The use of alternative electron acceptors in microbial peat decomposition

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Abstract

Peat drainage, performed to create usable land, accelerates microbial decomposition of OM, leading to an increase in CO_2 emissions from peat as well as land subsidence. While most studies focus on oxic decomposition, anoxic decomposition happening under the water-table is often not considered, leading to a potential underestimation of emission and subsidence rates. Recent studies proposed an "Iron gate theory" model in contrast to the traditional "Enzymic Latch" to explain peat loss regulatory processes. In this study, addition of iron and sulfate on peat originating from two anoxic peat sites in the Netherlands (Assendelft and Rouveen) was performed to evaluate if alternative electron acceptors other than oxygen could be used to decompose peat anoxically. Potential phenol oxidase activity in 15min was measured anoxically via L-DOPA assays. CO_2 emission rates from peat in a 48h time period were measured via a respirometer in anoxic conditions. Both basal respiration (BR) and substrate-induced respiration (SIR) rates were measured. The study highlighted an unknown interaction between L-DOPA and ferric iron, which is currently undocumented in soil enzymology literature. This interaction is probable to be causing biases in existing literature on POX activity estimates and peat loss models. CO_2 emission rates significantly decreased ($n=4$, df 3; $t= 3.6865$; $p = 0.0346$) with iron addition in Assendelft when a carbon donor (lactate) was added. Sulfate addition in Assendelft instead increased substrate-induced respiration rates. BR and SIR in Rouveen were not affected by addition of both iron and sulfate. Results suggest that sulfate may be used as a TEA in anoxic peat and interactions between iron and sulfate are regulating peat decomposition in anoxic environments.

Layman Summary

Peat is a type of slowly decomposing soil that is commonly drained for pastures and agriculture. This drainage causes the soil to release more carbon into the atmosphere and progressively sink lower into the ground. Most previous studies only looked at how peat decomposes when exposed to oxygen, without considering what happens under the water table. This research looked at phenol oxidase (POX), the main class of enzymes responsible for peat loss, as well as CO_2 emissions from peat soil. It was found that, when enough C to decompose is present, adding iron decreases emissions, while adding sulfate increases emissions. However, if not enough C is available, there is no effect on emissions by adding iron or sulfate. The research also found an interaction between ferric iron and L-DOPA, the compound used to measure POX. This interaction is not reported in enzymology literature and is likely to cause a bias in several different reports on POX activity and peat loss. Results from this research could help farmers and land managers better understand how to manage peat soil to reduce carbon emissions and prevent the land from sinking. Additionally, the research shows important new insight on how peat loss is regulated when oxygen is not available.

Introduction

Peat loss: an urgent challenge

Peatlands are a type of wetland ecosystem that covers over 3% of the Earth's surface (Kopansky, 2019). They are characterized by a layer of soft soil (peat) composed of slowly decomposing organic matter (OM) from plant origin. This slow decomposition, in comparison to the net primary production (NPP) rates in peatlands, is due to the presence of low oxygen levels under the water-table, complex organic compounds such as lignin, cellulose and hemicellulose which are difficult to degrade, low temperature and, in some cases, acidic conditions in the soil (Rydin et al., 2013). These factors limit the activity of microorganisms that degrade OM, resulting in the accumulation of partially decomposed plant material over time (Rydin et al., 2013). Because of the slow decomposition of OM, it is estimated that natural peatlands can store over 44% of global soil carbon and sequester over 0.37 gigatons of CO_2 from the atmosphere a year, thus acting as the largest carbon sink on land (IUCN, 2021). However, over the latest centuries, peatlands have been heavily exploited for both mining and agricultural purpose. In the Netherlands, peatlands currently cover 274,000 hectares (6-8% of the landscape) (Poppe et al., 2021), with most of the peat soil extensively being mostly used for dairy-farming pastures, urban expansion and in some cases for crop production.

Peat is often drained to create available land for anthropogenic activities, but it causes significant environmental problems. Drained peat soils become exposed to oxygen, which is a known trigger for decomposition of OM and leads to a decrease in soil carbon storage and a consequent increase in CO_2 emissions (Freeman et al, 2001). Moreover, peatland drainage also causes land subsidence, which can lead to increased risk of flooding, infrastructure damage and loss of available land (Galloway et al., 2016).

To address these environmental issues, water table restoration practices, such as rewetting, are progressively being promoted to protect peatlands (Meissner et al.,2008). However, previous studies have shown how post-draining rewetting practices do not restore peatlands to their natural state. A large study on European peatlands indicates that rewetted fen peatlands differ in biodiversity and ecosystem functioning from near-natural peatlands, especially regarding plant community composition and geochemical cycling of nutrients (Kreyling et al., 2021). Additionally, both draining and rewetting can create problems to peat stability (Kreyling et al., 2021; Meissner et al.,2008), as frequent fluctuations in the water table significantly alter the soil hydrology and biochemistry (Van Diggelen et al., 2020; Rydin et al., 2013). For instance, the distribution of soil microorganism dependent on accessibility to carbon and sensitive to changes in the surrounding environment, is highly affected by changes in the water table (Rydin et al., 2013). Methane (CH_4) emission rates from peat as well as water eutrophication have also been observed to increase during ongoing draining and rewetting (Van Diggelen et al., 2020). Also, water table fluctuations can lead to oxygen infiltration in the deep soil, ultimately affecting microbial metabolism and increasing the activity of enzymes that are directly linked to peat decomposition (Burns et. al, 2013; Rydin et al., 2013).

