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*Environmental Change and Ecosystems*

Thesis

The effect of fire regimes on carbon sequestration  
and stabilization in South African grasslands

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## **Abstract**

Grasslands are important ecosystems worldwide since they not only provide important ecosystem services such as biodiversity reserves and water catchments, but also play a large role in the cycling of carbon and nutrients. In general, the sequestration of carbon is in balance with respiration, but environmental conditions can dislodge this balance, resulting in a positive or negative impact on global climate change. A large portion of organic carbon is sequestered in soil organic matter (SOM) and the importance of SOM is recognized by science. The structure of carbon changes during the sequestration process, resulting in labile and recalcitrant carbon pools that determine the decomposition rate of the carbon. In South Africa, grasslands sequester a lot of carbon and have evolved over many centuries, creating unique ecosystems that can cope with disturbances. Fire regimes occur in these grasslands and influence the sequestration process of carbon. In this study, the effect of different fire treatments on soil carbon storage has been investigated. While fires increase the total amount of carbon stored in soils, it also affects the distribution of carbon and increases the amount of black carbon sequestered in soils. Combined, the changes in carbon distribution and the accumulation of black carbon increase the overall recalcitrance of carbon, resulting in longer residence times of carbon in soils. The role of fire in grassland ecosystems needs to be evaluated even further since fire could play a part in conserving grasslands and could aid as a potential climate mitigation option.

## **Preface and acknowledgements**

Before you lies the master thesis ‘The effect of fire regimes on carbon sequestration and stabilization in South African grasslands’, which is based on carbon data obtained during this research. The thesis has been written as a graduation requirement for the Sustainable Development Master’s programme at Utrecht University. The research started in February and was completed at the end of June.

The initial idea for this research topic came from Dr. Mariska te Beest. The research was conducted with the use of imported soil samples from South Africa. The samples had been obtained from an ongoing fire experiment (the Brotherton Trials) and were processed in Utrecht in order to obtain new data from the soil samples.

I would like to thank Dr. Mariska te Beest for providing me with such an interesting topic and for guiding me throughout the research. I’m very grateful that she kindled/stimulated my enthusiasm for research in general. I would also like to express my particular gratitude to Olli Hyvärinen for all his help. He was always available to exchange ideas with me and he made my research period even more enjoyable. I would also like to thank everyone who supported me mentally or helped me by answering questions related to my research. Finally, I would like to acknowledge the massive support from my mother, who is always there for me and helped me out more than she can imagine.

I hope you enjoy reading this thesis as much as I enjoyed doing research.

Victor Trouw

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## Introduction

Grasslands are important ecosystems worldwide, covering about 40% of the globe. They provide important ecosystem services such as biodiversity reserves and water catchments, but they also play a large role in the cycling of carbon and nutrients (te Beest et al., 2021). The global carbon cycle describes a closed system that is shaped by the continuous movement of carbon through the atmosphere and the earth's carbon sinks. Grasslands function as a terrestrial sink, taking up carbon through sequestration (Ward et al., 2016) and releasing carbon through respiration and decomposition in both vegetation and soils (Bikila, Tessema & Abule, 2016). In general, the sequestration of carbon is in balance (equilibrium) with the respiration through carbon-dioxide (CO<sub>2</sub>). However, environmental conditions can dislodge this balance, resulting in a positive or a negative impact on global climate change. Relative increased sequestration could have a positive effect on climate change since ecosystems can function as a carbon sink (Marschner et al., 2008; Carol Adair et al., 2009). The estimated carbon stock in grasslands is approximately three times larger in the soil than in the above ground vegetation (Bikila et al., 2016). Around the globe, more carbon is stored in soils than in the atmosphere and global vegetation combined, therefore small change in carbon contents in soils can result in large changes in the carbon balance (Marschner et al., 2008; Lehmann & Kleber, 2015; Yang et al., 2019).

A large portion of organic carbon is sequestered in soil organic matter (SOM). The amount of SOM in the soil relates the decomposition rate and the input of carbon from plant production and litter (Bikila, et al., 2016; Oliver et al., 2017). SOM has several important properties affecting the functioning of an ecosystem. The amount of SOM influences plant growth, since SOM directly affects soil properties important to plants, such as infiltration rates, porosity, bulk density and nutrient supply (Lehmann & Kleber, 2015; Bikila et al, 2016; Lavallee, Soong & Cotrufo, 2019; Viret & Grand, 2019). Furthermore, SOM is an important factor in global greenhouse gas emissions since decomposing organisms convert organic matter into greenhouse gasses such as methane (CH<sub>4</sub>) and CO<sub>2</sub> (Lehmann & Kleber, 2015).

The importance of SOM is recognized by science, but in the last couple of years the view on the nature of SOM has changed (Lehmann & Kleber, 2015). The classical approach to the nature of SOM is that biological, physical and chemical transformation processes are responsible for converting organic material into organic soil products. This transformation process is called 'humification', which produces nutritious, persistent soil compounds called 'humus'. However, modern analytical techniques can not find evidence for this process, therefore it is questioned more and more (Lehmann & Kleber, 2015; Lavallee et al., 2019). A more recent approach is to look at SOM from a more flexible point of view assuming that carbon sequestration is a continuous process in which the structure and the size of carbon fractions change during sequestration. These changes in structure and size result in different types of carbon pools within the soil. The type of carbon pool determines the accessibility of these carbon pools to decomposers and therefore the rate of decomposition (Lehmann & Kleber, 2015).

Land use change and management practices do not necessarily change the total amount of carbon within soils, but they can affect the distribution of carbon within the different carbon pools (Oliver et al., 2017). Several factors, such as the specifics of the soil system and the sequence of decomposing where the organic matter resides, decide into which carbon pools the SOM is sequestered. Specific system characteristics, such as spatial arrangement of the organic matter, temperature, moisture, grazing, fire regimes and available minerals, influence the decomposition pathway (Lehmann & Kleber, 2015; Bikila et al., 2016). Some characteristics will result in the sequestration of labile carbon fractions that have a short cycling rate and will be easily decomposed, other characteristics result in more stable and protected (recalcitrant) fractions that will remain in soils for decades (Plaza et al., 2012; Viret & Grand, 2019).

Fire is a relevant driver to study since it is a management tool that is often used to manage grasslands (Bikila et al., 2016). Moreover, fire regimes have changed and will continue to change due to population growth and further temperature rise (Pellegrini et al., 2018). Understanding the impact of fire on soil properties is therefore important. The effect of fire on ecosystems is very complex. In addition to reducing or removing above ground biomass, it influences below ground microbial, chemical and physical processes (Kuzyakov, Merino & Pereira, 2018). Fires can increase carbon sequestration based on the frequency of occurrence. In some experiments, decreases in SOM were found after fire occurrence, which was probably due to the sequestration of carbon into other carbon pools (Bikila et al., 2016). Fires also influence the type of carbon that is present in soils. Black (pyrogenic) carbon is formed when the incomplete combustion of biomass occurs (Preston & Schmidt, 2006). It has a very slow decomposition rate and can therefore play an important role in long-term carbon storage (Santín et al., 2016).

In order to properly manage carbon stocks, a better understanding of the formation, persistence and function of SOM is required, mainly since the storage potential of a soil relates to the capacity to store the carbon in a stable form which has a long cycling rate (Lavalley et al., 2019; Rumpel et al., 2020).

The impact of fire on the sequestration of specific carbon pools has yet to be properly investigated. The aim of this research is to gain insights into the sequestration process when affected by this driver, which led to the main research question:

*To what extent does fire influence the labile, stable and pyrogenic soil carbon pools in South African grasslands?*

Based on the research question, the following hypotheses have been formed:

1. Fire has a positive effect on soil carbon stocks in South African grasslands
2. Different fire treatments will result in a different soil carbon pool distribution
3. Fire treatments result in increased pyrogenic carbon in all carbon pools

## Relevance

It is relevant to study this topic since soil organic matter has a relation with greenhouse gas emissions, the water cycle and ecosystem functioning (Lehmann & Kleber, 2015). Current methods used to identify soil carbon stocks only focus on total amounts of soil carbon, while the composition is also very important (Manning et al., 2015). The knowledge obtained through this research can contribute to understanding the role of soil organic carbon (SOC) in processes that maintain the sustainability of a system. An increased understanding in SOC stocks can also aid the parameterization of current global carbon circulation models that are used for climate policy. The research will elaborate on the roles of fire on the carbon sequestration process in soils. The knowledge gained from this research will attempt to (partially) fill the current gap on sequestration pathways due to fire, providing new theory that can be used in future research.

The research is relevant for society since it will address several of the sustainability goals of the United Nations, which focus on improving environmental protection and the global climate (Rumpel et al., 2020). Carbon sequestration in soils can be considered as a mitigation option to reduce the impact on global warming (Veldman et al., 2015; Minasny et al., 2017; Lavallee et al., 2019; Rumpel et al., 2020; Viret & Grand, 2019). Since approximately a third of all natural vegetation occurs in grasslands, these ecosystems have large potential to function as a carbon sink (He et al., 2012). The main method to increase carbon sequestration in soils is restoring degraded ecosystems. It is estimated that approximately 60% of these grasslands is degraded to some extent, therefore the African grasslands have lots of potential for increased carbon sequestration (Minasny et al., 2017).

Furthermore, this research could contribute to the protection of ancient ecosystems. Grasslands are often targeted for above ground carbon sequestration projects through reforestation. Increased understanding of this ecosystem might contribute to its conservation (Veldman et al., 2015). A lot of grasslands are targeted for reforestation projects since they are misperceived as degraded or deforested due to lack of tree cover. Moreover, the effectiveness of reforestation projects is doubted. In drier grasslands, the invasion of woody species can slightly increase overall carbon storage (Jackson et al., 2002). However, in mesic grasslands the above ground biomass gained by woody plants is negligible in comparison to the below ground loss of carbon (Bond et al., 2019). These ancient grasslands have been shaped over the years by herbivory and long-term fire regimes and are therefore wrongly 'restored' (Bond et al., 2019; Silveira et al., 2020).

# Theory

## Tropical grassy ecosystems

Grasslands can be defined as ecosystems where graminoids, forbs and shrubs are present most of the time, resulting in a 'herbaceous layer'. The herbaceous species play a major role in species richness and the functioning of these ecosystems (Veldman et al., 2015). While grasslands occur all over the world, tropical grasslands have more significant ecosystem services incorporated in them. In addition to their large role in carbon, soil and water dynamics, the biodiversity in these grasslands is very species rich and a lot of these species are endemic (Buisson et al., 2019). Tropical grassy ecosystems are unique and have plant and animal life specifically adapted to an open habitat (Aleman et al., 2020; Bond, 2016). Endemic grass species in these ecosystems can be unique and in some regions over 500 different species are found (Bond, 2016).

In tropical regions, grasslands and tropical forests are two frequently occurring biomes. In general, forests occur more in wetter areas, whereas grasslands are more often located in dryer, seasonal areas. Depending on conditions such as climate, forests can transition into grasslands and vice versa. However, these alternative stable states are not influenced by climate alone (Aleman et al., 2020).

Grasslands have often been misperceived as early successional vegetation because of human deforestation, but this assumption neglects the existence of ancient grasslands (Buisson et al., 2019). Primary (ancient) grasslands have been shaped over the years and store more carbon, are more resistant to fire and are richer in species than secondary grasslands (Zaloumis & Bond, 2016), which are shaped by anthropogenic interference like fire and deforestation. Because these types of grassland look similar, it is difficult to distinguish between them (Silveira et al., 2020). However, primary grasslands are characterized by richer taxonomic, phylogenetic and endemic plant communities which are maintained by disturbances from fires, (mega-)herbivores and soil properties that limit the growth of trees (Buisson et al., 2019; Silveira et al., 2020; Yang et al., 2019). The increased species diversity and richness is relevant since it shows a positive relation with carbon storage in soils (Lange et al., 2015).

Ancient grasslands can only persist under certain conditions that prohibit the development of forests (Veldman et al., 2015). In edaphic grasslands, 'poor' soil properties such as shallow soils and low soil moisture prohibit tree development. Other grasslands are climate-determined, where factors such as low precipitation or low temperatures (due to altitude and/or latitude) prohibit tree development. Mesic grasslands are in areas where conditions are optimal for trees. The only suppression of tree growth in these grasslands are disturbances such as fire and herbivory (Veldman et al., 2015; Buisson et al., 2019). The impact of these disturbances on grass species is noticeable in certain plant traits. In Madagascar, grazing has resulted in high bulk density grasses with short wide thin leaves, while fire resulted in low bulk density grasses with longer, narrower and thicker leaves (Solofondranohatra et al., 2020).



## Fire regimes

The field of fire ecology focuses on '*the interactions of fire with the biotic and abiotic components of an ecosystem*' (Trollope, 2009, p. 63). Repeated fire patterns in a particular location can be described as a fire regime. The combination of elements such as the frequency, intensity, size, season and spread can be used to characterize a fire regime (Archibald et al., 2018).

The character of a fire regime is important since it determines the impact of the fire on an ecosystem. A fire with a low or medium intensity can be beneficial while a high intensity fire in the same ecosystem can be destructive. Fires release CO<sub>2</sub> and organic compounds into the atmosphere due to the combustion of vegetation. Charred compounds that remain can be sequestered into soils through carbon sequestration (Kuzyakov et al., 2018).

In tropical grassy ecosystems, fires can be crucial in maintaining the state of the ecosystem and even benefit soil properties (Bond, 2016). In systems that have been disrupted by humans, fire can even be used to restore grassland ecosystems and their biodiversity (Aleman et al., 2020). However, fire does not always have a positive effect. Changes in a fire regime, such as alterations in frequency, intensity and seasonality, can result in the degradation of a certain system. In Mediterranean systems, resilient shrubs can cope with high intensity fires at intervals of several decades, but they are lost when the frequency of fire increases (Keeley et al., 2011). In these systems, the altered fire regimes can eventually result in changes in vegetation, followed by soil degradation (Mayor et al., 2016). In ancient grasslands the altered fire regimes can lead to increased grassland encroachment by woody species, degrading the grassy ecosystem (Buisson et al., 2019). The seasonality of the fires plays an important role since the grass is least damaged when it is burned in a dormant state, at the start of the growing season when there is sufficient rain (Trollope, 2009).

