, is that the BSA concentration was not directly measured using this method. The colour change, which influenced the absorbance, occurred due to the reduction of copper in the working reagent. Therefore, the UV-vis spectrophotometer measured the absorbance of the colour induced by BSA's copper reduction, rather than directly measuring the BSA concentration.

The standard curve was produced by first creating a working reagent and BSA concentration standards, following the instructions of the kit. The different BSA standards had the following concentrations: 0,0 μg/ml, 0,5 μg/ml, 1 μg/ml, 2,5 μg/ml, 5,0 μg/ml, 10,0 μg/ml, 20,0 μg/ml, and 40,0 μg/ml. Thereafter 1 ml of working reagent was mixed with 1 ml of each BSA standard and then the samples were

incubated at 60 °C for 60 minutes. After the samples had cooled down to room temperature (21 °C) the absorbance was measured at 562 nm with the UV- visible spectrophotometer. Then, the absorbance values for each BSA standard were plotted against the known concentration of the sample, which created the standard curve.

2.2 Maximum adsorption of protein on ferrihydrite and goethite

The BSA concentration at which ferrihydrite and goethite are saturated was determined by first calculating the theoretical values for the maximum BSA adsorption onto ferrihydrite and goethite. These calculations were used to get a rough estimation of in what range the maximum adsorption is. The maximum BSA adsorption for ferrihydrite assumed a concentration of ferrihydrite of 1,23 g/l, a loading of 1,4g BSA/100m^2 (Krumina, 2017) and that the surface area was 300m^2/l (Schwertmann & Cornell, 2000). According to the calculation, the theoretical maximum adsorption of BSA was 51,66 mg BSA for 10 ml of ferrihydrite. In addition, the theoretical maximum adsorption for goethite was 12,68 mg BSA for 10 ml of goethite. This theoretical value was based on a surface area of 20 m^2/l (Schwertmann & Cornell, 2000), a concentration of 4,53 g goethite/l, and a loading of BSA of 1,4 g/100m^2 (Krumina, 2017).

Thereafter a range of different BSA concentrations based on the theoretical values were tested. The samples were tested by measuring the concentration of BSA that was not adsorbed. This was conducted by first adding the desired quantity of BSA to 10 ml of either goethite or ferrihydrite and then vortexing the samples and putting them on a rotator for 60 minutes. The samples were then centrifuged for 10 minutes to precipitate all the created mineral-protein complexes. Then the supernatant was passed through a poly ether sulphone filter. This filter was chosen since it has a low binding capacity for protein. Thereafter the BSA concentration in the supernatant (the concentration of BSA that was not adsorbed) was measured by using the Micro BCA Protein Assay Kit and a UV- visible spectrophotometer. This was achieved by first adding 1 ml of working reagent with 1 ml of each sample and then incubating them at 60 °C for 60 minutes. After the samples had cooled down to room temperature (21 °C) the absorbance was measured at 562 nm with the UV- visible spectrophotometer. For all measurements the absorbance of a blank was subtracted from each sample. The blank consisted of 1 ml of working reagent and 1 ml of milli-Q water. The BSA concentration was then ultimately determined by using the equation of the BSA standard curve.

The first measurements for goethite and ferrihydrite, based on the theoretical values, were then evaluated to determine the new BSA concentrations that should be

tested. The saturation level was found by continuously evaluating the results and adjusting the measurement ranges. This process gradually narrowed down the span and eventually revealed the precise BSA saturation concentration. The goal was to be able to pinpoint an exact concentration that corresponded to the saturation level. The saturation level meant that the BSA concentration in the supernatant would be 0 mg/ml and that an addition of BSA would result in that the BSA concentration in the supernatant increased.

3. Results

3.1 BSA concentration standard curve

The equation for the BSA standard curve was $y=26{,}823x + 0{,}5606$ (figure 2). The high R^2 value ($R^2=0{,}9992$) indicated that the linear regression was highly reliable within the tested concentrations. However, something worth noting is that the BSA concentration could not be calculated for samples with an absorbance above 1,472 au (arbitrary unit), since it is not appropriate to extrapolate outside of the BSA standards that were measured (figure 2).