Current trends of climate change also pose a significant threat to Dutch peatlands (Van Diggelen et al., 2020). Recent estimates suggest that summer droughts will become more frequent in the Netherlands in the near future (Spinoni et. al, 2018). With increased drought periods, saturated peat is likely to become to exposed to oxygen due to decline in the water table, resulting in higher GHGs emissions and subsidence rates. Moreover, increased CO_2 emissions from peat are expected to generate a positive-feedback with the already worsening changes in the climate, ultimately accelerating the peat decomposition process (Rydin et al., 2013). Similarly, due to increased subsidence in drained peatlands, global rising sea level are also expected to increase flooding risks in coastal peatlands (Van Diggelen et al., 2020). Therefore, it is crucial to understand the mechanisms affecting peat decomposition in both oxic and anoxic conditions to accurately estimate peat loss rates and GHGs emissions from peat, as well as ensure that anthropogenic activities and upcoming changes in the climate do not compromise the important ecosystem services of peatlands.

OM decomposition and peat loss

While the exact biogeochemical pathways leading to peat degradation are still unclear, microbial decomposition of organic matter (OM) is known to be the major cause of peat loss (de Jong et al., 2020, Rydin and Bennett, 2013). Fungi, bacteria and archaea degrade OM for their respiration, releasing CO_2, methane (CH_4) and nitrous oxide (N_2O) and as byproducts of their metabolism. In the Netherlands, recent emission models estimate that 4.0 to 8.0 Mton of CO_2 are released ever year from Dutch peatlands (Erkens et al., 2016), meaning that 3% of the total yearly CO_2 emissions in the Netherlands are due to peat loss (Boonman et al., 2022). Oxic decomposition rates of peat soils are often more than 10 to 30 times higher than anoxic decomposition rates (Scanlon and Moore, 2000). Therefore, the focus on most studies and emission models on peatlands has been on greenhouse gases emissions from the unsaturated zone, neglecting the contribution of decomposition processes happening in anoxic, waterlogged conditions. Although oxic emissions are higher, most of peat exists in saturated conditions under the water table. Peat layers thickness can reach up to 8m underground (Rydin et al., 2013). However, peat drainage can reach up to 1-1.5m depths (Van Diggelen et al., 2020), meaning that a large amount of water-saturated peat where anoxic decomposition is occurring is also present and often not consider in emission models. Additionally, frequent drainage increases oxygenation of the saturated peat layers (de Jong et al, 2020), increasing overall emissions. Therefore, focusing on oxic process could lead to an underestimation of peat loss rates since it is only considering less than 30% of the overall peat profile.

Phenol oxidase and the enzymic latch theory

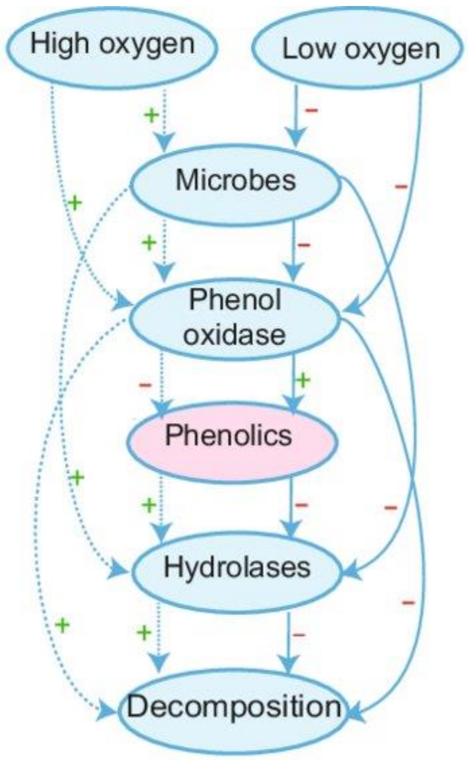


Figure 1: An overview of the enzymic latch mechanisms retrieved from Saraswati et. al (2016). Green arrows **(+)** show positive relationships, red arrows (-) show negative relationships.