Tropical grassy biomes are often characterized by their grass-fire cycle. During a fire, both the herbaceous layer and the young trees are burned (Parr et al., 2014). The absence of fire can temporarily reduce the species diversity due to competitive exclusion. As a result, dominant grasses, shrubs and eventually trees will outcompete most of the other species. Furthermore, short term fire exclusion leads to increased fire intensity (in the next fire) due to the accumulation of biomass (Buisson et al., 2019).

In the long run, fire exclusion can lead to more catastrophic results. Changes in fire regimes can result in abrupt shifts in the ecosystem composition since both tropical forests and grasslands rely on positive feedbacks to maintain their state (Parr et al., 2014; Buisson et al., 2019). Dense tree cover maintains the forest state by limiting herbaceous growth, since grasses in these systems require a lot of light for photosynthesis. The lack of grasses will then exclude herbivores and fires (Parr et al., 2014). Grasslands are characterized by open fields that are grazed and burned often, making it difficult for trees to sprout (Buisson et al., 2019).

In areas where the climate is more favourable to forests, fire feedbacks are one of the main mechanisms that enable grasslands to exist (Zaloumis & Bond, 2016; Aleman et al., 2020). In tropical grassland ecosystems, the fire exclusion is therefore a bigger threat than an increase in fire frequency (Buisson et al., 2019).

Fires in grasslands are often misperceived since they are thought to be created and maintained by anthropogenic sources. Grasslands are often ancient and have evolved to cope with or even thrive under the influence of lightning induced fires regimes (Gordijn, Everson & O'Connor, 2018; Bond et al., 2019). In general, tropical grassy ecosystems are highly flammable due to extensive seasonal changes. During rain seasons, grasslands are often highly productive, generating a lot of 'fuel' for the long lasting dry seasons, resulting in high fire probability (Bond, 2016).

### Species adapted to fire

Some tropical and subtropical grasslands are estimated to be at least 3 million years old. Over time the species have evolved to cope with fire occurrence (Bond, 2016). Species do not adapt to fire in general, but to a fire regime such as frequency or intensity. Changes in the fire regime can threaten the species (Keeley et al., 2011).

Species have different ways to cope with fire regimes. Some trees develop insulating barks, other species will use the soil as a heat insulator (Pausas et al., 2018). Plant parts that are important for growth are protected beneath the surface to resist disturbances such as fire, allowing plants to quickly regenerate their photosynthetic tissues after being disturbed. This strategy is even more effective due to the temporary lack of competition. Species that would normally shade tillers are now temporarily excluded from the system allowing grasses to flourish (Gordijn et al., 2019).

These plants mainly resprout, rather than reseed, quickly after disturbances due to their investment in underground organs that store resources such as water and starch. This strategy is mainly used by plants in mesic grasslands and relies on clonal growth from buds. This has some advantages compared to sprouting from seeds since disturbances like water stress and seed predation do not occur as much (Veldman et al., 2015; Bond, 2016; Buisson et al., 2019). Plants store the below ground buds in suitable places such as roots, root crowns, rhizomes, basal burls, fleshy swellings and below ground caudexes (Pausas et al., 2018).

Seeds do play a role in these systems. Flowering species produce excessive amounts of seeds, but most of them do not establish (Buisson et al., 2019). When the grassland's structure is temporarily changed due to fires, resulting in a temporarily reduced canopy from herbaceous species, these seeds do get a chance to grow (Gordijn et al., 2018).

Fire impacts several earth system's processes including the carbon cycle. In the short term, fire influences the combustion flux of CO<sub>2</sub>. However, most of this CO<sub>2</sub> is taken up by vegetation shortly after. Therefore, fire only influences the carbon cycle if there is a mismatch between uptake and combustion (Archibald et al., 2018). Carbon fluxes as a

result of fire regimes have often been studied, and these studies often focus on the combustion of plant biomass. However, the impact of fire on soil carbon is still uncertain (Pellegrini et al., 2018).

The soil carbon pool can be negatively influenced by fire if soil carbon (such as peat) is burned (Archibald et al., 2018), but fire can also have a positive effect on the production of black carbon (such as charcoal and soot) and carbon sequestration in general. Even though fire decreases many of the above ground carbon stocks incorporated in trees and shrubs, it increases the carbon sequestered in grass species. The increase in grass biomass is often due to temporary factors such as the exclusion of herbivores and competitive advantage over tree and shrub species (Bikila et al., 2016).

## Carbon sequestration and stabilization in soils

The global carbon cycle revolves around photosynthesis, which fixates atmospheric CO<sub>2</sub>, and the release of carbon through respiration and decomposition. Carbon is cycled through some of the earth's reservoirs such as the ocean, atmosphere and the biosphere (te Beest et al., 2021). The amount of carbon is determined by the balance of input, such as plant residuals and root litter, and output, such as root exudates and microbial decomposition. When carbon sequestration increases, this is either due to enhanced primary production or due to slower decomposition, which extends the residence time of organic materials in the soil. The accumulation of plant litter might raise microbial activity, increasing decomposition rates and therefore reducing stored soil carbon. On the other hand, increased inputs might result in more recalcitrant organic compounds being formed, therefore increasing the amount of stored soil carbon (Lange et al., 2015).

Carbon in soils is influenced by several drivers. The climate affects metabolism rates and therefore the eventual litter production. Abiotic soil properties can affect the pH and texture of the soil, which in their turn affect microbial activity and plant growth. On local levels, certain land use types can also affect the carbon in soils (Manning et al., 2015). When organic matter ends up in soils it becomes soil organic matter (SOM). SOM is a key player in the global carbon cycle. It consists of a combination of different organic molecules derived from decomposed organisms (Oliver et al., 2017). The SOM balance in soils relates to the input (carbon material such as plant litter) and the output (decomposition rate) (Bikila et al., 2016). SOM is diverse due to differences in chemical structure and the interactions with other particles. The rate in which it is decomposed depends on the SOM characteristics and environmental conditions such as the composition of the microbial community and soil acidity (Oliver et al., 2017).

The composition of carbon fractions is important for the cycling rate of carbon and can change as a result of disturbances (Tan et al., 2007; He et al., 2012; Oliver et al., 2017), such as land management and microbiological activity (Tokarski et al., 2020). Through the process of decomposition organic matter is constantly subjected to change. Based on the state of decomposition, organic compounds can vary in size and energy level. Decomposed carbon can only be taken in by micro-organisms if organic fragments are

small enough to pass through the walls of their cells. Depending on the state of decomposition, carbon fractions decrease in size which results in new fragments with their own characteristics. Some carbon groups will become polarized or ionized, which will allow them to dissolve in water (Marschner et al., 2008). Other carbon groups are more resistant to degradation since they are protected by protection mechanisms. Physical protection occurs when carbon group cluster together into aggregates, reducing the spatial accessibility of the carbon. Chemical protection stabilizes carbon through interactions with minerals. Biochemical protection makes carbon more stable through improved carbon structures that are less prone to degradation (Tokarski et al., 2020). These processes increase the protection against decomposition and stabilize the carbon in the soil (Marschner et al., 2008; Lehmann & Kleber, 2015).

Previous studies have shown that increases or decreases in soil carbon do not necessarily give an indication for turnover rates. While the total carbon content may be unaffected after a disturbance, it may affect the SOM distribution (Oliver et al., 2017). Measuring the total organic carbon content may therefore give biased information on cycling rates since carbon in soils may increase, while the availability of this carbon decreases (Mayor et al., 2016).

After fire occurrence there might be an increase in some carbon fractions and a decrease in other fractions. This difference can be explained by specific soil characteristics such as the vertical SOM distribution. Overall, fire can increase or decrease the net carbon sequestration rate in grasslands (Michelsen et al., 2004; Carol Adair et al., 2009; Bikila et al., 2016; Pellegrini et al., 2018). For instance, if a lot of SOM is stored in the fire-prone topsoil, net carbon rates can decrease after a fire (Michelsen et al., 2004; Bikila et al., 2016). Furthermore, fire is most likely to rearrange the distribution of SOM into other particles, depending on SOM depth, fire intensity and soil properties such as mineral availability, water and SOM availability (Michelsen et al., 2004; Chen & Shrestha, 2012). Oliver et al. (2017) describes that fire and herbivory experiments in Peruvian montane grasslands resulted in no significant changes in total carbon stocks. The experiments did however change the distribution of carbon fractions within the soil since the treatments led to a decrease of the labile fraction and increased the resistant occluded fraction.

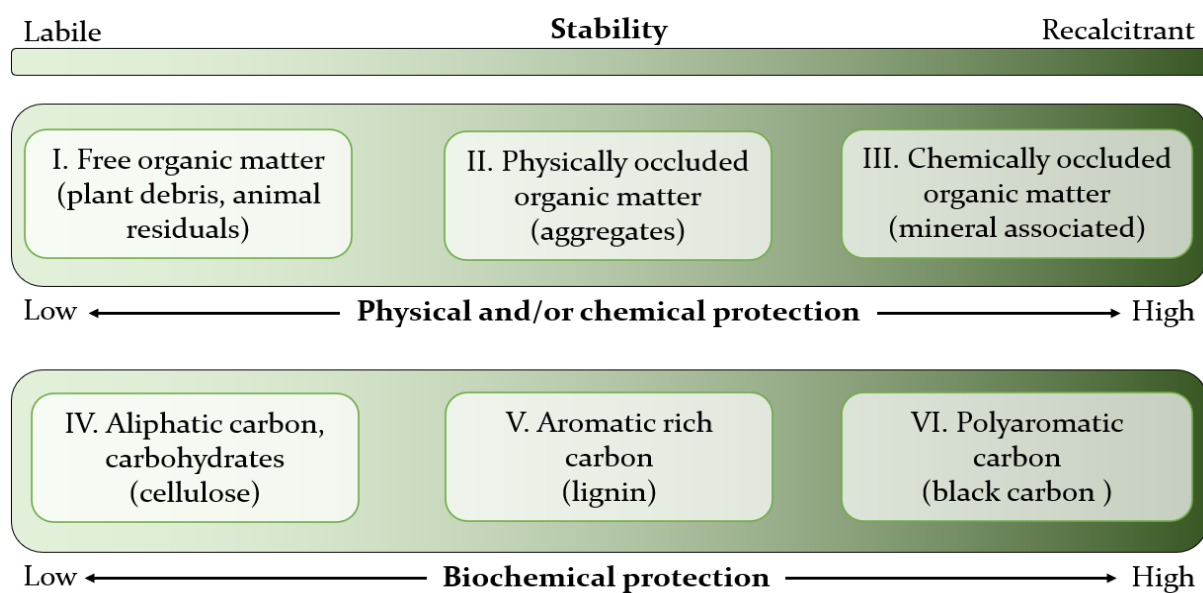
## Carbon pools

Carbon in soils occurs in different fractions that vary in size, chemical properties and cycling rates (Tokarski et al., 2020). Fractions can be labile (easy to decompose) or recalcitrant (difficult to decompose). The degree of decomposability influences the cycling rate of carbon in ecosystems (Marschner et al., 2008; Plaza et al., 2012). The persistence of organic matter depends on the molecular structure and protection mechanisms (Plaza et al., 2013). The carbon in the soil can be classified into three general pools that have different cycling rates.

The first pool is the unprotected or active carbon pool (Oliver et al., 2017). This pool consists of freely accessible carbon, making it the most labile (Plaza et al., 2012; Oliver et al., 2017; Lavalley et al., 2019). It is made up of plant debris, animal residual, seeds, pollen and micro-organisms (figure 1: I & IV). In general, this pool is often composed of large organic fragments not or barely affected by chemical and/or physical processes (Tan et al., 2007; Cerli et al., 2012). The main components in this pool are sugar structures and amino acid structures, which often contain physically available carbon that can be chemically mineralized. It plays an important role in short term nutrient cycling, since its cycling rate ranges from days to years (Oliver et al., 2017).

The second pool of carbon is the resistant pool (Oliver et al., 2017) which is physically and/or chemically protected (figure 1: II & III). This pool is composed of the same elements as the first pool, but these elements have undergone some degree of physical and/or chemical change, resulting in smaller organic particles that can cluster into aggregates (Cerli et al., 2012). Aggregate structures limit the decomposition rate of carbon through decreased spatial accessibility and reduced enzyme diffusion. Smaller aggregates are more resistant to decomposition and therefore more recalcitrant (Plaza et al., 2012). The chemically protected carbon stems from interactions between carbon fractions and minerals. Most soils contain a wide range of minerals, some of which have a large sorption (binding) capacity with SOM, which is determined by the mineral's relative surface area and the reactivity of its surface (Viret & Grand, 2019). The interactions between carbon and minerals increase the recalcitrance of the carbon by reducing the capacity of micro-organisms to aid in the decomposition (Plaza et al., 2012; Lavalley et al., 2019). This pool is important for several soil functions such as water-holding capacity and has a cycling rate of decades (Oliver et al., 2017).

The final pool is the inert pool, which consists of carbon that is protected by biochemical structures (figure 1: V & VI). Molecules in this pool can be highly carbonized, meaning that they are composed of high carbon molecular structures such as aromaticity. These structures are very recalcitrant and therefore more difficult to decompose and dissolve. This pool is most relevant for long-term carbon storage since it has a cycling rate from decades to millennia (Oliver et al., 2017). The different carbon pools also occur in combinations. Biochemically protected carbon fractions may cluster into aggregates, which also gives them physical protection (Plaza et al., 2012; Oliver et al., 2017; Lavalley et al., 2019).



**Figure 1.** Recalcitrance of different carbon compounds based on physical, chemical and biochemical protection mechanisms.

## Dissolved organic carbon (DOC)

An important factor in the decomposition of SOM is the solubility of organic matter in water, since mineralization often occurs in a soluble state (Marschner et al., 2008). Dissolved organic carbon is a transitional stage between the solid organic matter in soil and the eventual release of CO<sub>2</sub> (Don & Schulze, 2008). When the carbon gets dissolved in water, it becomes mobile, allowing it to be transported through the soil. Through this process the carbon can be mineralized, stabilized or leached into groundwater (Marschner et al., 2008; Don & Schulze, 2008). Whether DOC is leached or consumed (mineralized) depends on environmental and biological conditions. Soil characteristics such as pH, texture – which influences contact time between DOC and decomposers – and aggregates relate to the environmental conditions that can influence the mineralization process. Characteristics of the microbial community such as size and composition also determine this process (Don & Schulze, 2008).