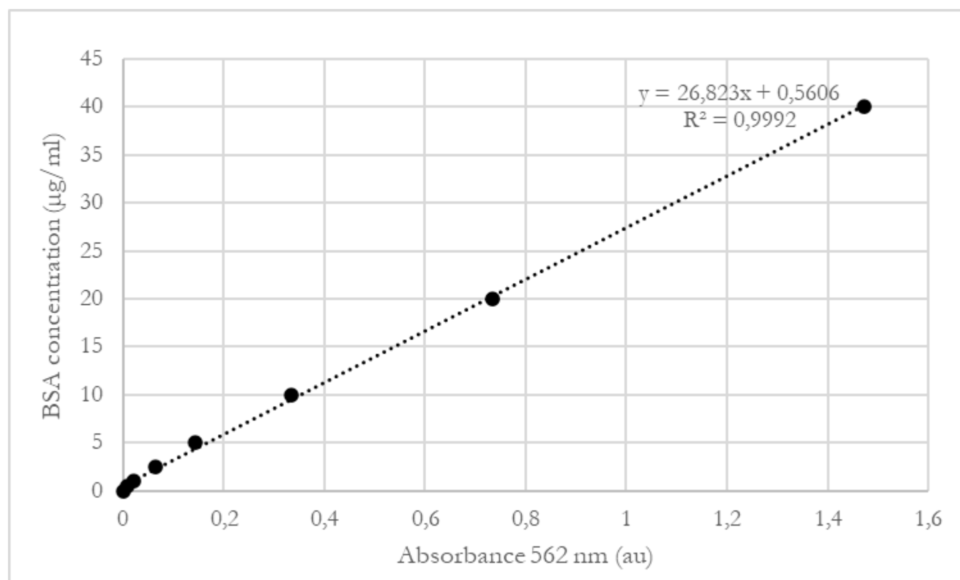

Figure 2: BSA (Bovine Serum Albumin) concentration standard curve with the BSA concentration (µg/ml) in relation to light absorbance at 562 nm arbitrary unit (au).

3.2 What is the maximum adsorption of BSA on ferrihydrite?

Using the theoretical value of BSA adsorption on ferrihydrite (5,166 mg BSA/ml ferrihydrite), as a reference, the following BSA concentrations were tested: 2,60 mg/ml, 5,20 mg/ml, 7,42 mg/ml and 10,16 mg /ml. Following, the addition of the working reagent, all samples had dark purple colour. This implied that these samples surpassed the saturation level, as samples at saturation would have had a clear colour. Thus, the absorbance was not measured with the UV-vis spectrophotometer.

During the second measurement, a range between 2,04 mg/ml and 0,12 mg/ml was tested (table 1). The absorbance for all samples except two, were above 1,472 au (table 1). Hence, the BSA concentration was only calculated for two of the samples (table 1). The sample with a total BSA concentration of 1,050 mg/ml had a measured absorbance value of 0,010 au which corresponded to that the concentration of BSA that was not adsorbed was 0,829 µg/ml (table 1). However, for the next sample (1,500 mg/ml), the BSA concentration that was not adsorbed increased drastically to 15,823 µg/ml. This result indicated that the saturation level was in between these two concentrations.

Table 1: The absorbance value and the BSA concentration that was adsorbed and not adsorbed for samples with different total concentrations of BSA. Note that three samples have the symbol "-" in two of the columns. This is due to that the absorbance was outside of the tested standards.

Concentration of added BSA (µg/ml)	Absorbance at 562 nm (au)	Concentration of not adsorbed BSA (µg/ml)	Concentration of adsorbed BSA (µg/ml)
120	2,179	-	-
510	2,899	-	-
1050	0,010	0,829	1049,171
1500	0,569	15,823	1484,177
2040	>3,000	-	-

In the third measurement, a range between 1,200 mg/ml and 0,800 mg/ml was tested (table 2). All samples had a concentration of BSA that was not adsorbed, close to 0 µg/ml (table 2). Thus, to find the saturation level, higher total BSA concentrations needed to be tested.

Table 2: The absorbance value and the BSA concentration that was adsorbed and not adsorbed for samples with different total concentrations of BSA.