The decomposition process is facilitated, or driven by, the microbial secretion of hydrolytic and oxidative exoenzymes into the peat matrix (Freeman et. al, 2001; Rydin & Bennett, 2013).
Exoenzymes production has been observed in both terrestrial and marine biomes (Kamalanathan et al., 2020) and is highly linked to biogeochemical cycles such as turnover of carbon, phosphorus and nitrogen (Burns et al., 2013). In the context of peatlands, microbial decomposition of OM happens via the action of two classes of exoenzymes that are secreted into the peat matrix by the microbial community: hydrolytic and oxidative exoenzymes (Burns et al., 2013; ; Rydin & Bennett, 2013). Hydrolases are directly responsible for degrading OM into easily engulfables particles that the microbes can absorb. Depending on the type of hydrolase, different molecules are targeted and degraded. For instance, β-glucosidase, proteases and lipases are respectively responsible for the hydrolysis of glucose polysaccharides (e.g. cellulose), peptide bonds in proteins and ester bonds lipids

and fatty acids in OM (Shukla et al., 2022). In addition, many of hydrolases have been shown to decompose OM under both oxic and anoxic conditions (Ortega et al., 2021).

In contrast to easily degradable particles, larger organic compounds such as lignin and phenolics, are first broken down by the action of oxidases before being targeted by the hydrolases (Sinsabaugh, 2010). Oxidases catalyze the oxidative decomposition of larger compounds by transferring electrons to, generally, oxygen molecules (Duran et al., 2000). In the case of peatlands, phenol oxidases (POX) have been shown to be essential in the decomposition process in peatlands as they are able to degrade phenolic compounds (Rydin & Bennett, 2013), secondary metabolites found in plants which can strongly inhibit the activity of hydrolases (Freeman et al., 2001). This inhibition can occur through several mechanisms, including direct toxicity to microbes, binding to active site of the hydrolases, or formation of phenolics-nutrients complexes which leads to depriving microbes of essential resources for survival.

Due to the indirect inhibiting role of phenolics on the activity of hydrolases, POX activity is considered to be the main indicator of on-going decomposition in peat, as POX activity and hydrolases activity have been shown to be positively linked (Figure 1). According to this model developed by Freeman (2001), when oxygen is present, oxidation of phenolics via POX activity increases activity of hydrolases, resulting in higher rates of peat loss. In the study, POX activity increased 7-fold in oxic conditions, and it was concluded that the inhibitory activity of phenolics on hydrolases stops OM decomposition under anoxic conditions (Freeman et. al, 2001). Hence, one of central point of this "Enzymic Latch Theory" (Figure 1) states that POX require oxygen to function, contributing to the high focus in recent years on oxic peat decomposition processes. Additionally, the Enzymic Latch theory also proposes a continued degradation of anoxic peat via activity of hydrolases after short-time oxygenation (Freeman et al., 2001). This means that when phenolics are removed from the system, and when the water-table is raised after rewetting, there is still a risk of leftover OM decomposition. Over time, contrasting results on the Enzymic Latch model have been observed when looking at POX activity in both oxic and anoxic conditions, with some studies reporting no significant differences between the two (Urbanová and Hájek, 2021). For example, Coulas et al. (2021) indicate that with increasing phenolics, POX activity will also increase in oxic conditions, contradicting the Enzymic Latch theory. This could suggest that there are more variables affecting POX activity in addition to oxygen availability and variables such as temperature changes, pH, moisture content and DOC availability are among some of the options that are currently being evaluated as regulating factors for peat loss (Chen et al., 2020; de Jong et al., 2020; Wen et al., 2019).

Terminal electron acceptors (TEAs) in peat

OM decomposition is known to be an oxidative reaction where electrons are transferred from compounds with low electron affinity (OM) to high electron affinity (generally oxygen) (Rydin et al., 2013). Therefore, one hypothesis to explain discrepancies in literature proposes the presence of alternative terminal electron acceptors (TEAs) for the microbes in the deep anoxic layers of the soil. A terminal electron acceptor is defined as last element or compound to receive an electron in an electron transport chain. While oxygen (O_2) is the most favorable electron acceptor for biogeochemical reactions, elements such as nitrate (NO_3^-), ferric iron (Fe^{3+}), manganese (Mn^{2+}) and sulfate (SO_4^{2-}) may also be used as TEAs when oxygen is limited, or not available at all (Balk, 2007). This has been observed in several microbial species such as *E. coli* (Unden & Bongaerts, 1997), extremophile species (Balk, 2007; Martín-Rodríguez et al., 2021) as well as some protists (Glock et al., 2019). In the context of peat loss, this is important as anoxic pathways could have been overlooked by the strong focus on oxic process of previous studies. Considering that most of peat exists under saturated conditions, only focusing on oxic process could in fact result in an underestimation of overall decomposition rates in peatlands. Peat loss in anoxic conditions could be the result of alternative redox pathways and alternative TEAs being reduced by microbial oxidases to catalyze the oxidative breakdown of OM into CO_2 and water. In order to have a full picture of peat loss dynamics, it is therefore important to also consider microbe-OM matter interactions when oxygen is not available.