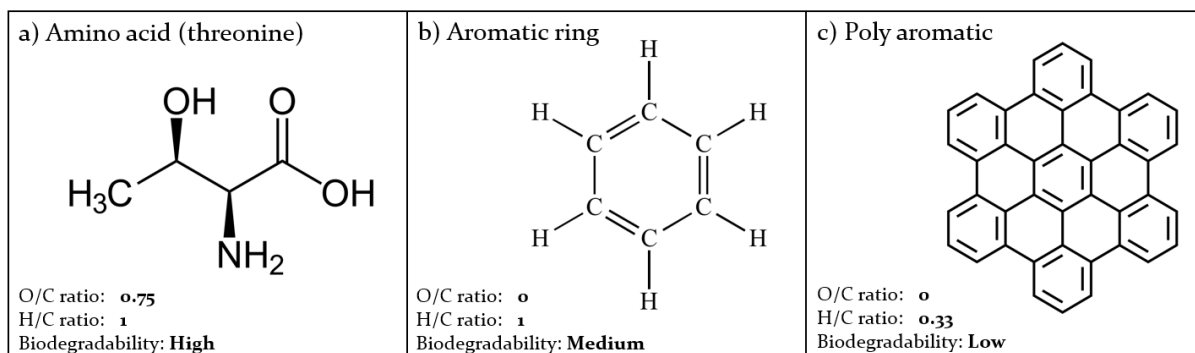
The recalcitrance of DOC is very diverse, since the degradability can vary a lot. Labile DOC can have a residence time of days, while stable aromatic pools can last for years (Marschner et al., 2008). Microbial degradation of DOC occurs in two steps. First the microbes release enzymes that cleave the more complex molecules into smaller compounds. When these compounds are small enough, they are incorporated into the microbes, where they are used as an energy source and are mineralized (Don & Schulze, 2008). This process allows DOC to be consumed, but also to be produced. Unpalatable solid carbon can be solubilized by microbes dissolving – but not mineralizing – the carbon, therefore adding to the DOC (Don & Schulze, 2008).

## Black carbon / Pyrogenic carbon (BC/PYC)

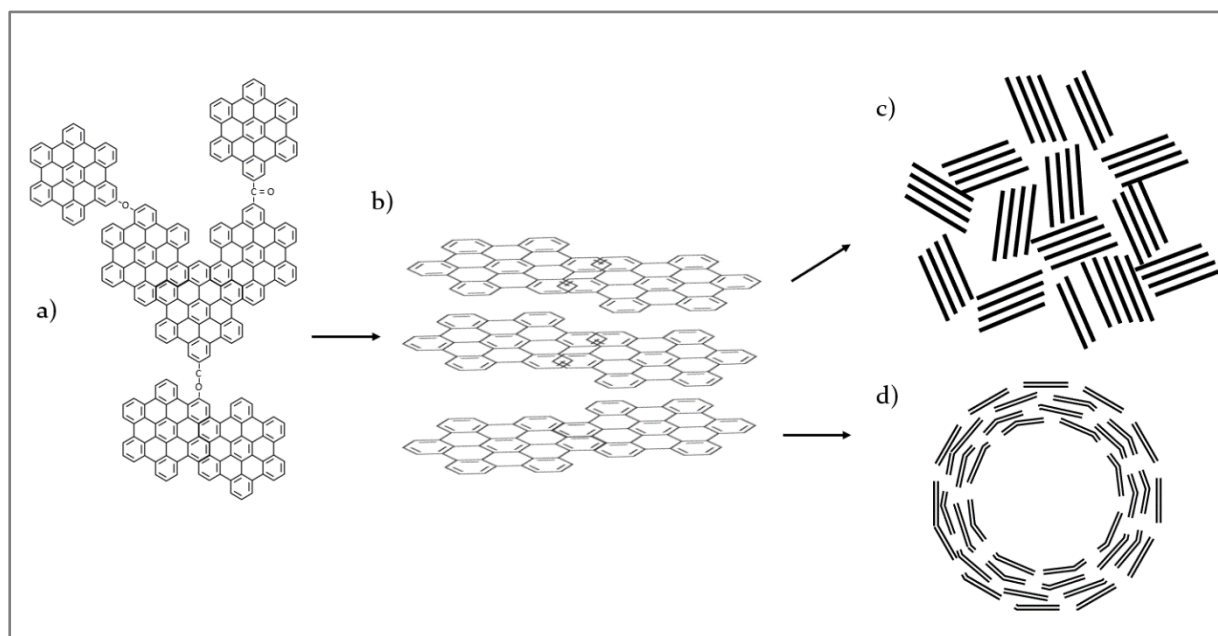
When fires occur, small portions of biomass can be converted into black (pyrogenic) carbon (De La Rosa et al., 2008). This is charred residue that is created whenever incomplete combustion of vegetation and detrital material occurs (Preston & Schmidt, 2006). It is often found in nature and has a very slow decomposition rate (Kuzyakov et al., 2018; Santín et al., 2016). Due to its lower degradability, the cycling rate of black carbon is increased which influences long-term carbon storage and nutrient cycling (Marschner et al., 2008; Pellegrini et al., 2018). However, black carbon is not indestructible and will not remain in the soil forever. The rough estimated half time is about 6600 years when the black carbon is not disturbed, since it is prone to oxidation when it is exposed. Subsequent fires and movement through soil profiles (due to uprooting or other disturbances) can degrade the black carbon more easily (Preston & Schmidt, 2006).

Black carbon can be described as a wide ranged spectrum of different charred plant materials. Char results in charcoal, whereas volatiles during a fire result in soot. Graphite, the most rare and robust type of black carbon, is only formed when heat is combined with pressure (Preston & Schmidt, 2006). The incomplete combustion of biomass leads to charring, which allows the occurrence of condensation reactions. During these reactions, two molecules get attached to each other leading to the release of another molecule such as water (Santín et al., 2016). These condensation reactions result in an increase in aromaticity (increased aryl and O-aryl structures) and a decrease in carbohydrate and lignin structures (De La Rosa et al, 2008). The loss of water results in a decrease in functional groups which are mainly oxygen (O) and hydrogen (H). The biodegradability of an organic compound is determined by the number of functional groups. Therefore, the ratio between carbon and function groups (O/H or O/C ratio) is an indicator for its degradability. The ratio of condensed structures is closer to zero, therefore it is more recalcitrant (figure 2).

When this condensation process lasts long enough, cross-linked aromatic structures (figure 3: a)) result in aromatic clusters that form graphene sheets (figure 3: b)). The functional groups are polarized and responsible for the binding of the structures. In soils, the groups randomly stack (figure 3: c)). Air is a-polar and therefore the aromatic groups align in an onion shape, stacking aromatic layers on top of each other (figure 3: d)) (Preston & Schmidt, 2006).



**Figure 2.** Decrease in functional groups as a result of condensation reactions from fire. Amount of functional group in a) amino acids (O/C= 3/4, H/C = 4/4), b) aromatic ring (O/C= 0/6, H/C = 6/6) and c) poly aromatic (O/C= 0/54, H/C = 18/54).



**Figure 3.** Consecutive black carbon (c) and soot (d) structure formation. Based on the original figure from (Santín et al., 2016).

During vegetation fires, most of the black carbon remains on site within the soil, the ash layer and charred plant tissue. Approximately <20% is emitted into the atmosphere but these values can vary depending on the environmental factors such as slopes and wind (Santín et al., 2016). Black carbon found deep within soils is likely to originate from vertical mobilization processes since fire temperatures do not exceed the charring temperature in deeper soils (with the exception of organic soils, roots and peat soil that can char beneath the surface). The vertical movement of black carbon within soils depends on water flows, intrinsic carbon properties (small particles move faster), soil characteristics and bioturbation processes. Black carbon in soils can interact with water to move faster or stabilize by interacting with minerals or forming aggregates (Santín et al., 2016).



## Methodology

### Research site

The soil samples were collected in an ongoing experiment in the uKhahlamba-Drakensberg Park in South Africa (figure 4), located on the Brotherton plateau at Cathedral Peak, at an altitude of approximately 1900 metres (Manson, Jewitt & Short, 2007). The mean annual precipitation is approximately 1400 mm (Gordijn et al., 2018) and falls mostly during the summer months (Manson et al., 2007). The plateau has a basalt surface, covering the underlying sandstone. The vegetation in the park can be described as moist montane grassland (Manson et al., 2007) or basalt grassland, with the *Themeda Triandra* as dominant species (Gordijn et al., 2018).

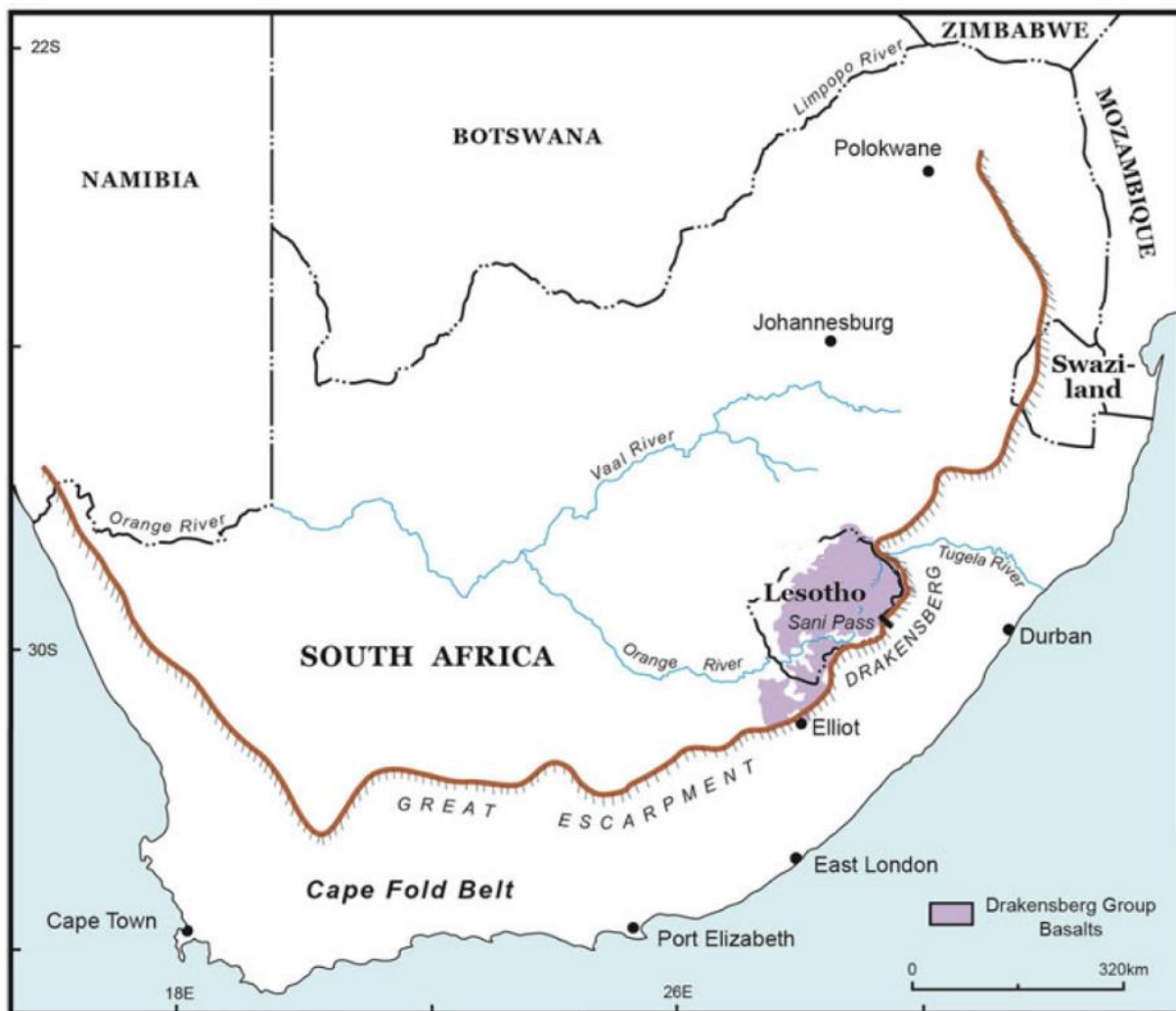


Figure 4. Drakensberg in South Africa (Modified from: Knight & Grab, 2015).

## Experimental setup

The Brotherton fire experiment was established in 1980 in order to study the effect of regular burning on grassland communities. The experiment is laid out in a randomized block design and fire treatments manipulate both fire frequency (annual, biennial, quinquennial and no burn) and seasonality (spring and autumn burns), resulting in a full factorial experiment with four levels of fire frequency, each replicated four times (Manson et al., 2007). Each fire treatment was applied to plots of 25 x 25 metres with four replicates. The soil samples were collected in April 2019. Sampling points were cleared of vegetation, and by using an auger ( $\varnothing$ : 5cm) 20 subsamples were collected at two depths on each fire treatment plot (40 cores per plot). After the collection, the subsamples were mixed into a total sample of the plot (Manson et al., 2007). The soil from three treatments (Annual Spring, Biennial Spring, Five Year Rotation) and control plots was transferred to Utrecht where it was processed and analyzed (figure 5).

	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Plot nr.	3	12	21	45
Treatment	AS	AS	AS	AS
Depths	0-5 cm + 5-15 cm	0-5 cm + 5-15 cm	0-5 cm + 5-15 cm	0-5 cm + 5-15 cm
Plot nr.	2	15	19	31
Treatment	BS	BS	BS	BS
Depths	0-5 cm + 5-15 cm	0-5 cm + 5-15 cm	0-5 cm + 5-15 cm	0-5 cm + 5-15 cm
Plot nr.	6	13	17	37
Treatment	FYR	FYR	FYR	FYR
Depths	0-5 cm + 5-15 cm	0-5 cm + 5-15 cm	0-5 cm + 5-15 cm	0-5 cm + 5-15 cm
Plot nr.	11	24	35	44
Control	NB	NB	NB	NB
Depths	0-5 cm + 5-15 cm	0-5 cm + 5-15 cm	0-5 cm + 5-15 cm	0-5 cm + 5-15 cm

**Figure 5.** Experimental setup: Plots with four levels of fire frequency and four replicates. Abbreviations: AS (Annual Spring burn treatment), BS (Biennial Spring burn treatment), FYR (Five Year Rotation burn) and NB (No Burn control).

## Data collection

The data was collected according to the assumption that SOM interacts with soil particles into different fractions. Therefore, the SOM fractions can be isolated by looking at each fraction's different properties (Tokarski et al., 2020). Density fractionation broadly corresponds with the cycling rates of carbon (Manning et al., 2015) and should result in soil recovery rates of approximately 90% (Viret & Grand, 2019). Therefore, density fractionation is an appropriate method to use for this study.