Concentration of added BSA (µg /ml)	Absorbance at 562 nm (au)	Concentration of not adsorbed BSA (µg/ml)	Concentration of adsorbed BSA (µg/ml)
800	0,014	0,936	799,064
900	0,013	0,909	899,091
1000	0,011	0,856	999,144
1100	0,010	0,829	1099,171
1200	0,009	0,802	1199,198

In the last measurement, slightly higher BSA concentrations were tested (table 3). The result from this measurement indicated that ferrihydrite was saturated between the BSA concentrations 1,25 mg/ml and 1,3 mg/ml since the concentration of BSA that was not adsorbed increased slightly from a value close to 0 µg/ml (table 3). However, more measurements around these BSA concentrations are required to determine a more precise BSA saturation concentration. Unfortunately, the UV-vis spectrophotometer malfunctioned before these measurements could be carried out. Therefore, it was only possible to conclude that the BSA saturation concentration was between 1,250 mg/ml and 1,300 mg/ml. This result is illustrated in the graph below (figure 4), were data from all measurements except for those with absorbance values above 3,0 au were included.

Table 3: The absorbance value and the BSA concentration that was adsorbed and not adsorbed for samples with different total concentrations of BSA.

Concentration of added BSA (µg/ml)	Absorbance at 562 nm (au)	Concentration of not adsorbed BSA (µg/ml)	Concentration of adsorbed BSA (µg/ml)
1250	0,007	0,748	1249,252
1300	0,018	1,043	1298,957
1350	0,051	1,929	1348,071
1400	0,348	9,895	1390,105
1450	0,439	12,336	1437,664

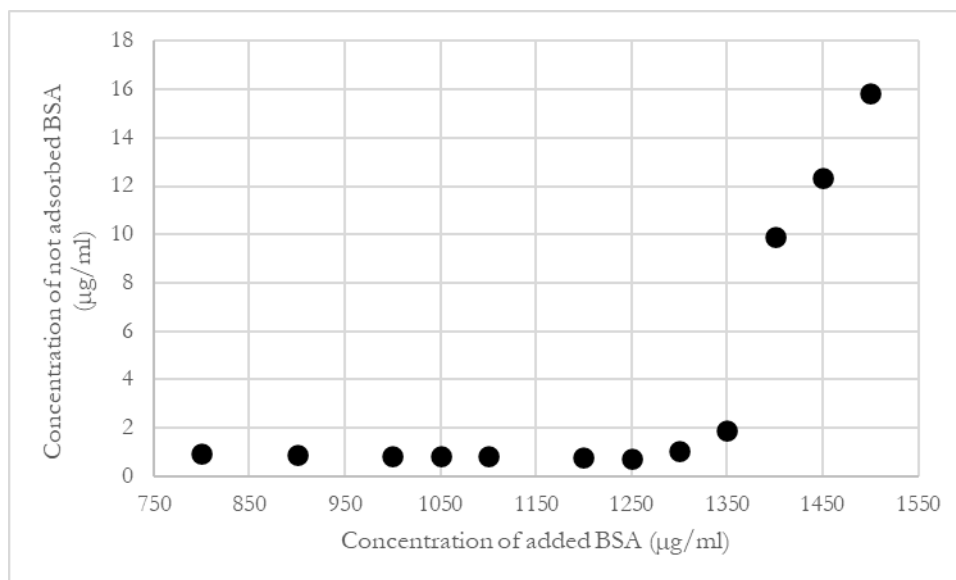

Figure 4: The maximum adsorption of BSA (Bovine Serum Albumin) on ferrihydrite with the concentration of not adsorbed BSA (µg/ml) in relation to the concentration of added BSA (µg/ml). The graph indicates that the BSA concentration corresponding to maximum BSA adsorption is between 1250 µg BSA/ml and 1300 µg BSA/ml. This is due to the fact that when saturation is attained, the concentration of unadsorbed BSA increases from zero.

3.3 What is the maximum adsorption of BSA on goethite?

The initial BSA concentration range that was tested, was determined based on the theoretical maximum adsorption value (1,268 mg BSA/ml goethite) (table 4). In this measurement, the following BSA concentrations were tested: 0,490 mg/ml, 1,040 mg/ml, 1,480 mg/ml, and 2,130 mg/ml. All samples except the one with a BSA concentration of 0,490 mg/ml had an absorbance higher than 3,0 au which implied that these samples were above saturation (table 4). However, the sample with 0,490 mg BSA/ml had a significantly lower absorbance value of approximately zero (0,016 au) (table 4). Thus, these results indicated that the BSA concentration corresponding to the maximum adsorption was between 0,490 mg/ml and 1,040 mg/ml.

Table 4: The absorbance value and the BSA concentration that was adsorbed and not adsorbed for samples with different total concentrations of BSA. Note that three samples have the symbol "-" in two of the columns. This is due to that the absorbance value was outside of the tested standards.