The iron gate theory

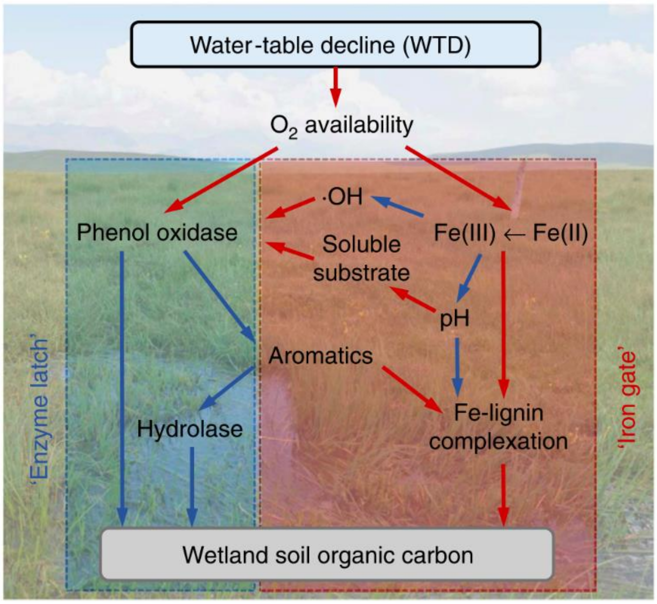


Figure 2: Visual Comparison of the enzymic latch theory and the iron gate hypothesis. Retried from Wang et al. (2017). Red arrows show positive relationships. Blue arrows show negative relationships.

One of the proposed theories that challenges the classic enzymic latch view, proposes that redox-induced transformation of iron in peat could act as a regulatory mechanism decreasing OM decomposition in peatlands.

This "*iron gate*" theory (Figure 2) states that, when oxygen enters the peat soil after drainage, ferrous iron (Fe^{2+}) is oxidized to ferric iron (Fe^{3+}) via biotic or abiotic processes. This reaction decreases POX activity due to decreasing hydroxyl (OH-) radicals in the peat matrix as well as by limiting available substrate for the enzymes by lowering soil pH (Wen et. al, 2019; Wang et al., 2017). Additionally, Wang et. al (2017) also observed that the increasing concentrations of phenolics and aromatic structures due to a lower POX activity stimulates C stabilization in the form of Lignin-Fe complexes and other C-rich compounds, showing how Fe oxidation could have protective role on peat derived OM during oxygenation events (Wen et. al, 2019). In other words, the theory proposes that iron oxidation acts as a gate against the enzymic latch during oxic conditions. However, it is still unclear if similar reactions involving the redox chemistry of iron are affecting OM decomposition either positively (as alternative TEA) or negatively (Fe-OC complexation & pH) when oxygen is absent. For these reasons, as well as contrasting results in follow-up studies, the iron gate model is also being debated among researchers. Wen et al. (2019) indicate that both the enzymic latch and iron gate mechanisms may be regulating POX activity and soil organic carbon mineralization in peat, with the dominant mechanism depending on trade-offs between O_2 and Fe^{2+} availability. Li et. al (2023) found no correlation between increasing Fe^{2+} concentrations and POX activity but also reported a significant effect of Fe oxides on SOC concentrations in non-waterlogged soils.

Iron and Sulfate in Dutch peatlands

Previous research has recorded the presence of both Fe^{2+} and Fe^{3+} in anoxic peat soil (Bhattacharyya et al., 2018), which is unusual as Fe^{3+} is commonly associated with oxic environments. In the Netherlands, due to saltwater intrusion in coastal peatlands, moderate and high levels of both iron and sulfate are progressively being recorded in several different peatlands in the country, particularly in polders in the provinces of North Holland (Zak et. al, 2010; Vermaat et al., 2016), South Holland (Smolders et al., 2006) and Utrecht (Vermaat et al., 2016). Moreover, iron fertilizers are also regularly applied on pastures and, less frequently, on agricultural crop field built on peat soil (Nieminen et al., 2011). The presence of sulfate especially causes a major problem in drained peatlands, as it has been observed to easily leech and enhance eutrophication of surrounding surface water. Therefore, it is important to understand how OM decomposition and POX activity are affected when oxygen is absent and high concentrations of iron and sulfate are present in the peat matrix. The biogeochemical cycles of iron and sulfur are highly interconnected. For examples, the high evapotranspiration during drought period, as well as peat drainage, increases aerobic oxidation of FeSx minerals (iron sulfides) ,which mobilizes Fe^{2+} and SO_4^{2-} in the unsaturated peat layers (Van Diggelen et al., 2020). While sulfate ions are easily transported out of the system by water flow, ferrous iron becomes oxidized and precipitates as poorly soluble ferric Fe oxides and hydroxides (FeOOH), leading to a large accumulation of Fe in the unsaturated zone (Van Diggelen et al., 2020). In contrast, when the water-table is raised, groundwater concentration of SO_4^{2-} tend to be low and Fe^{2+} concentration increase (Van Diggelen et al., 2020). Additionally, iron and sulfate ions mutually affect each other's geochemistry indirectly by interacting with phosphate (PO_4^{3-}) and other P compounds. (Kraal et al; Van Diggelen et al., 2020) For instance, it has been observed than the presence of hydrogen sulfide (H_2S), a resulting product of sulfate being used as an electron acceptor, can decouple Fe-P interactions in peat when the water table is lowered (Van Diggelen et al., 2020).