The soil samples from the experiment were separated into the different carbon pools based on the (modified) method of Plaza et al. (2012), which separates the carbon based on the density of carbon fractions (density fractionation) and allows fractions to be isolated mostly intact (figure 6). During this process light and heavy fractions are separated since minerals and organic material have different densities and physical dispersion (clustering). This method should result in three different fractions: the labile light fraction (L<sub>1</sub>), the occluded light fraction (L<sub>2</sub>) and the recalcitrant heavy fraction (H) with little loss of material during the process (Cerli et al., 2012; Viret & Grand, 2019). The step by step breaking down of the SOM and the aggregates (by means such as sonication) resulted in different measurable carbon pools.

### Sample preparation

The soil from each plot was divided into three sub-samples of 10 grams each, with a maximum deviation of 1% of the weight, obtaining 30 grams of sample per plot in total. The sub-samples were stored in 50 ml polypropylene conical centrifuge tubes. The tubes were then filled with 40 ml sodium polytungstate (SPT) with a density of 1.6 g/ml. This density was chosen since it results in a maximum yield of organic matter (Cerli et al., 2012). The SPT was prepared according to the protocol from Viret & Grand (2019).

### Dissolved organic carbon (DOC) collection

The DOC was obtained according to the modified protocol from Houba et al. (2000), which measures organic carbon by looking at CO<sub>2</sub> that resulted from acidification reactions. For each plot, an additional centrifuge tube was filled with 3 mg of bulk soil. The centrifuge tubes were then filled with 30 ml of CaCl<sub>2</sub> solution (1.11 g/L). The centrifuge tubes were placed in an overhead shaker for 2 hours at 120 rpm and centrifuged afterwards for 30 min at 2500 rpm. The supernatant liquid was then extracted with a pipette (5 ml) and put in 30 ml glass vials, and diluted with 15 ml of ultra pure water. The carbon contents were then analyzed with the use of a Shimadzu TOC-VCP. This device uses infrared adsorption, measuring the amount of infrared adsorbed by gasses (in this case CO<sub>2</sub>).

### The light fraction (L<sub>1</sub>) collection

The first step was to obtain the lightest fraction consisting of the unprotected carbon pool in the soil. This was done by dissolving the soil into a heavy liquid that allows the separation of the lightest fractions through sieving and filtration. After filling the tubes with SPT, they were shut and gently shaken, suspending the soil. The tubes were then put upright and left to settle for 30 minutes, before centrifuging the samples at 2500

rpm for 120 minutes. After the centrifugation, the samples were carefully removed from the centrifuge, not resubmerging the floating fractions, or resuspending the heavy fractions into the SPT.

The L<sub>1</sub> fraction was then removed from the samples with a vacuum ultrafiltration set-up. The suspended material was carefully poured onto a 1.6 µm filter, sucking the SPT through the filter, leaving the light fraction on the filter. The vacuum pump was then detached to repour the SPT back into the centrifuge tubes of the same sample. Since the loss of SPT was approximately 2%, this deficit was supplemented with additional SPT to ensure all the centrifuge tubes contained 40 ml of SPT.

After the repouring of the SPT, the vacuum filtration was reattached. The light fraction was rinsed 3 times with deionized water (approximately 5 ml per rinse ) to remove any remaining SPT from the filtered sample. The vacuum pump was then detached again and the filter with the light fraction was carefully removed and put into glass vials (20 ml, weighed in advance). This was done by pouring deionized water on the filter while holding it over a funnel. The remaining light fraction from the vacuum pump was also rinsed into the glass vial. The glass vial was then covered with aluminum foil to prevent contamination of any sort and perforated with a needle to allow water to evaporate. The vials were stored in an oven at 60 °C to dry the samples completely until constant weight.

### **The occluded fraction (L<sub>2</sub>) collection**

After removing the light fraction, the centrifuge tubes were refilled with SPT to prepare them for the next step. The tubes were firmly shaken to resuspend the pellet into the SPT to prepare them for the sonication process. A sonication device has a probe that resonates at very high frequencies and applies ultrasonic energy to particles, freeing the occluded fraction. This process breaks down stable macroaggregates and larger microaggregates, releasing the occluded organic matter. The amount of energy required in order to break down the aggregates varies based on the soil type and has to be optimized (Viret & Grand, 2019). The calibration was done by using six different types of soil across a texture gradient and measuring the amount of occluded fraction after different sonication levels. The optimal energy input was selected when the amount of occluded light fraction no longer increased, indicating that all the aggregates had been broken down. The tubes were sonicated with the obtained optimal energy amount of 111.7 J/ml over a duration of 10 minutes. To prevent overheating during the sonication, the tubes were put into a refrigerator overnight to lower the temperature of the sample. After the sonication process the samples were centrifuged, once again at 2500 rpm for 120 minutes. The occluded light fraction was then collected via vacuum ultrafiltration, in a way similar to the collection of the light fraction. The only difference compared to the light fraction collection was that the residual SPT was not poured back into the centrifuge tubes. Instead, the residual SPT was stored in a plastic container to be recycled (see p. 22: *the recycling of SPT*). The glass vials were covered with perforated aluminum foil and stored in an oven at 60 °C to dry the samples completely.

### **The heavy fraction (H) collection**

After removing the occluded fraction from the samples, the pellet with the heavy fraction remained in the tube. In order to obtain the heavy fraction, the remaining SPT had to be rinsed out of the tubes. The tubes were filled with 40 ml of deionized water and firmly shaken to resuspend the pellet. The tubes were then centrifuged for 30 minutes at 2500 rpm and the deionized water was poured out. This step was repeated two more times in order to remove all the remaining SPT. The samples were then covered with aluminum foil, perforated and stored in an oven at 60 °C to dry the samples completely.

### **Fraction weighing and analysis preparation**

After the collection of the different fractions all samples were dried. The samples were weighed daily and were declared completely dried when the weight did not change over 3 days. The sample data was then obtained by subtracting the mass of the empty vial or centrifuge tube from the total mass of the sample. The samples were then prepared for the C/N elemental analyzer (Thermo Scientific EA Isolink CN) to determine the carbon content. The elemental analyzer is a highly accurate measuring device that measures the carbon content in a very small samples, weighing from 500 µg up to 8 grams. A small twig could make the sample biased, therefore the samples were individually grinded in a pestle and mortar for approximately 10 minutes until a heterogenous powder was obtained, which would provide a representative reading of the sample.

### **Thermogravimetric analysis**

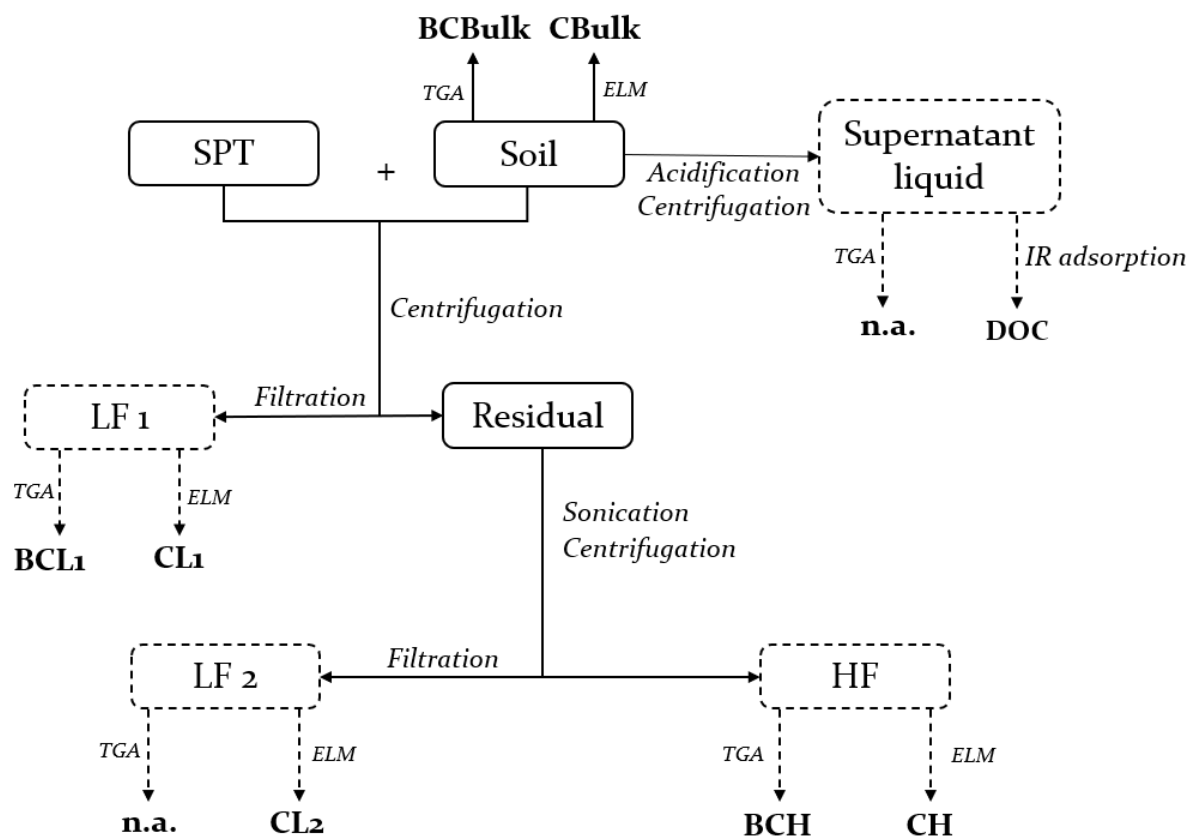
Thermogravimetric analysis (TGA) is a method that describes weight loss based on increases in temperature. With the use of a highly accurate scale, the samples' weight is constantly monitored while the samples are gradually heated (Lopez-Capel et al., 2005; Plante, Fernández & Leifeld, 2009). The samples were processed in a manner similar to the one described in the protocol from Lopez-Capel et al. (2005).

During the TGA, the weight loss at certain temperatures corresponds with the loss of specific fractions. Labile organic matter is lost between 200-380 °C, recalcitrant organic matter is lost between 380-475 °C and refractory organic matter (ROM) is lost between 475-650 °C. Within ROM, material lost in the temperature range between 550-650 °C indicates black carbon and/or kerogen. However, depending on the origin of the black carbon the combustion of black carbon might start at 500 °C (De La Rosa et al., 2008). Therefore, the data extracted from the TGA analysis was extracted for two different temperature ranges: T<sub>1</sub>, ranging from 550-650 °C and T<sub>2</sub>, ranging from 500-650 °C.

Even though TGA does not give a complete overview of soil composition, it does work as an indicator of the thermal stability of SOM, thus being an indicator for labile fractions and biochemically stable fractions (Plante et al., 2009). De La Rosa et al. (2008) found that Mediterranean burned soils subjected to TGA lost less overall weight when were they exposed to temperatures up to 500 °C. However, they lost more weight in temperature ranges that are associated with refractory organic matter (ROM). This is most likely due to a reduction of labile carbon fractions and increases in recalcitrant fractions such as black carbon due to the fire events.

## The recycling of SPT

SPT has many advantages in comparison with other high-density fluids, such as less toxicity, lower viscosity at high concentrations and high-density diversity. The disadvantage of this fluid is that it is rather expensive and that immediate reuse can result in biased results. For those reasons, it has to be recycled (Six et al., 1999). The SPT was recycled by guiding it through a column of activated carbon, which removed the dissolved carbon from the liquid and resulted in clean SPT with lower density. In this study the SPT density was restored to its original density of 1.6 g/ml by heating the residual SPT. It was then cooled down and reused for new samples.



**Figure 6.** Schematic overview of the method used for this study. Abbreviations: DOC (Dissolved Organic Carbon), BC (Black Carbon), C (Total Carbon), SPT (Sodium Polytungstate), LF<sub>1</sub> (Light fraction), LF<sub>2</sub> (Occluded fraction), HF (Heavy fraction), TGA (Thermogravimetric Analysis), ELM (Elemental Analysis).

## Statistical analysis

In this research, two statistical analyses were conducted. The first statistical analysis was used to identify the effect of burning treatments on the total amount of carbon stored in five different carbon pools – DOC, bulk soil, light fraction (L<sub>1</sub>), occluded fraction (L<sub>2</sub>) and heavy fraction (H) – at two different depths, 0-5 cm and 5-15 cm. The data that was obtained was standardized prior to the analysis. During the sample preparation, sample weights deviated slightly. This deviation was corrected by calculating the average amount of carbon per gram of soil for all the measured variables. This data set was then analyzed using linear models. Treatments were compared for the total amount of carbon in each carbon fraction at both depths. Post hoc Tukey tests were then used to identify differences between treatments.

The second statistical analysis was used to identify the effects of burning treatments on formation of black carbon within three different carbon pools (bulk soil, L<sub>1</sub> and H) at two depths (0-5 cm and 5-15 cm) and two temperature ranges (T<sub>1</sub>: 550-650 °C and T<sub>2</sub>: 500-650 °C). The DOC and the L<sub>2</sub> fraction were excluded from this analysis due to lack of data, since insufficient material was obtained to perform a representative TGA analysis. The data that was obtained was standardized prior to the analysis. The sample weight used in the TGA analysis differed per sample. To correct these differences, the average amount of black carbon per gram of sample was calculated. This data set was analyzed using linear models. Treatments were compared for the total amount of black carbon in the bulk soil, the L<sub>1</sub> fraction and the H fraction. This was done for two temperature ranges and both depths. Post hoc Tukey tests were then used to identify the differences between treatments.

Both statistical analyses were conducted with R, version 4.0.4. The chosen level of significance was 0.10. The normality of distributions was checked visually through histograms and statistically through a Shapiro-Wilk test (appendix 1). The linear models were constructed using the 'nlme'-package, and the paired post hoc Tukey tests were conducted with the 'emmeans'-package (complete R-script, appendix 2).

## Results

The effectiveness of the fractionating procedure can be determined by looking at the soil recovery rate. This study had an average soil recovery rate of 98%. The recovery rate is calculated by looking at the mass balance, which is composed of the mass of the bulk soil compared to the combined mass of all fractions. Mass imbalances between 10-15% are common and do not result in biased results.