Concentration of added BSA (µg/ml)	Absorbance at 562 nm (au)	Concentration of not adsorbed BSA (µg/ml)	Concentration of adsorbed BSA (µg/ml)
490	0,016	0,990	489,010
1040	>3,000	-	-
1480	>3,000	-	-
2130	>3,000	-	-

In the second measurement, the following concentrations were tested: 0,390 mg/ml, 0,490 mg/ml, and 0,590 mg/ml (table 5). With the exception of the sample containing a total BSA concentration of 0,590 mg/ml, all other samples adsorbed almost all of the added BSA (table 5). These findings suggests that the saturation was within the range of 0,490 mg/ml and 0,590 mg/ml.

Table 5: The absorbance value and the BSA concentration that was adsorbed and not adsorbed for samples with different total concentrations of BSA.

Concentration of added BSA (µg/ml)	Absorbance at 562 nm (au)	Concentration of not adsorbed BSA (µg/ml)	Concentration of adsorbed BSA (µg/ml)
390	0,013	0,909	389,091
490	0,009	0,802	489,198
590	0,409	11,531	578,469

In the last measurement, several concentrations between 0,510 mg/ml and 0,580 mg/ml were tested. All samples had a concentration of not adsorbed BSA of approximately 0 µg/ml (table 6). Since this value increased drastically for the sample with a total BSA concentration of 0,590 mg/ml (table 5) this result suggests that the BSA concentration corresponding to the maximum adsorption was 0,580 mg/ml. This is also illustrated the graph below (figure 5), where all values from the two last measurements were included.

Table 6: The absorbance value and the BSA concentration that was adsorbed and not adsorbed for samples with different total concentrations of BSA.

Concentration of added BSA (µg/ml)	Absorbance 562 nm (au)	Concentration of not adsorbed BSA (µg/ml)	Concentration of adsorbed BSA (µg/ml)
510	0,005	0,698	509,305
520	0,005	0,695	519,305
530	0,006	0,722	529,278
540	0,007	0,748	539,252
550	0,008	0,775	549,225
560	0,010	0,829	559,171
570	0,009	0,802	569,198
580	0,008	0,775	579,225

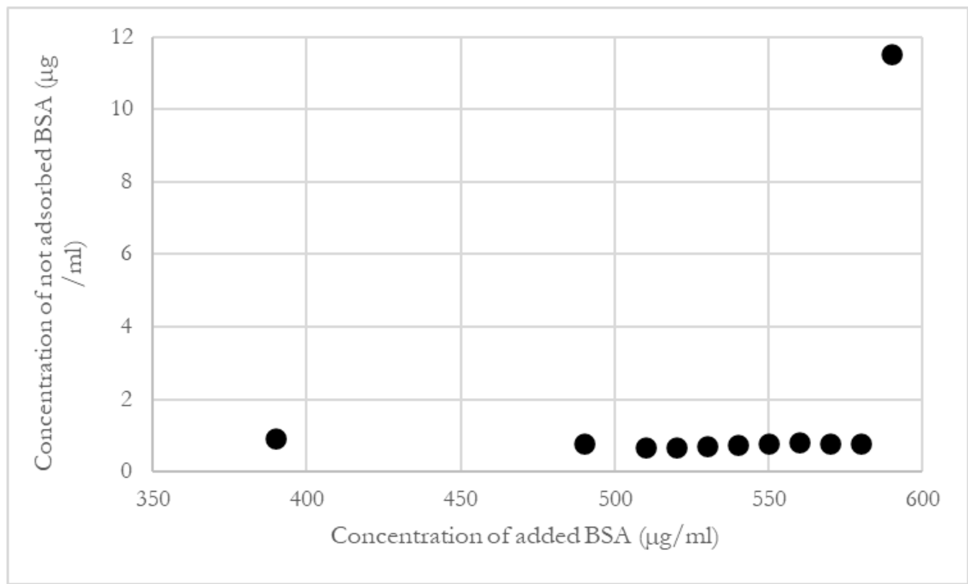

Figure 5: Maximum adsorption of BSA (Bovine Serum Albumin) on goethite with the concentration of not adsorbed BSA (µg/ml) in relation to the concentration of added BSA (µg/ml). The graph indicates that the BSA concentration corresponding to maximum BSA adsorption is 580 µg/ml. This is due to the fact that when saturation is attained, the concentration of unadsorbed BSA increases from zero.