Aims of the research

The aim of this paper is to determine how availability of iron and sulfate affects peat decomposition under anoxic conditions. To answer this main research question, two sub questions were drafted, focusing respectively on the cause (increasing POX-activity) and consequence (CO_2 emission rates) of peat decomposition.

Q1: How does addition of different oxidation states of iron (Fe^{3+}/ Fe^{2+}/ mixture) in peat affect potential phenol oxidase (POX) activity under anoxic conditions?

Q2: How does addition of iron or sulfate affect basal respiration rate (in terms of CO_2 emission rates) in peat under anoxic conditions?

In this research, the potential phenol oxidase activity and basal respiration rate of the peat soil were chosen as the two indexes to evaluate the effects of TEAs on decomposition rates. Basal respiration (BR) is defined as the steady rate of respiration in soil on a predetermined timescale and is often considered to be a good indicator of potential on-going decomposition of OM in peat (Pell et al., 2006). For Q1, it is hypothesized that Fe^{3+} will act as an alternative electron acceptor (Qin et al, 2022), therefore stimulating decomposition of peat by increasing POX activity, while Fe^{2+} addition hypothesized that may inhibit POX and have a protecting role on peat (Wen et al, 2019). For Q2 it is hypothesized that addition of iron and sulfate will stimulate OM decomposition and result in higher anoxic basal respiration rates (Gosch et al., 2019; Wen et al., 2019).

Material and Methods

Overview of the research

The research project consisted of two main experiments. The first experiment studied the impact of iron addition and different iron oxidation states (Fe^{2+}/Fe^{3+}/Fe mixture) on potential POX activity in anoxic conditions. The second experiment focused on how addition of alternative electron acceptors (Fe^{3+} and SO_4^{2-}) in anoxic conditions affects microbial respiration rates due to decomposition of peat.

Sample collection, preparation and storage

<u>Peat collection</u>

Peat samples were collected from two research locations of the Nationaal Onderzoeksprogramma Broeikasgassen Veenweiden (Erkens et al., 2021): Assendelft, North-Holland (ASD), and Rouveen, Overijssel (ROU). Additional details and exact locations of the collection sites can be found in Table 1. These sites were considered as representative for the Dutch peatlands. The study areas have distinct geochemical properties, with Assendelft contain high concentrations of sulfate (1.0×10^4 μmol/l) and Rouveen presenting high concentrations of Fe^{2+} (150 μmol/l). At each location, five field peat samples were collected. In both study areas, peat was collected from the permanently water saturated layer (120-140cm depth in Assendelft and 140-160cm depth in Rouveen. An Edelman auger (Eijkelkap, Breda, the Netherlands) was used to remove the unsaturated surface clay layers. Peat was then collected via a wide gaughe (6cm) (Eijkelkap, Breda, the Netherlands). Once extracted, a 20cm peat section was cut and quickly packed into a sealed anoxic bag containing a gas-tight incubation bag (Anaerocult, A mini, Merck, the Netherlands). To remove any residual oxygen from the bag and prevent peat oxidation as much as possible. All samples were then stored in a cool box during transportation to the laboratory and then placed into a +4°C laboratory storage fridge unit at Utrecht University, Utrecht, the Netherlands until further use.

Table 1: Summary of the chosen peatland's sites used in the study. Concentrations of TEAs were estimated from Erkens et al. (2021).

	Assendelft	**Rouveen**
Province	Noord-Holland, NL	Overijssel, NL
Location	52°28'30.4"N 4°44'29.3"E	52°38'02.0"N 6°05'20.6"E
Peat type	Sedge/Reed	Sedge
Use	Dairy farming	Dairy farming
Hydrology	Salt water intrusion	Seepage
Dominant TEA present (porewater)	SO_4^{2-} 1.0×10^4 µmol/l *Erkens et al. (2021)*	Fe^{2+} 150 µmol/l *Erkens et al. (2021)*

Anoxic condition

The peat samples were maintained in anoxic conditions throughout the experiment. This was achieved via the use of an anoxic chamber, gas-tight bags (Anaerocult, A mini, Merck, the Netherlands) and anoxically sealed containers (Merck, the Netherlands). The demi water and MILLI-Q water used were purged of dissolved oxygen and headspace oxygen by bubbling with nitrogen gas for 30 minutes before use.

Anoxic Chamber

The vinyl anoxic chamber (Coy Laboratory Products Inc, Grass Lake, Michigan, United States) consists of a sealed environment with a two-door airlock used for transferring material to the outside. The chamber's environment (figure 2A) is kept anoxic via the use of a background (purge) gas and a hydrogen gas mix, which is continuously circulated through palladium catalysts inside the chamber to remove left-over oxygen. To establish the initial anaerobic conditions, the oxygen-rich air is removed via a vacuum pump. Then, the chamber is filled with the background gas. After the oxygen is purged from the chamber, the chamber is filled with the hydrogen gas mix until hydrogen levels reached 2-5%. This level of hydrogen is optimal on-going oxygen removal as air inside the chamber will circulate through the palladium catalyst. Purging is also repeated when material is transferred inside or outside of the chamber via the airlock. Whenever hydrogen gas levels in the chamber decreased under 2%, additional flushing of the chamber with the gas mix was performed to restore optimal H_2 levels. In a later stage of the project, a hydrogen sulfide removal was also installed inside the chamber to avoid interaction between sulfur compounds and hydrogen gas inside the chamber.