### Bulk soil

The total amount of carbon in the soil samples varied between 84.4 mg and 155.2 mg per gram of soil. On average there was more carbon in the topsoil (128.7 mg) than in the deeper soil (106.2 mg) (figure 7). For the topsoil, the treatments did not result in a significant difference in carbon contents ( $p = 0.4086$ ). For the deeper soil the treatments

have a significant effect on the carbon content within the soil ( $p = 0.0308$ ) (table 1). The main difference in the deeper soil was explained by a significant increase in carbon content as a result of the Annual Spring treatment (AS) compared to the No Burn treatment (NB) (AS-NB:  $p = 0.0934$ ), and as a result of the Biennial Spring treatment (BS) compared to NB (BS-NB:  $p = 0.0503$ ) (table 2).

The black carbon (BC) in the bulk soil varied between 0.8 mg and 1.4 mg per gram of soil for the first temperature range (T<sub>1</sub>: 550-650 °C). For the second temperature range (T<sub>2</sub>: 500-650 °C) the BC content varied between 0.6 mg and 0.9 mg. For both T<sub>1</sub> and T<sub>2</sub>, the average amount of BC was higher in the topsoils compared to the average amount of BC in the deeper soils (figure 8). The treatments only resulted in significant increases in BC in deeper soils for both T<sub>1</sub> and T<sub>2</sub> (Bulk<sub>0-5cm</sub>T<sub>1</sub>:  $p = 0.4663$ , Bulk<sub>0-5cm</sub>T<sub>2</sub>:  $p = 0.6158$ , Bulk<sub>5-15cm</sub>T<sub>1</sub>:  $p = 0.03227$ , Bulk<sub>5-15cm</sub>T<sub>2</sub>:  $p = 0.0623$ ) (table 3). The main differences are found between the AS-NB and BS-NB (AS-NB:  $p = 0.0973$ , BS-NB:  $p = 0.0470$ ) (table 4).

### DOC

The dissolved organic carbon (DOC) values varied between 2.5 and 4.9 mg per gram of soil. The samples from the 0-5 cm depth had a slightly lower average value than the 5-15 cm depth (respectively 3.465 mg to 3.594 mg) (figure 7). No significant treatment effects were found for this carbon pool (DOC<sub>0-5cm</sub>:  $p = 0.1348$ , DOC<sub>5-15cm</sub>:  $p = 0.3116$ ) (table 1).

### Light fraction (L<sub>1</sub>)

The amount of carbon in the light fraction (L<sub>1</sub>) varied between 1.4 mg and 28.1 mg per gram of soil. On average the topsoil contained more light fraction (18.1 mg per gram of soil) than the deeper soil (5.975 mg per gram of soil). The standard deviation is relatively wide for different treatments (figure 7). For both depths, increased fire-recurrence intervals lead to increases in carbon contents in the light fraction. The treatments resulted in significant effects on carbon contents of the L<sub>1</sub> fraction at both depths (L<sub>10-5cm</sub>:  $p = 0.046$ , L<sub>15-15cm</sub>:  $p = 0.012$ ) (table 1).

In the topsoil, increases in carbon content are found as a result of the AS treatment compared to the five year rotation treatment (FYR) (AS-FYR:  $p = 0.0571$ ), and as a result of the AS treatment compared to the NB treatment (AS-NB:  $p = 0.0794$ ). For the deeper soil, increases in carbon content are found as a result of the Annual or Biennial Spring treatment compared to the low-intensity or No Burn treatments (AS-FYR:  $p = 0.0980$ , BS-FYR:  $p = 0.0214$ , BS-NB:  $p = 0.0393$ ) (table 2). Both the AS and BS treatments result in two to three times the amount of carbon stored in comparison with the carbon stored as a result of the FYR and NB treatments. Both AS and BS treatments result in increased carbon storage in the L<sub>1</sub> fraction, the difference is that the AS treatment stores slightly more in the topsoil and the BS treatment stores slightly more in the deeper soil.

The amount of BC within the light fraction varied between 0.30 and 0.47 mg per gram of soil for T<sub>1</sub>. For T<sub>2</sub>, the BC contents varied between 1.7 and 2.7 mg per gram of soil. For both T<sub>1</sub> and T<sub>2</sub>, the average topsoil BC content was slightly higher than the deeper BC



content (T1: 0.46 mg compared to 0.39 mg, and T2: 1.7 mg compared to 1.3 mg) (figure 8). Significant differences in BC content based on the treatment were only found for the topsoil within T1 ( $L_{10-5cm}T1: p = 0.06069$ ) while the other ranges showed no significant differences ( $L_{15-15cm}T1: p = 0.1662$ ,  $L_{10-5cm}T2: p = 0.3800$ ,  $L_{15-15cm}T2: p = 0.6003$ ) (table 3). The differences in treatments for the BC in T1 are mainly between the AS and BS treatments ( $p = 0.0439$ ), where BC contents in the AS treatment are much larger than in the BS treatment (table 4).

### **Occluded fraction (L2)**

Carbon within the occluded fractions (L2) was only present in small quantities, varying between 0.4 and 4.2 mg per gram of soil. On average, topsoils contained a higher occluded carbon content (2.9 mg of carbon per gram of soil) compared to deeper soils (1.0 mg of carbon per gram of soil). The standard deviation is very small, mainly for the deeper soil (figure 7). For both depths, no significant treatment effects on carbon sequestration in the occluded fraction have been found ( $L_{20-5cm}: p = 0.3444$ ,  $L_{25-15cm}: p = 0.4035$ ) (table 1).

### **Heavy fraction (H)**

Comparing fractions, the largest quantities of carbon were found in the heavy fraction, varying between 73.1 and 125.2 mg per gram of soil. On average, more carbon was found in topsoils (99.5 mg of carbon per gram of soil) than in deeper soils (91.1 mg of carbon per gram of soil). The standard deviation is larger in deeper soils (figure 7).

For the topsoil, treatments did not result in significant differences in carbon contents ( $H_{0-5cm}: p = 0.4726$ ). In deeper soils, the treatment does influence the total amount of carbon sequestered in the heavy fraction ( $H_{5-15cm}: p = 0.06552$ ) (table 1). Increases in carbon content are mainly found as a result of the BS treatment compared to the NB treatment (BS-NB:  $p = 0.0978$ ) and the almost significant AS treatment compared to the NB treatment (AS-NB:  $p = 0.1298$ ). In the deeper soils, carbon contents in the AS and BS treatments were generally higher compared to the FYR and NB treatments (table 2).

The amount of BC within the heavy fraction varied between 0.25 mg and 0.45 mg per gram of soil for T1. For T2 this range varied between 0.47 mg and 0.81 mg of BC per gram of soil. For both T1 and T2, the average BC content was slightly larger in the topsoil (T1: 0.36 mg, T2: 0.66 mg) compared to the deeper soil (T1: 0.33 mg, T2: 0.60 mg) (figure 8). Significant treatment effect on BC was found for almost all depths and temperature ranges except for topsoil HT1 ( $H_{0-5cm}T1: p = 0.1018$ ,  $H_{5-15cm}T1: p = 0.04874$ ,  $H_{0-5cm}T2: p = 0.08301$ ,  $H_{5-15cm}T2: p = 0.0456$ ) (table 3). For the topsoil, the difference is mainly explained due to larger BC contents within the AS treatment compared to the NB treatment (AS-NB:  $p = 0.0761$ ). In the deeper soils, both the AS and the BS treatments result in increased BC content in comparison with the NB treatment (T1. AS-NB:  $p = 0.0752$ , BS-NB:  $p = 0.1127$ ; T2. AS-NB:  $p = 0.0990$ , BS-NB:  $p = 0.1020$ ) (table 4).

**Table 1.** Results of the linear effects model, testing the effect of treatments (fixed effect) on different carbon fractions, analyzed over two depths. Statistically significant effects ( $p < 0.1$ ).

Treatment	df	Bulk soil		DOC		L1, light fraction	
		<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>
Depth 0-5cm	3	1.0437	0.4086	2.2511	0.1348	3.591	<b>0.04641</b>
Depth 5-15cm	3	4.1665	<b>0.0308</b>	1.3264	0.3116	5.6777	<b>0.01174</b>
Residuals	12						

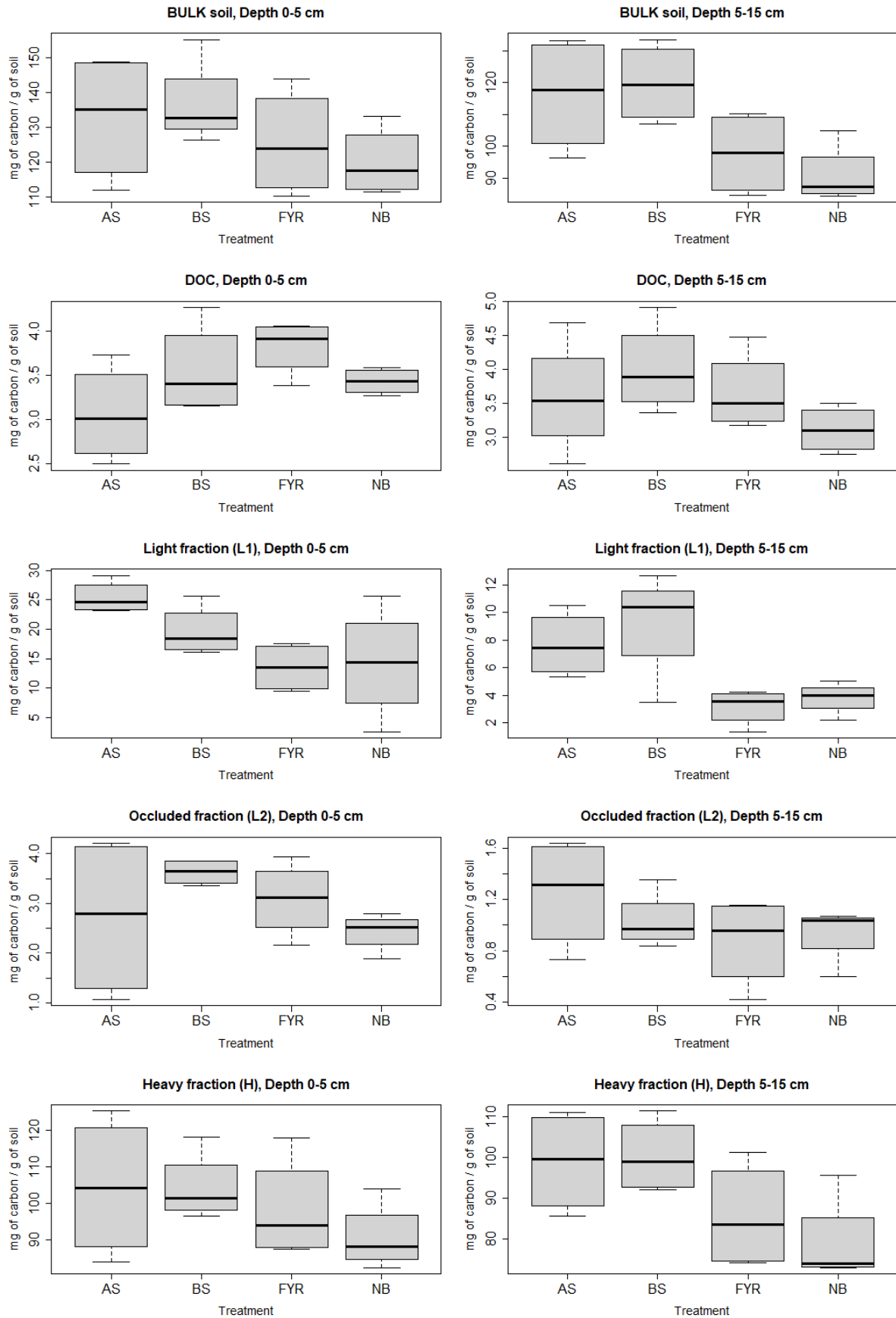
Treatment	df	L2, occluded fraction		H, heavy fraction	
		<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>
Depth 0-5cm	3	1.2214	0.3444	0.8934	0.4726
Depth 5-15cm	3	1.0566	0.4035	3.1343	<b>0.06552</b>
Residuals	12				

**Table 2.** Results of post hoc Tukey test, testing the difference in carbon sample means, analyzed over two depths. Abbreviations: AS = Annual Spring burn treatment, BS = Biennial Spring burn treatment, FYR = Five Year Rotation burn treatment, NB = No Burn control. Statistically significant ( $p < 0.1$ ).

Depth 0-5 cm						
Treatments	df	Bulk	DOC	L1	L2	H
		<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>
AS - BS	12	0.9805	0.3822	0.5220	0.5366	1.0000
AS - FYR	12	0.8938	0.1013	<b>0.0571</b>	0.9420	0.9237
AS - NB	12	0.6149	0.6091	<b>0.0794</b>	0.9712	0.5136
BS - FYR	12	0.7059	0.8111	0.4725	0.8462	0.9279
BS - NB	12	0.4045	0.9749	0.5817	0.3153	0.5209
FYR - NB	12	0.9484	0.5786	0.9973	0.7548	0.8548

Depth 5-15 cm						
Treatments	df	Bulk	DOC	L1	L2	H
		<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>
AS - BS	12	0.9826	0.7906	0.8136	0.7758	0.9981
AS - FYR	12	0.2743	0.9984	<b>0.0980</b>	0.3853	0.4102
AS - NB	12	<b>0.0934</b>	0.7248	0.1717	0.5301	0.1298
BS - FYR	12	0.1587	0.8685	<b>0.0214</b>	0.8944	0.3282
BS - NB	12	<b>0.0503</b>	0.2490	<b>0.0393</b>	0.9721	<b>0.0978</b>
FYR - NB	12	0.8987	0.6309	0.9848	0.9926	0.8564



**Figure 7.** Boxplots density fractionation and the DOC analysis. Abbreviations: AS (Annual Spring), BS (Biennial Spring), FYR (Five Year Rotation) & NB (No Burn).

**Table 3.** Results of the linear effects model, testing the effect of treatments (fixed effect) on the formation of black carbon in different carbon fractions, analyzed over two depths and two different temperature ranges (T<sub>1</sub> = 550-650 °C, T<sub>2</sub>= 500-650 °C). Statistically significant effects ( $p < 0.1$ ).