4. Discussion

4.1 Maximum adsorption of BSA on ferrihydrite and goethite

According to the results of this study, the maximum adsorption of BSA on gothite was 0,58 mg/ml. Unfortunately, it was not possible to provide an exact BSA concentration of when ferrihydrite is saturated. This was due to that the UV-Vis spectrophotometer malfunctioned before the last set of measurements could be completed. But based on the tests that were conducted, the results indicated that the saturation was between 1,25 mg/ml and 1,3 mg/ml. The obtained results demonstrate that ferrihydrite can adsorb more BSA than goethite, which is consistent with the hypothesis. However, in a comparison with the theoretical calculations and the result, it appears that these values differ. This could indicate that some source of error has affected the result or that the theoretical values used for the calculations are incorrect and/or that there might be some variation in different batches of iron oxides. To investigate this, the measurements should first and foremost be repeated. Note however, that this should be done regardless before certain conclusions can be drawn since several replicates are needed to reduce the effect of possible sources of error. In addition, the theoretical values should be investigated to ensure that no mistakes were made during previous experiments when values for the concentration of ferrihydrite and goethite and the maximum loading of 1,4 g of BSA per 100 m^2 were derived. But as mentioned above, these differences in the results might also be due to, for instance, variations of iron oxides concentrations in the different batches. Therefore, these results might indicate that the values needed for calculations that depend on a batch of iron oxides needs to be investigated prior of doing calculations. But regardless of this, the results of this study can still be used as an indication of how much BSA that can adsorb on ferrihydrite and goethite (on the condition that the suspensions of the minerals are prepared using the same methods).

In table 1, the two samples with the lowest concentrations of BSA (0,120 mg/ml and 0,510 mg/ml) both had high absorbance values which indicated that there was a high excess of BSA which was not adsorbed on the minerals. However, the validity of this result is questionable, as all BSA should get adsorbed until saturation is reached. An explanation could be that the remaining iron, which was not adsorbed by protein

and precipitated, in fact reduced the copper in the working reagent and resulted in "false" high absorbance.

During the course of this study, different mineral-protein complexes of varying sizes were observed within the samples. This observation raised the question as to whether complexes with different ratios of protein:minerals can be created, for instance, 1:1, 2:1, 3:1 and so forth. This in turn, leads to the following question: How do these complexes affect the accessibility of the protein to degradation? Could it be possible that in the presence of large protein complexes, where a substantial amount of protein is adsorbed onto a mineral surface, the proteins themselves might offer physical protection against degradation. Additionally, it is worth considering whether larger complexes protect the iron oxide mineral and make it less accessible to be involved in the production of hydroxyl radicals. However, before investigating this, it is necessary to study if different complexes can be created.

The potential formation of various complexes implies that all BSA can adsorb onto all iron oxide minerals and precipitate at multiple BSA concentrations (with the assumption that the amount of iron oxide minerals is the same). Therefore, one practical way to explore this, is to measure the remaining iron oxides in suspension and the BSA concentration prior to reaching saturation. The BSA concentration can be measured using the same method as described in the study's methodology. The iron concentration can be determined by removing the supernatant, adjusting the pH to solubilize the iron, and then measure the concentration using Inductively Coupled Plasma (ICP) Spectroscopy. If there are multiple BSA concentrations at which neither BSA nor iron oxide minerals are detected in the supernatant, this finding would provide support for the hypothesis that various complexes can be formed.

4.2 The study's relevance from an environmental and social science perspective

Understanding the role of ECM fungi in decomposing SOM and releasing nitrogen is important from several perspectives. For example, it provides insights into the impact of ECM fungi on soil carbon storage in boreal forests (Averill et al., 2014). Moreover, through a better understanding of the role of ECM fungi in SOM dynamics it is also possible to provide a more accurate representation of ECM fungi's role in global biogeochemical models which is crucial for more accurately projecting ecosystems responses to climate change (Terrer et al., 2016). Additionally, ECM fungi's ability to decompose SOM and liberate nitrogen is also of great importance for plant growth since nitrogen is a limiting nutrient for tree growth (Högberg et al., 2017; Tamm, 1991). By identifying which mechanisms that contribute to nitrogen availability for trees, it is

then possible to know which mechanisms that are important for an increased timber production.