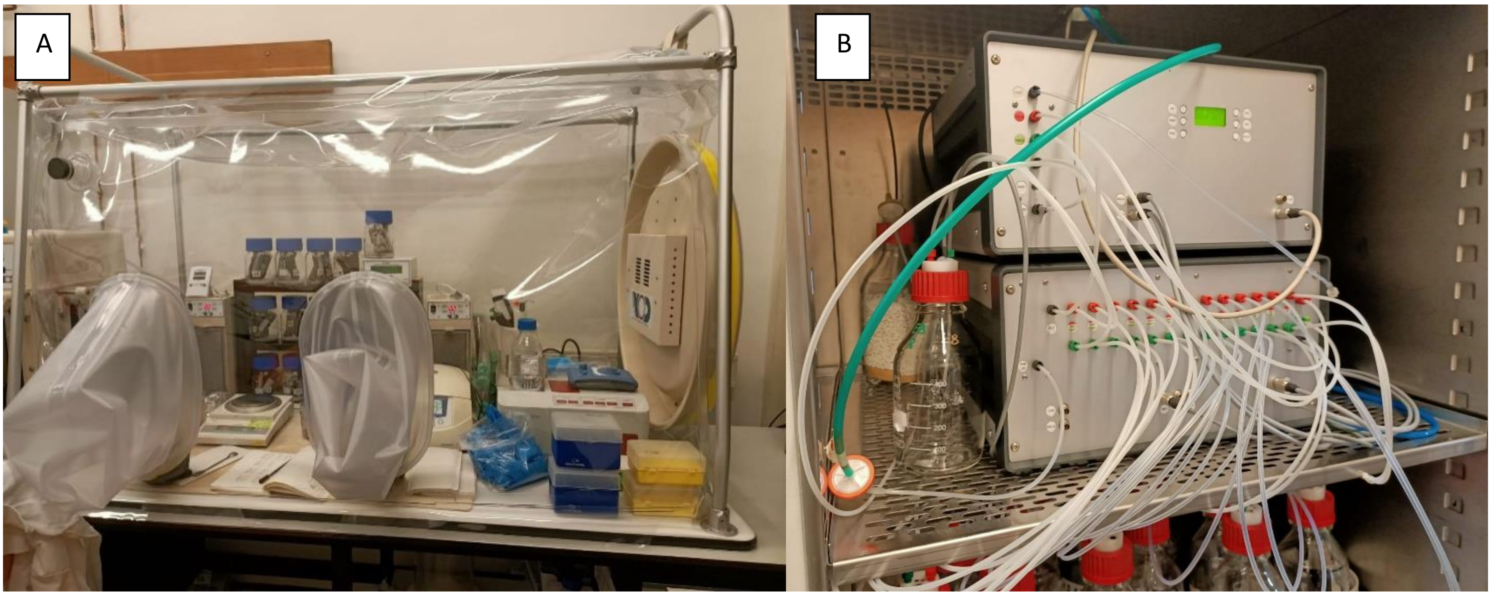


Figure 2*:* A) The vinyl anoxic chamber in which the experiment was conducted B) Example of the respirometer equipment used for the respiration rate experiment

<u>Iron and sulfate sources</u>

Iron and sulfate were added to the peat in solutions. For the POX experiment, three 0.1M solutions of were prepared made respectively of Fe^{2+}, Fe^{3+} and a 1:1 mixture of $Fe^{2+/3+}$ based on Iron(II)-Chloride (13478-10-9, Merck, the Netherlands) and Iron(III)-chloride (7705-08-0, Merck, the Netherlands). Potassium sulfate (0.1M, 7778-80-5, Merck, the Netherlands) was chosen as a source of sulfate. All solutions were prepared anoxically and within 24h before the start of the incubation experiment.

Experiment 1: Potential POX activity

POX was measured according to a protocol revised from Dunn et al. (2014) and was only performed on the experiment with iron addition due to time constraints. This experiment was conducted entirely within the anoxic chamber. Before the start of the experiment, peat samples were taken from the fridge and acclimated to room temperature for 24h. Soil was then mixed, and large fragments were removed. In the experiment, 10 mL of 10 mM 3,4-Dihydroxy-L-phenylalanine (L- DOPA) solution (Sigma-Aldrich, St. Louis, Missouri, United States) were added to 1gr fresh peat samples together with 5ml of the respective iron solution. 10 mL MilliQ water were used for controls instead of L-DOPA. Each sample was mixed for 15 seconds with an Ultra Turraxx Tube Drive mixer (IKA, Staufen, Germany). Samples were then incubated for 15min and subsequently placed in 2ml Eppendorf which were centrifuged at 14000 rpm for 5 min. 300 ul of the resultant supernatant from each tube was transferred to a transparent 96-well plate (Merck, the Netherlands). POX activity was measured in the supernatant with a spectrometer (Spectrostar BMG Labtech, Ortenberg, Germany) at 475 nm (also under anoxic conditions) .