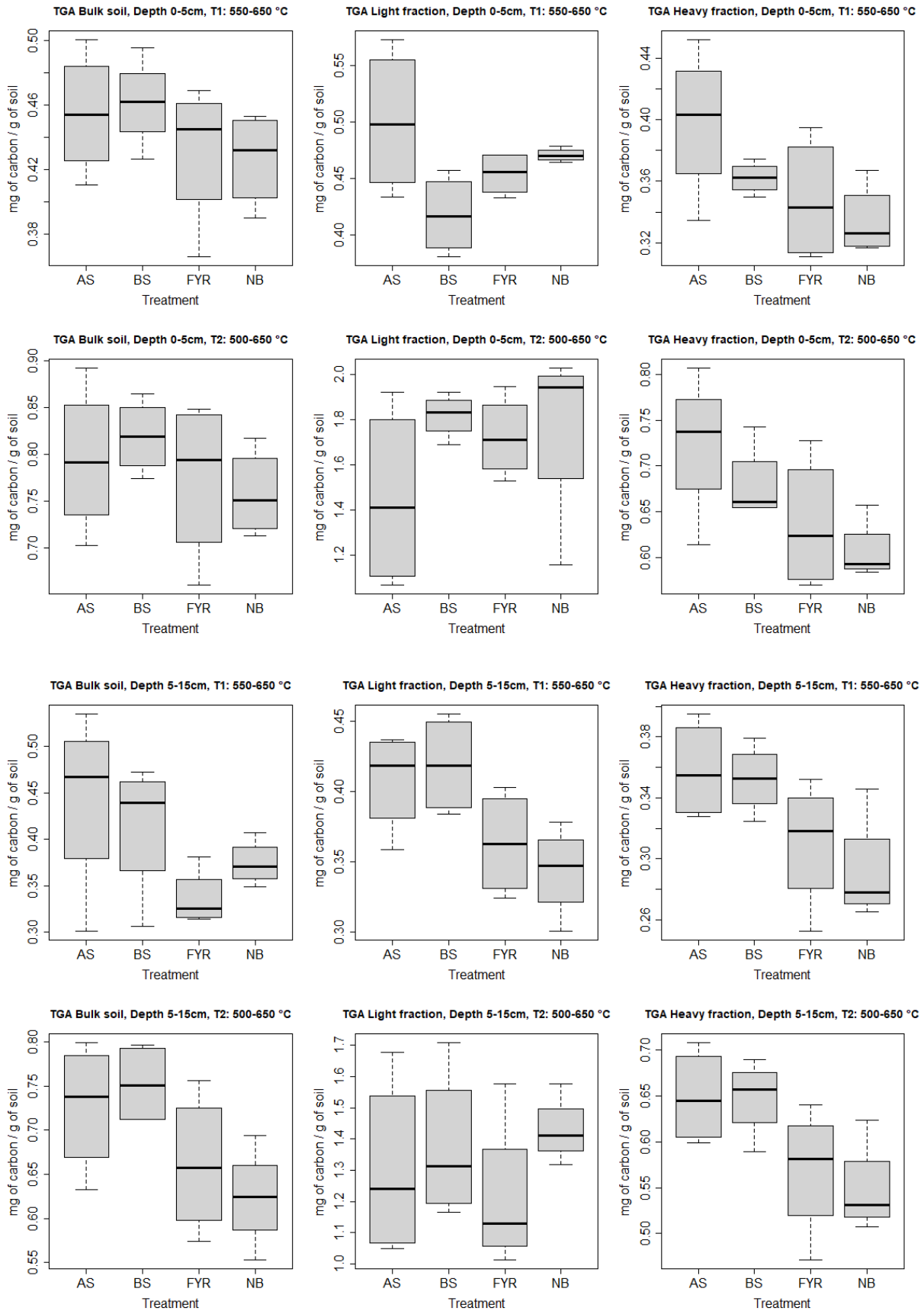
Treatment	df	Bulk T <sub>1</sub>		L <sub>1</sub> T <sub>1</sub>		H T <sub>1</sub>	
		<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>
Depth 0-5cm	3	0.9072	0.4663	3.2279	<b>0.06069</b>	2,5834	0.1018
Depth 5-15cm	3	4.0996	<b>0.03227</b>	2.0109	0.1662	3,5245	<b>0.04874</b>
Residuals	12						

Treatment	df	Bulk T <sub>2</sub>		L <sub>1</sub> T <sub>2</sub>		H T <sub>2</sub>	
		<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>
Depth 0-5cm	3	0.6193	0.6158	1.1189	0.38	2.8342	<b>0.08301</b>
Depth 5-15cm	3	3.1995	<b>0.0623</b>	0.646	0.6003	3.6151	<b>0.0456</b>
Residuals	12						

**Table 4.** Results of post hoc Tukey test, testing the difference in black carbon sample means, analyzed over two depths and two temperature ranges (T<sub>1</sub> = 550-650 °C, T<sub>2</sub>= 500-650 °C). Abbreviations: AS = Annual Spring burn treatment, BS = Biennial Spring burn treatment, FYR = Five Year Rotation burn treatment, NB = No Burn control. Statistically significant ( $p < 0.1$ ).

Depth 0-5 cm							
Treatments	df	Bulk T <sub>1</sub>	Bulk T <sub>2</sub>	L <sub>1</sub> , T <sub>1</sub>	L <sub>1</sub> , T <sub>2</sub>	H, T <sub>1</sub>	H, T <sub>2</sub>
		<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>
AS - BS	12	0.9930	0.9513	<b>0.0439</b>	0.3758	0.4756	0.7402
AS - FYR	12	0.7920	0.9738	0.3603	0.6154	0.1543	0.2285
AS - NB	12	0.6975	0.8686	0.7035	0.5003	0.1493	<b>0.0761</b>
BS - FYR	12	0.6431	0.7812	0.5607	0.9706	0.3731	0.7443
BS - NB	12	0.5431	0.5858	0.2569	0.9950	0.8208	0.3646
FYR - NB	12	0.9981	0.9853	0.9227	0.9970	0.8462	0.5723

Depth 5-15 cm							
Treatments	df	Bulk T <sub>1</sub>	Bulk T <sub>2</sub>	L <sub>1</sub> , T <sub>1</sub>	L <sub>1</sub> , T <sub>2</sub>	H, T <sub>1</sub>	H, T <sub>2</sub>
		<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>
AS - BS	12	0.9726	0.9432	0.9272	0.9712	0.9947	1.0000
AS - FYR	12	0.3239	0.5244	0.1543	0.9457	0.2519	0.2234
AS - NB	12	<b>0.0973</b>	0.1773	0.4793	0.8678	<b>0.0752</b>	<b>0.0990</b>
BS - FYR	12	0.1734	0.2584	0.3731	0.7620	0.3523	0.2294
BS - NB	12	<b>0.0470</b>	<b>0.0716</b>	0.8208	0.9868	0.1127	<b>0.1020</b>
FYR - NB	12	0.8592	0.8499	0.8462	0.5723	0.8705	0.9541



**Figure 8.** Boxplots the TGA analysis. Abbreviations: AS (Annual Spring), BS (Biennial Spring), FYR (Five Year Rotation) & NB (No Burn).

## Discussion

Different fire treatments influence the total amount of carbon sequestered in soils. In bulk soils, the amount of carbon increases as a result of increased fire frequency. The distribution of carbon is also affected by fire treatments. The lightest, unprotected carbon fraction is increased by fire regimes with higher frequency in both top and deeper soils. Annual recurring fires mainly result in unprotected carbon in topsoils, whereas biennial recurring fires result in unprotected carbon increases in deeper soils. In deeper soils, the recalcitrant heavy fraction is also increased by frequent fires, thereby increasing the amount of carbon that is protected against decomposition. None of the treatments seem to affect carbon fractions dissolved in water and carbon fractions that have occluded into aggregates. Increases in black carbon content are mainly found in the deeper soils for the bulk and the heavy fraction, which means an increase in the biochemical recalcitrance of the carbon within the soil.

This study shows that increased fire frequency positively affects carbon stored within soils. Disturbances such as fire are beneficial and can increase carbon sequestration in grasslands similar to the grasslands from this study (Michelsen et al., 2004; Carol Adair et al., 2009; Bikila et al., 2016; Pellegrini et al., 2018). Even very frequent fire regimes such as annual burns will not result in decreases of the soil carbon stock, which was confirmed by previous studies (Manson et al., 2007). However, these results are only representative for areas with similar environmental and biophysical conditions. Other fire prone regions might encounter degradation of soil carbon stocks due to ecosystem shifts that result in losses of soil carbon (Carol Adair et al., 2009; Pellegrini et al., 2018).

The fire treatments partially influence the distribution of soil carbon within the different carbon fractions. In the topsoils, the total amount of carbon did not appear to change significantly as a result of frequent fires, while the amount of labile carbon as a result of annual burns increased.

The increase in labile carbon could be an indication of a redistribution of carbon among the different fractions. This significant increase in the light fraction might be explained by either a small carbon increase in the bulk soil or a small carbon decrease in the heavy fraction. However, changes in the bulk soil and the heavy fraction were not significant. The fact that there was only a significant increase found in the light fraction can be explained by the relatively low standard error for the light fraction and the relatively large standard error for the heavy fraction and the bulk soil. This corresponds with the findings of Oliver et al. (2017), who indicated that the carbon distribution in soils might change while total carbon stocks remain unchanged.

In deeper soils, increased fire frequency influenced the total amount of carbon and some carbon fractions. Apart from an increase in the total amount of carbon in the bulk soil, the labile carbon fraction and the recalcitrant carbon fraction increased. However, the occluded fraction did not increase, which might indicate a redistribution of carbon. It is difficult to attribute the increase in these fractions solely to a redistribution of carbon

pools, since an increase in the total amount of carbon will most likely also increase the carbon in each fraction.

Frequent fire treatments result in increases in some of the carbon fractions, but the deeper soil carbon fractions increase more as a result of biennial burns than as a result of annual burns. This can probably be explained by soil mobilization, which processes matter vertically through soils after burns. This process is most active in the first years after a fire disturbance (Kuzyakov et al., 2018). Annual burns can limit deeper soil carbon sequestration since too frequent fires can result in erosion of the topsoil, therefore disturbing the mobilization process, not allowing carbon to reach the deeper soils (Michelsen et al., 2004), while biennial burns allow more matter to be processed.

This study also found that increased fire frequency affects the occurrence of black carbon within different depths and carbon fractions. Different treatments of the topsoil affected the black carbon contents in the light fraction significantly. The Annual Spring treatment resulted in much larger black carbon contents than the Biennial Spring treatment, which is notable since this study found that the effects of the annual and biennial regimes were not that different for the total amount of carbon in the carbon fractions. This difference could be explained by the ash layer, which is often not considered in pyro carbon research. Ash can be rapidly redistributed after fires vertically (by water) and horizontally (by wind erosion). It is likely that samples taken after annual burns contain more remaining ash than samples taken after biennial burns, therefore increasing the black carbon contents found in them (Santín et al., 2016).

This study also reveals increases in black carbon contents in deeper soils. In deeper soils the difference is mainly explained by the fact that a high frequency of burn treatments leads to an accumulation of black carbon. Low frequency burn treatments or no burn treatments will not lead to the accumulation black carbon. Most vegetation fires do not exceed temperatures that are needed for charring in deeper soils, but these soils still contain significant amounts of black carbon. The black carbon in deeper soils most likely originates from the combustion of above ground vegetation (and litter) that has accumulated through processes such as bioturbation (Santín et al., 2016). The accumulation of black carbon in these soils is most likely also reinforced by climate conditions in the area. Wet seasons allow black carbon to become much more mobile due to increased water contents in soils.

This research could have been limited by the loss of samples during the fractionation procedure. Average soil recovery rates (98%) were even larger than the acceptable ranges (85-90%), therefore the fractionation did not limit this research (Viret & Grand, 2019). Future studies could continue and expand this research by including more treatments and depths. The only treatments studied for this research were spring burns that occur just before the growing season. The burning season significantly influences the growth of vegetation (Trollope, 2009) and could therefore also influence the carbon sequestration and carbon distribution in soils. This research focused on two different soil depths: 0-5 cm and 5-15 cm. Normally, a 15-30 cm depth would also be included, but time constraints made this impossible. Even deeper soils are not often included. Ward

et al. (2016), did however find considerable carbon stocks in soils at depths of 30 cm to 100 cm. Based on different treatments, the amount of carbon varied significantly, indicating the potential to research these soil depths.

Carbon sequestration is currently considered as a climate mitigation option (Veldman et al., 2015; Lavallee et al., 2019; Viret & Grand, 2019). Since the potential of soils to store carbon increases when the carbon is more recalcitrant, influencing the carbon sequestration process through treatments that increase long-term carbon storage has lots of potential (Lavallee et al., 2019; Rumpel et al., 2020). This study indicates that fire in the region studied contributes to the formation of recalcitrant carbon, thus increasing the carbon storage potential. Hopefully these findings will be used to further the insight that fire regimes can have a positive effect on carbon sinks in grasslands and that they may contribute to the conservation and understanding of these unique ancient ecosystems.

## **Conclusion**

This study shows that burning treatments not only affect the total amount of carbon stored within soils, but also affect the distribution of carbon in the different carbon fractions. In South African grasslands, frequent fires are beneficial for the carbon sequestration process since they increase the total amount of soil carbon stored and redistribute the carbon into different carbon pools. In these grasslands the enhancement of the carbon's chemical stability, especially in deeper soils, will increase long term carbon storage. The biochemical stability also contributes to the long-term carbon storage, since fires result in the formation of highly recalcitrant black carbon. Therefore, adequate fire regimes can be a driver in climate mitigation by creating a larger stable soil carbon pool in South African grasslands.