However, it is important to point out that there are still several gaps in the knowledge regarding to what extent ECM fungi enable plants to acquire organic nitrogen bound in SOM (Näsholm et al., 2009; Pellitier & Zak, 2018). An example of a knowledge gap is whether ECM fungi can decompose SOM and liberate nitrogen while forming mycorrhiza (Pellitier & Zak, 2018). Additionally, the ability for ECM to transfer organic derived nitrogen from SOM to the plant host also needs to be investigated further (Pellitier & Zak, 2018). One study has shown that this transfer may depend on if the soil is nitrogen limited or if there are larger amounts of nitrogen available (Näsholm et al., 2013). These examples represent only a few of the knowledge gaps that must be addressed in order to gain a comprehensive understanding of the impact of ECM fungi on plant growth and carbon storage.

This study focused on protein-mineral complexes which store a large part of the organic soil nitrogen (Jilling et al., 2018). But this study alone cannot provide an answer as to whether these protein-mineral complexes offer protection against ECM fungal decomposition, or if it instead accelerates it. However, the study does supply valuable information required for the development of a film method that can investigate this.

If ECM fungi can easily access the nitrogen present in iron oxide minerals and they accomplish this through the production of hydroxyl radicals, it suggests that this mechanism might play a role in soil carbon storage and plant growth within boreal soils. However, if the findings reveal that ECM fungi are unable to perform this function or have limited capability, or if it is discovered that adsorption provides physical protection against degradation, it could imply that these minerals indeed stabilize SON. However, it is crucial to conduct further investigation before drawing such definitive conclusions. For example, the knowledge gaps described above must first be answered. Furthermore, the ability of ECM fungi to access nitrogen from these protein mineral complexes must also be studied under field conditions to ensure that these possible processes take place in reality. Additionally, it is also essential to study the capacity of different ECM fungi to acquire nitrogen from these complexes through the production of hydroxyl radicals. It is important to point out that there currently only is evidence of that the *one* type of ECM fungus *may* have the ability to use hydroxyl radicals produced by the Fenton reaction to facilitate nitrogen acquisition (Op De Beeck et al., 2018). Hence, it is necessary to study if other types of ECM fungi have this ability as well before exploring if it is common that ECM fungi can acquire nitrogen from these mineral complexes by using a non-enzymatic mechanism. In addition, as the film method is currently described, only two types of iron oxides and one type of protein are examined. But in future studies, different types of protein and iron oxide minerals should also be studied to gain a deeper and broader understanding of ECM fungi's ability to acquire nitrogen from iron oxide minerals.

To summarize, there is currently a lack of knowledge regarding the role of ECM fungi in carbon dynamics. While this study adds a valuable piece to the puzzle, it is

crucial to continue investigating this topic to unravel the mechanisms underlying soil carbon storage in boreal forests.

5. Conclusion

According to the results of this study, the maximum adsorption of BSA on goethite is 0,58 mg BSA/ml goethite and for ferrihydrite the maximum adsorption is between 1,25-1,30 mg BSA/ml ferrihydrite. These results are consistent with the hypothesis which stated that ferrihydrite can adsorb more protein than goethite due to its larger surface area. However, several replicates are needed to reduce the effect of possible sources of error. Furthermore, since results yielded lower values for the maximum adsorption than those predicted by the theoretical calculations, it is necessary to review the values used in those calculations to ensure that no errors were made in previous studies. Additionally, the possibility of variations between different batches of iron oxides were also highlighted due to the difference between the theoretical values and the results. Thus, this might indicate that values needed for calculations that might depend on a batch of iron oxides, for instance concentration, needs to be investigated prior of doing calculations. Nevertheless, the result of this study can still serve as a reference point for future studies (on the condition that the suspensions of the minerals are prepared using the same methods).

In this study, several other areas of further investigation were also identified. For instance, this study highlighted the potential formation of protein-mineral complexes of varying sizes. By first exploring whether this is possible, future studies can subsequently examine the potential effect this has on how ECM fungi obtain nitrogen from these complexes. Furthermore, it was highlighted that there is a significant knowledge gap regarding the extent to which ECM fungi enable plants to acquire organic nitrogen bound in SOM. This is crucial to investigate in order to understand the potential effects of ECM fungal nitrogen acquisition on carbon storage and tree growth. Additionally, it was also emphasized that there is currently only evidence for one type of ECM fungus having the ability to use hydroxyl radicals produced by the Fenton reaction to facilitate nitrogen acquisition. Therefore, it is necessary to study whether other types of ECM fungi have this ability as well before investigating the possibility of ECM fungi commonly acquiring nitrogen from these mineral complexes through a non-enzymatic mechanism.

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