Table 2: Overview of potential POX activity experiment. The experiment tested addition of Fe^{2+}, Fe^{3+} and mixture of Fe^{2+} and Fe^{3+} on peat. L-DOPA(10mM) was used as a substrate to estimate potential POX activity in 15min at 475nm.

Treatment	*L-DOPA (10mM)*	*Fe^{2+} (0.1M sol.)*	*Fe^{3+} (0.1M sol.)*
Peat (control)	x	-	-
Fe^{2+} + peat	x	x	-
Fe^{3+} + peat	x	-	x
Fe^{2+} + Fe^{3+} + peat	x	x	x

Experiment 2: Respiration rate experiment

The Respiration rate experiment aimed at evaluating the effects that iron or sulfate addition as TEA have on peat decomposition. To do this, basal respiration rate, in terms of CO_2 production from samples, was measured via a respirometer (Figure 2B). An overview of the experiment can be found in table 3. In a second stage of the experiment, substrate-induced respiration rate (SIR rate) was also measured. To do this, lactate was chosen as a substrate and added to peat samples as C source.

Table 3: Overview of the respiration rate experiment. This experiment measured basal respiration (BR) and substrate-induced respiration using lactate (SIR) on peat treated with iron addition (1:1 Fe^{2+}/Fe^{3+} mixture) and sulfate addition. Data are missing for SIR on sulfate treated Rouveen peat.

	Basal Respiration		***Substrate-Induced Respiration (Lactate)***	
	Iron	Sulfate	Iron	Sulfate
Assendelft	x	x	x	x
Rouveen	x	x	x	-

Sample preparation for the respiration rate experiment was performed in the anoxic chamber. Peat samples were taken out of the fridge and acclimatized to room temperature 24h prior to incubation of the samples. For samples preparation, 30g of fresh peat was weighted and placed into 30ml tubes. 15 ml of either iron solution (1:1 Fe^{2+}/Fe^{3+}), sulfate solution or demi water (control) was then added to the tube, according to the experimental series (Table 3 above). Each tube was then shaken for 10sec on a shaker to ensure correct mixture of the solutions. Tubes were later placed each into a 500ml glass bottle containing 50ml of perlite, which was added to ensure stable moisture content in the samples throughout the whole experiment. Lastly, each container was sealed and attached to the respirometer. CO_2 production was measured at intervals of 20 min for 48 hr.

Data analysis

Data analysis was performed in Rstudio version 4.2.0 and Microsoft Excel. Raw data from the respiration rate experiment were converted into basal respiration (BR) data using a custom R script. This conversion utilized the dry weight of samples (obtained via oven drying (70°C, 48h) to estimate CO_2 production rates. BR data were then analyzed via a paired t-test. An equivalent non-parametric test (Wilcoxon signed rank), was instead used for the analysis when BR data did not follow a normal distribution.

POX activity data were converted from absorbance values into potential POX concentration following the Beer-Lambert law. Initially, data analysis of the potential POX experiment samples was going to be compared via a one-way analysis of variance (ANOVA). The aim of this test was to compare iron treated samples and control samples among each other to look for statistical differences in mean potential POX activity. However, as discussed in a later section of this paper, it was not possible to analyze POX data due to unexpected interactions between L-DOPA and ferric iron which affected results. This interaction made it impossible to quantitatively separate between the effect of iron and effect of POX on absorbance rates of L-DOPA. Therefore, since analyzing the potential POX activity data was not possible, raw absorbance data from this experiment are also presented and discussed in this paper.

Results

Potential POX activity

The potential POX activity measured in the peat samples ranged between 0.288 and 18.63 µmol $gDW^{-1}min^{-1}$. No significant differences between sites were observed (figure 3). Enzymic activity increased in both study sites when any form of iron was added to peat. Fe^{2+} addition showed the lowest increase in potential POX activity compared to control. Instead Fe^{3+} addition resulted in the highest activity in both Assendelft and Rouveen samples. As shown in figure 3, Fe mix addition to peat resulted in a much larger increase in potential POX activity than the Fe^{2+} treatment, but lower than the Fe^{3+} treatment. Additionally, results for both the Fe mix treatment and the Fe^{3+} treatment showed higher enzymic activity in Rouveen than in Assendelft. The potential POX activity in the Fe^{2+} added treatments was instead similar between the two sites and ranged from 0.288 to 0.650 63 µmol $gDW^{-1}min^{-1}$.