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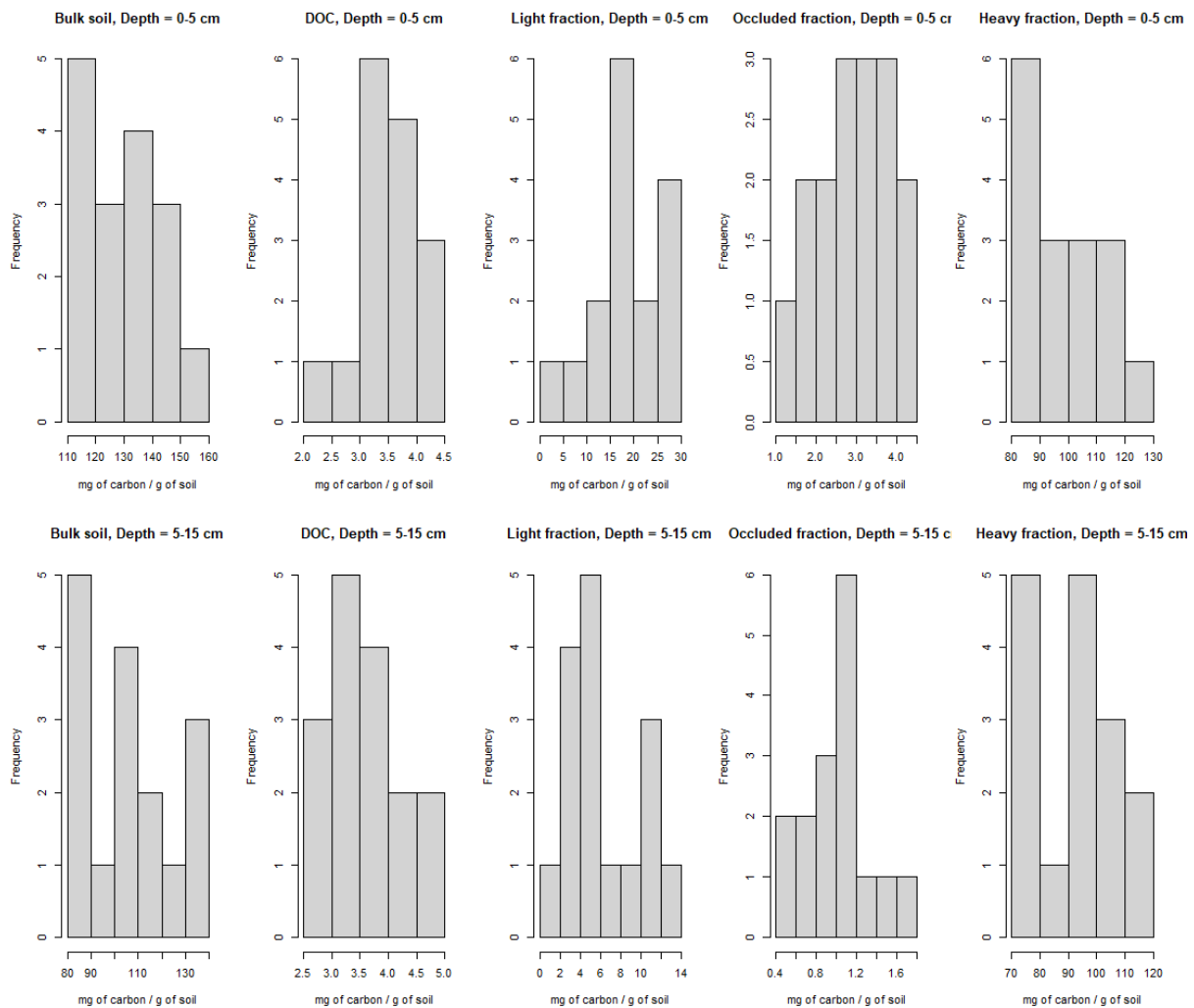
# Appendix

## 1. Distribution of data

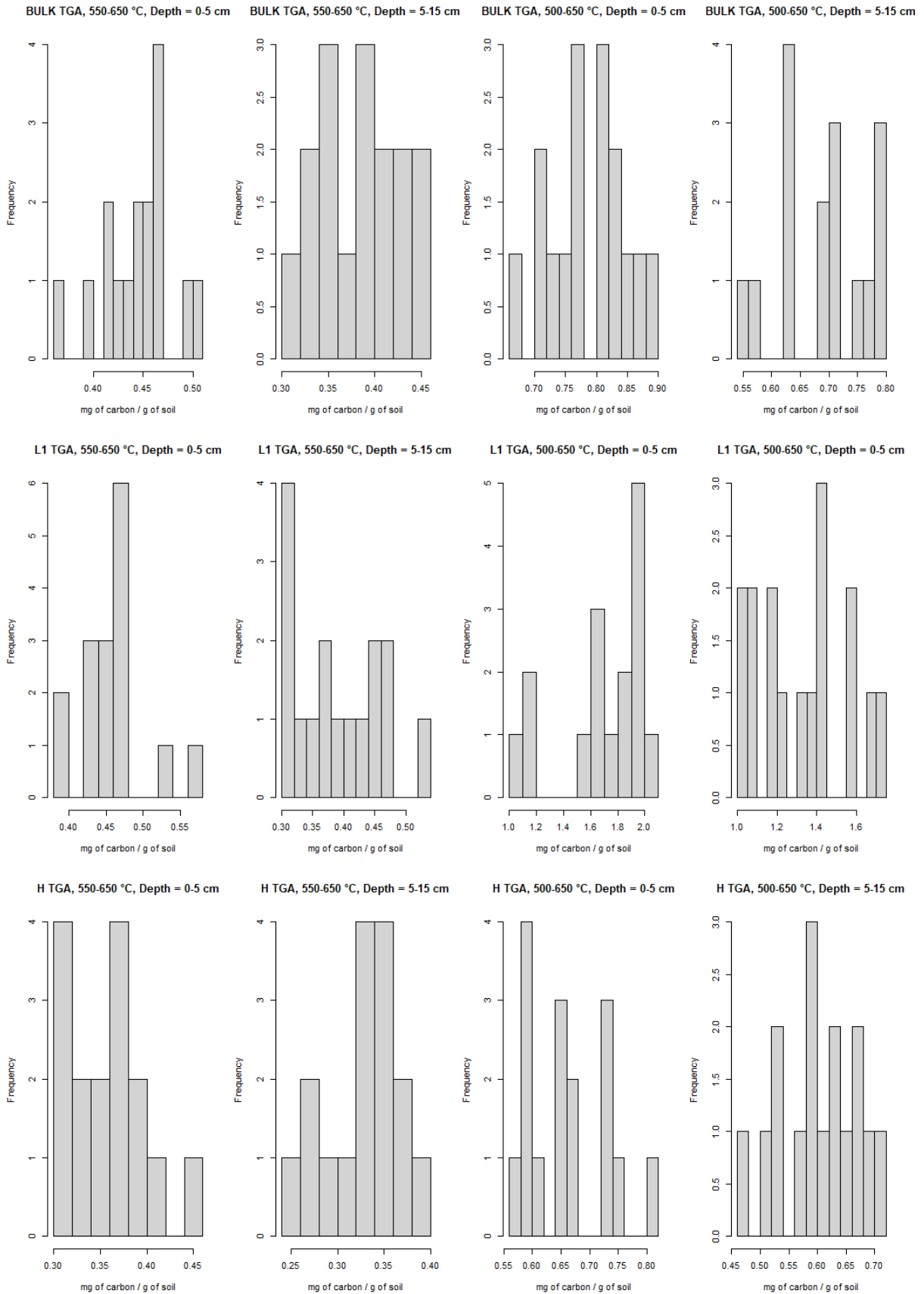
All the samples are normally distributed within their range, except for SCTL<sub>1</sub> T<sub>2</sub>.

**Table 5.** Results Shapiro-Wilk test. Abbreviations: SC (Soil Carbon), DOC (Dissolved Organic Carbon), SCTB (Soil Carbon TGA Bulk), SCTL (Soil Carbon TGA L<sub>1</sub>), SCTL<sub>1</sub> (Soil Carbon TGA L<sub>1</sub> Heavy fraction). Normally distributed if  $p = > 0.05$ .

Depth	SC Bulk	DOC	SC L <sub>1</sub>	SC L <sub>2</sub>	SC H	SCTB T <sub>1</sub>	SCTB T <sub>2</sub>	SCTL <sub>1</sub> T <sub>1</sub>	SCTL <sub>1</sub> T <sub>2</sub>	SCTH T <sub>1</sub>	SCTH T <sub>2</sub>
Depth 0-5											
P-value	0.19	0.92	0.61	0.40	0.14	0.79	0.97	0.12	<b>0.0085</b>	0.33	0.17
Normal?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
Depth 5-15											
P-value	0.066	0.38	0.072	0.77	0.08	0.86	0.31	0.34	0.30	0.59	0.88
Normal?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes



**Figure 9.** Distribution of total carbon content in different fractions, measured for two depths.



**Figure 10.** Distribution of black carbon content in different fractions. Measured for two depths and two temperature ranges.

## 2. R-script

```
## SCRIPT MASTER THESIS VICTOR TROUW, 6312918, SUSD-ECE, UTRECHT UNIVERSITY ####
## THE IMPACT OF FIRE ON SOIL CARBON SEQUESTRATION IN SOUTH AFRICAN GRASSLANDS #
##### STEP 1 - SET UP #####

### STEP 1a: Clear previous environment, plots and console ###
rm(list = ls())      # Clear environment
dev.off()           # Clear previous plots
cat("\014")         # Clear Console

### Step 1b: Set up work directory ###
setwd("C:/00 DATA/Documents/R/Masterthesiscarbon")

### STEP 1c: Download required packages ###
install.packages("AICcmodavg") # Statistical diagnostics
install.packages("emmeans")   # Estimated marginal means for linear model
install.packages("dplyr")     # Data set manipulation
install.packages("nlme")      # Linear and non linear mixed effects models
install.packages("rlang")     # Reading excel files
install.packages("readxl")    # Reading excel files
install.packages("tidyverse") # General data analysis
install.packages("ggplot2")   # Data visualization

### STEP 1d: Set required library's ###
library(readxl)
library(emmeans)
library(dplyr)
library(nlme)
library(rlang)
library(ggplot2)
library(tidyverse)

### STEP 1e: Load data set ###
DF <- read.csv(file = "00 Complete final data sheet, master thesis Victor
Trouw.csv", header=TRUE, sep=";", dec = ",")
DFD05 <- DF[1:16,]          # Dataframe Depth 0-5
DFD515 <- DF[17:32,]       # Dataframe Depth 5-15
View(DF)
summary(DF)

##### STEP 2 - DESCRIPTIVE STATISTICS #####

### Step 2a: Checking distribution and normality of data, normal if p > 0.05 ###
shapiro.test(DFD05$SCBulK1)
shapiro.test(DFD515$SCBulK1)
shapiro.test(DFD05$DOC)
shapiro.test(DFD515$DOC)
shapiro.test(DFD05$SCL1g)
shapiro.test(DFD515$SCL1g)
shapiro.test(DFD05$SCL2g)
shapiro.test(DFD515$SCL2g)
shapiro.test(DFD05$SCHg)
shapiro.test(DFD515$SCHg)

shapiro.test(DFD05$SCTBT1mg)
shapiro.test(DFD515$SCTBT1mg)
shapiro.test(DFD05$SCTBT2mg)
shapiro.test(DFD515$SCTBT2mg)
shapiro.test(DFD05$SCTL1T1mg)
shapiro.test(DFD515$SCTL1T1mg)
shapiro.test(DFD05$SCTL1T2mg)
shapiro.test(DFD515$SCTL1T2mg)
shapiro.test(DFD05$SCTHT1mg)
shapiro.test(DFD515$SCTHT1mg)
shapiro.test(DFD05$SCTHT2mg)
shapiro.test(DFD515$SCTHT2mg)
```

```

### Step 2b: Visualisation of distributions ###
# Setting figure size, 2 rows, 5 colloms
par(mfrow=c(2,5))
# HISTOGRAMS DENSITY CARBON VALUES #
hist(DFD05$SCBulk1, freq = TRUE, main = "Bulk soil, Depth = 0-5 cm",
     xlab = "mg of carbon / g of soil", ylab = "Frequency")
hist(DFD05$DOC, freq = TRUE, main = "DOC, Depth = 0-5 cm",
     xlab = "mg of carbon / g of soil", ylab = "Frequency", breaks = 5)
hist(DFD05$SCL1g, freq = TRUE, main = "Light fraction, Depth = 0-5 cm",
     xlab = "mg of carbon / g of soil", ylab = "Frequency")
hist(DFD05$SCL2g, freq = TRUE, main = "Occluded fraction, Depth = 0-5 cm",
     xlab = "mg of carbon / g of soil", ylab = "Frequency")
hist(DFD05$SCHg, freq = TRUE, main = "Heavy fraction, Depth = 0-5 cm",
     xlab = "mg of carbon / g of soil", ylab = "Frequency")
hist(DFD515$SCBulk1, freq = TRUE, main = "Bulk soil, Depth = 5-15 cm",
     xlab = "mg of carbon / g of soil", ylab = "Frequency")
hist(DFD515$DOC, freq = TRUE, main = "DOC, Depth = 5-15 cm",
     xlab = "mg of carbon / g of soil", ylab = "Frequency", breaks = 8)
hist(DFD515$SCL1g, freq = TRUE, main = "Light fraction, Depth = 5-15 cm",
     xlab = "mg of carbon / g of soil", ylab = "Frequency")
hist(DFD515$SCL2g, freq = TRUE, main = "Occluded fraction, Depth = 5-15 cm",
     xlab = "mg of carbon / g of soil", ylab = "Frequency")
hist(DFD515$SCHg, freq = TRUE, main = "Heavy fraction, Depth = 5-15 cm",
     xlab = "mg of carbon / g of soil", ylab = "Frequency")

# Setting figure size, 2 rows, 6 colloms
par(mfrow=c(3,4))

# HISTOGRAMS TGA CARBON VALUES #
hist(DFD05$SCTBT1mg, freq = TRUE, breaks = 10, main = "BULK TGA, 550-650 °C, Depth = 0-5cm", xlab = "mg of carbon / g of soil", ylab = "Frequency")
hist(DFD515$SCTBT1mg, freq = TRUE, breaks = 10, main = "BULK TGA, 550-650 °C, Depth = 5-15cm", xlab = "mg of carbon / g of soil", ylab = "Frequency")
hist(DFD05$SCTBT2mg, freq = TRUE, breaks = 10, main = "BULK TGA, 500-650 °C, Depth = 0-5cm", xlab = "mg of carbon / g of soil", ylab = "Frequency")
hist(DFD515$SCTBT2mg, freq = TRUE, breaks = 10, main = "BULK TGA, 500-650 °C, Depth = 5-15cm", xlab = "mg of carbon / g of soil", ylab = "Frequency")
hist(DFD05$SCTL1T1mg, freq = TRUE, breaks = 10, main = "L1 TGA, 550-650 °C, Depth = 0-5 cm", xlab = "mg of carbon / g of soil", ylab = "Frequency")
hist(DFD515$SCTL1T1mg, freq = TRUE, breaks = 10, main = "L1 TGA, 550-650 °C, Depth = 5-15cm", xlab = "mg of carbon / g of soil", ylab = "Frequency")
hist(DFD05$SCTL1T2mg, freq = TRUE, breaks = 10, main = "L1 TGA, 500-650 °C, Depth = 0-5cm", xlab = "mg of carbon / g of soil", ylab = "Frequency")
hist(DFD515$SCTL1T2mg, freq = TRUE, breaks = 10, main = "L1 TGA, 500-650 °C, Depth = 0-5cm", xlab = "mg of carbon / g of soil", ylab = "Frequency")
hist(DFD05$SCTHT1mg, freq = TRUE, breaks = 10, main = "H TGA, 550-650 °C, Depth = 0-5cm", xlab = "mg of carbon / g of soil", ylab = "Frequency")
hist(DFD515$SCTHT1mg, freq = TRUE, breaks = 10, main = "H TGA, 550-650 °C, Depth = 5-15cm", xlab = "mg of carbon / g of soil", ylab = "Frequency")
hist(DFD05$SCTHT2mg, freq = TRUE, breaks = 10, main = "H TGA, 500-650 °C, Depth = 0-5cm", xlab = "mg of carbon / g of soil", ylab = "Frequency")
hist(DFD515$SCTHT2mg, freq = TRUE, breaks = 10, main = "H TGA, 500-650 °C, Depth = 5-15cm", xlab = "mg of carbon / g of soil", ylab = "Frequency")

# Restore figure settings #
par(mfrow=c(1,1))

##### STEP 3 - ANALYZING STATISTICS #####

### Step 3a: Constructing linear models for Density frac. ###
M.CBulk_0_5 <- lm(SCBulk1 ~ Treatment, data = DFD05)
M.CBulk_5_15 <- lm(SCBulk1 ~ Treatment, data = DFD515)
M.CDOC_0_5 <- lm(DOC ~ Treatment, data = DFD05)
M.CDOC_5_15 <- lm(DOC ~ Treatment, data = DFD515)
M.CL1_0_5 <- lm(SCL1g ~ Treatment, data = DFD05)
M.CL1_5_15 <- lm(SCL1g ~ Treatment, data = DFD515)
M.CL2_0_5 <- lm(SCL2g ~ Treatment, data = DFD05)
M.CL2_5_15 <- lm(SCL2g ~ Treatment, data = DFD515)
M.CH_0_5 <- lm(SCHg ~ Treatment, data = DFD05)
M.CH_5_15 <- lm(SCHg ~ Treatment, data = DFD515)

anova(M.CBulk_0_5)
anova(M.CBulk_5_15)
anova(M.CDOC_0_5)
anova(M.CDOC_5_15)
anova(M.CL1_0_5)
anova(M.CL1_5_15)

```



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anova(M.CL2_0_5)
anova(M.CL2_5_15)
anova(M.CH_0_5)
anova(M.CH_5_15)

### Step 3b: Post hoc tests ###
emmM.CBulk_0_5 <- emmeans(M.CBulk_0_5, ~ Treatment)
pairs(emmM.CBulk_0_5)
emmM.CBulk_5_15 <- emmeans(M.CBulk_5_15, ~ Treatment)
pairs(emmM.CBulk_5_15)
emmM.CDOC_0_5 <- emmeans(M.CDOC_0_5, ~ Treatment)
pairs(emmM.CDOC_0_5)
emmM.CDOC_5_15 <- emmeans(M.CDOC_5_15, ~ Treatment)
pairs(emmM.CDOC_5_15)
emmM.CL1_0_5 <- emmeans(M.CL1_0_5, ~ Treatment)
pairs(emmM.CL1_0_5)
emmM.CL1_5_15 <- emmeans(M.CL1_5_15, ~ Treatment)
pairs(emmM.CL1_5_15)
emmM.CL2_0_5 <- emmeans(M.CL2_0_5, ~ Treatment)
pairs(emmM.CL2_0_5)
emmM.CL2_5_15 <- emmeans(M.CL2_5_15, ~ Treatment)
pairs(emmM.CL2_5_15)
emmM.CH_0_5 <- emmeans(M.CH_0_5, ~ Treatment)
pairs(emmM.CH_0_5)
emmM.CH_5_15 <- emmeans(M.CH_5_15, ~ Treatment)
pairs(emmM.CH_5_15)

### Step 3c: Visualization ###
par(mfrow=c(5,2))

boxplot(SCBulk1 ~ Treatment, data = DFD05, main = "BULK soil, Depth 0-5cm",
        xlab = "Treatment", ylab = "mg of carbon / g of soil",
        cex.lab=1.3, cex.main = 1.5, cex.axis=1.5)
boxplot(SCBulk1 ~ Treatment, data = DFD515, main = "BULK soil, Depth 5-15cm",
        xlab = "Treatment", ylab = "mg of carbon / g of soil",
        cex.lab=1.3, cex.main = 1.5, cex.axis=1.5)
boxplot(DOC ~ Treatment, data = DFD05, main = "DOC, Depth 0-5cm",
        xlab = "Treatment", ylab = "mg of carbon / g of soil",
        cex.lab=1.3, cex.main = 1.5, cex.axis=1.5)
boxplot(DOC ~ Treatment, data = DFD515, main = "DOC, Depth 5-15cm",
        xlab = "Treatment", ylab = "mg of carbon / g of soil",
        cex.lab=1.3, cex.main = 1.5, cex.axis=1.5)
boxplot(SCL1g ~ Treatment, data = DFD05, main = "Light fraction (L1),Depth 0-5cm",
        xlab = "Treatment", ylab = "mg of carbon / g of soil",
        cex.lab=1.3, cex.main = 1.5, cex.axis=1.5)
boxplot(SCL1g ~ Treatment, data = DFD515, main = "Light fraction (L1),Depth 5-15cm",
        xlab = "Treatment", ylab = "mg of carbon / g of soil",
        cex.lab=1.3, cex.main = 1.5, cex.axis=1.5)
boxplot(SCL2g ~ Treatment, data = DFD05, main = "Occluded fraction (L2),Depth 0-5cm",
        xlab = "Treatment", ylab = "mg of carbon / g of soil",
        cex.lab=1.3, cex.main = 1.5, cex.axis=1.5)
boxplot(SCL2g ~ Treatment, data = DFD515, main = "Occluded fraction(L2),Depth 5-15cm",
        xlab = "Treatment", ylab = "mg of carbon / g of soil",
        cex.lab=1.3, cex.main = 1.5, cex.axis=1.5)
boxplot(SCHg ~ Treatment, data = DFD05, main = "Heavy fraction (H), Depth 0-5cm",
        xlab = "Treatment", ylab = "mg of carbon / g of soil",
        cex.lab=1.3, cex.main = 1.5, cex.axis=1.5)
boxplot(SCHg ~ Treatment, data = DFD515, main = "Heavy fraction (H), Depth 5-15cm",
        xlab = "Treatment", ylab = "mg of carbon / g of soil",
        cex.lab=1.3, cex.main = 1.5, cex.axis=1.5)

### Step 3d: Constructing linear models for TGA ###
M.CTGABulkT1_0_5 <- lm(SCTBT1mg ~ Treatment, data = DFD05)
M.CTGABulkT1_5_15 <- lm(SCTBT1mg ~ Treatment, data = DFD515)
M.CTGAL1T1_0_5 <- lm(SCTL1T1mg ~ Treatment, data = DFD05)
M.CTGAL1T1_5_15 <- lm(SCTL1T1mg ~ Treatment, data = DFD515)
M.CTGAHT1_0_5 <- lm(SCTHT1mg ~ Treatment, data = DFD05)
M.CTGAHT1_5_15 <- lm(SCTHT1mg ~ Treatment, data = DFD515)
M.CTGABulkT2_0_5 <- lm(SCTBT2mg ~ Treatment, data = DFD05)
M.CTGABulkT2_5_15 <- lm(SCTBT2mg ~ Treatment, data = DFD515)
M.CTGAL1T2_0_5 <- lm(SCTL1T2mg ~ Treatment, data = DFD05)
M.CTGAL1T2_5_15 <- lm(SCTL1T2mg ~ Treatment, data = DFD515)
M.CTGAHT2_0_5 <- lm(SCTHT2mg ~ Treatment, data = DFD05)
M.CTGAHT2_5_15 <- lm(SCTHT2mg ~ Treatment, data = DFD515)

```

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anova(M.CTGABu1kT1_0_5)
anova(M.CTGABu1kT1_5_15)
anova(M.CTGAL1T1_0_5)
anova(M.CTGAL1T1_5_15)
anova(M.CTGAHT1_0_5)
anova(M.CTGAHT1_5_15)
anova(M.CTGABu1kT2_0_5)
anova(M.CTGABu1kT2_5_15)
anova(M.CTGAL1T2_0_5)
anova(M.CTGAL1T2_5_15)
anova(M.CTGAHT2_0_5)
anova(M.CTGAHT2_5_15)

### Step 3e: Post hoc tests ##
emmM.CTGABu1kT1_0_5 <- emmeans(M.CTGABu1kT1_0_5, ~ Treatment)
pairs(emmM.CTGABu1kT1_0_5)
emmM.CTGABu1kT1_5_15 <- emmeans(M.CTGABu1kT1_5_15, ~ Treatment)
pairs(emmM.CTGABu1kT1_5_15)
emmM.CTGAL1T1_0_5 <- emmeans(M.CTGAL1T1_0_5, ~ Treatment)
pairs(emmM.CTGAL1T1_0_5)
emmM.CTGAL1T1_5_15 <- emmeans(M.CTGAL1T1_5_15, ~ Treatment)
pairs(emmM.CTGAL1T1_5_15)
emmM.CTGAHT1_0_5 <- emmeans(M.CTGAHT1_0_5, ~ Treatment)
pairs(emmM.CTGAHT1_0_5)
emmM.CTGAHT1_5_15 <- emmeans(M.CTGAHT1_5_15, ~ Treatment)
pairs(emmM.CTGAHT1_5_15)
emmM.CTGABu1kT2_0_5 <- emmeans(M.CTGABu1kT2_0_5, ~ Treatment)
pairs(emmM.CTGABu1kT2_0_5)
emmM.CTGABu1kT2_5_15 <- emmeans(M.CTGABu1kT2_5_15, ~ Treatment)
pairs(emmM.CTGABu1kT2_5_15)
emmM.CTGAL1T2_0_5 <- emmeans(M.CTGAL1T2_0_5, ~ Treatment)
pairs(emmM.CTGAL1T2_0_5)
emmM.CTGAL1T2_5_15 <- emmeans(M.CTGAL1T2_5_15, ~ Treatment)
pairs(emmM.CTGAL1T2_5_15)
emmM.CTGAHT2_0_5 <- emmeans(M.CTGAHT2_0_5, ~ Treatment)
pairs(emmM.CTGAHT2_0_5)
emmM.CTGAHT2_5_15 <- emmeans(M.CTGAHT2_5_15, ~ Treatment)
pairs(emmM.CTGAHT2_5_15)

### STEP 3f: Visualisation ###
par(mfrow=c(2,3))

boxplot(SCTBT1mg ~ Treatment, data = DFD05,
        main = "TGA Bulk soil, Depth 0-5cm, T1: 550-650 °C",
        xlab = "Treatment", ylab = "mg of carbon / g of soil",
        cex.lab=1.5, cex.main = 1.3, cex.axis=1.5)
boxplot(SCTL1T1mg ~ Treatment, data = DFD05,
        main = "TGA Light fraction, Depth 0-5cm, T1: 550-650 °C",
        xlab = "Treatment", ylab = "mg of carbon / g of soil",
        cex.lab=1.5, cex.main = 1.3, cex.axis=1.5)
boxplot(SCTHT1mg ~ Treatment, data = DFD05,
        main = "TGA Heavy fraction, Depth 0-5cm, T1: 550-650 °C",
        xlab = "Treatment", ylab = "mg of carbon / g of soil",
        cex.lab=1.5, cex.main = 1.3, cex.axis=1.5)
boxplot(SCTBT2mg ~ Treatment, data = DFD05,
        main = "TGA Bulk soil, Depth 0-5cm, T2: 500-650 °C",
        xlab = "Treatment", ylab = "mg of carbon / g of soil",
        cex.lab=1.5, cex.main = 1.3, cex.axis=1.5)
boxplot(SCTL1T2mg ~ Treatment, data = DFD05,
        main = "TGA Light fraction, Depth 0-5cm, T2: 500-650 °C",
        xlab = "Treatment", ylab = "mg of carbon / g of soil",
        cex.lab=1.5, cex.main = 1.3, cex.axis=1.5)
boxplot(SCTHT2mg ~ Treatment, data = DFD05,
        main = "TGA Heavy fraction, Depth 0-5cm, T2: 500-650 °C",
        xlab = "Treatment", ylab = "mg of carbon / g of soil",
        cex.lab=1.5, cex.main = 1.3, cex.axis=1.5)
boxplot(SCTL1T1mg ~ Treatment, data = DFD515,
        main = "TGA Bulk soil, Depth 5-15cm, T1: 550-650 °C",
        xlab = "Treatment", ylab = "mg of carbon / g of soil",
        cex.lab=1.5, cex.main = 1.3, cex.axis=1.5)
boxplot(SCTBT1mg ~ Treatment, data = DFD515,
        main = "TGA Light fraction, Depth 5-15cm, T1: 550-650 °C",
        xlab = "Treatment", ylab = "mg of carbon / g of soil",
        cex.lab=1.5, cex.main = 1.3, cex.axis=1.5)
boxplot(SCTHT1mg ~ Treatment, data = DFD515,
        main = "TGA Heavy fraction, Depth 5-15cm, T1: 550-650 °C",
        xlab = "Treatment", ylab = "mg of carbon / g of soil",

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      cex.lab=1.5, cex.main = 1.3, cex.axis=1.5)
boxplot(SCTBT2mg ~ Treatment, data = DFD515,
      main = "TGA Bulk soil, Depth 5-15cm, T2: 500-650 °C",
      xlab = "Treatment", ylab = "mg of carbon / g of soil",
      cex.lab=1.5, cex.main = 1.3, cex.axis=1.5)
boxplot(SCTL1T2mg ~ Treatment, data = DFD515,
      main = "TGA Light fraction, Depth 5-15cm, T2: 500-650 °C",
      xlab = "Treatment", ylab = "mg of carbon / g of soil",
      cex.lab=1.5, cex.main = 1.3, cex.axis=1.5)
boxplot(SCTHT2mg ~ Treatment, data = DFD515,
      main = "TGA Heavy fraction, Depth 5-15cm, T2: 500-650 °C",
      xlab = "Treatment", ylab = "mg of carbon / g of soil",
      cex.lab=1.5, cex.main = 1.3, cex.axis=1.5)

### FIN ###
```