Raw absorbance data measured at 475nm (Figure 4) ranged from 0.048 to 3.968 and showed different patterns than the potential POX activities expressed as µmol $gDW^{-1}min^{-1}$. This deviating pattern is partly caused by the differences in dry bulk density between the two peat types. Analyzing the raw absorbance data without weight correction revealed that absorbance rates of the Fe^{3+} treatment for both peat types was surpassing the detection limit of the plate reader, which resulted in identical absorbance (3.968) across all replicates and both study sites. Therefore, the potential POX activity in µmol $gDW^{-1}min^{-1}$ for this treatment is considered to be unreliable. For the other treatments within the Assendelft series, control samples generally had the lowest absorbance, followed respectively by the Fe^{2+} treatment, the Fe mix. In contrast , Rouveen showed the lowest absorbance with the Fe^{2+} addition treatment, followed by control samples, treatments with Fe mix resulted in higher absorbance in Rouveen than in Assendelft.

Figure 3: Potential POX activity (µmol $gDW^{-1}min^{-1}$) measured for respectively Assendelft and Rouveen peat. Treatments (left to right) are respectively control (in black), Fe^{2+} addition (in blue), Fe^{3+} addition (in red), Fe mixture addition (in yellow)

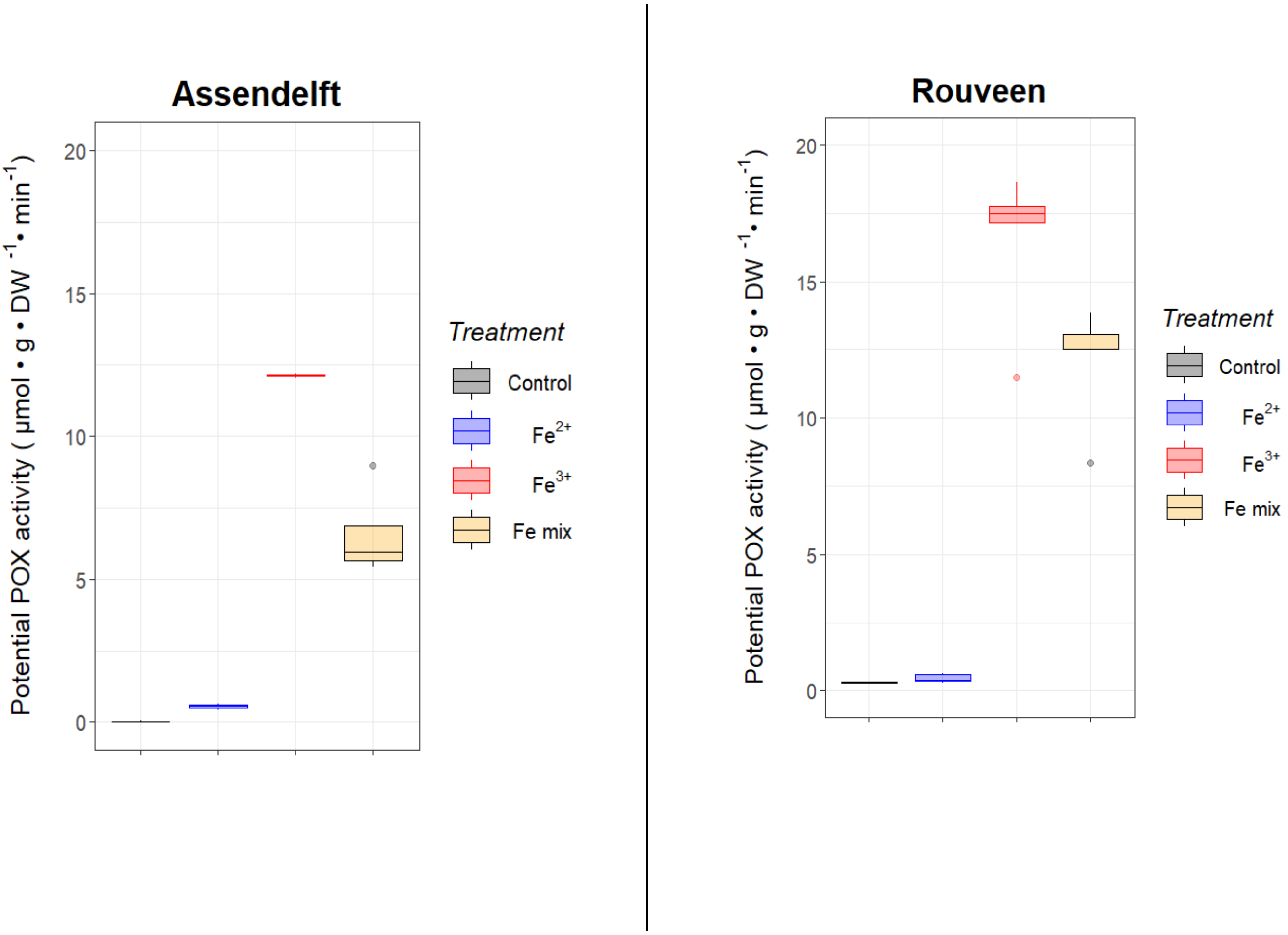
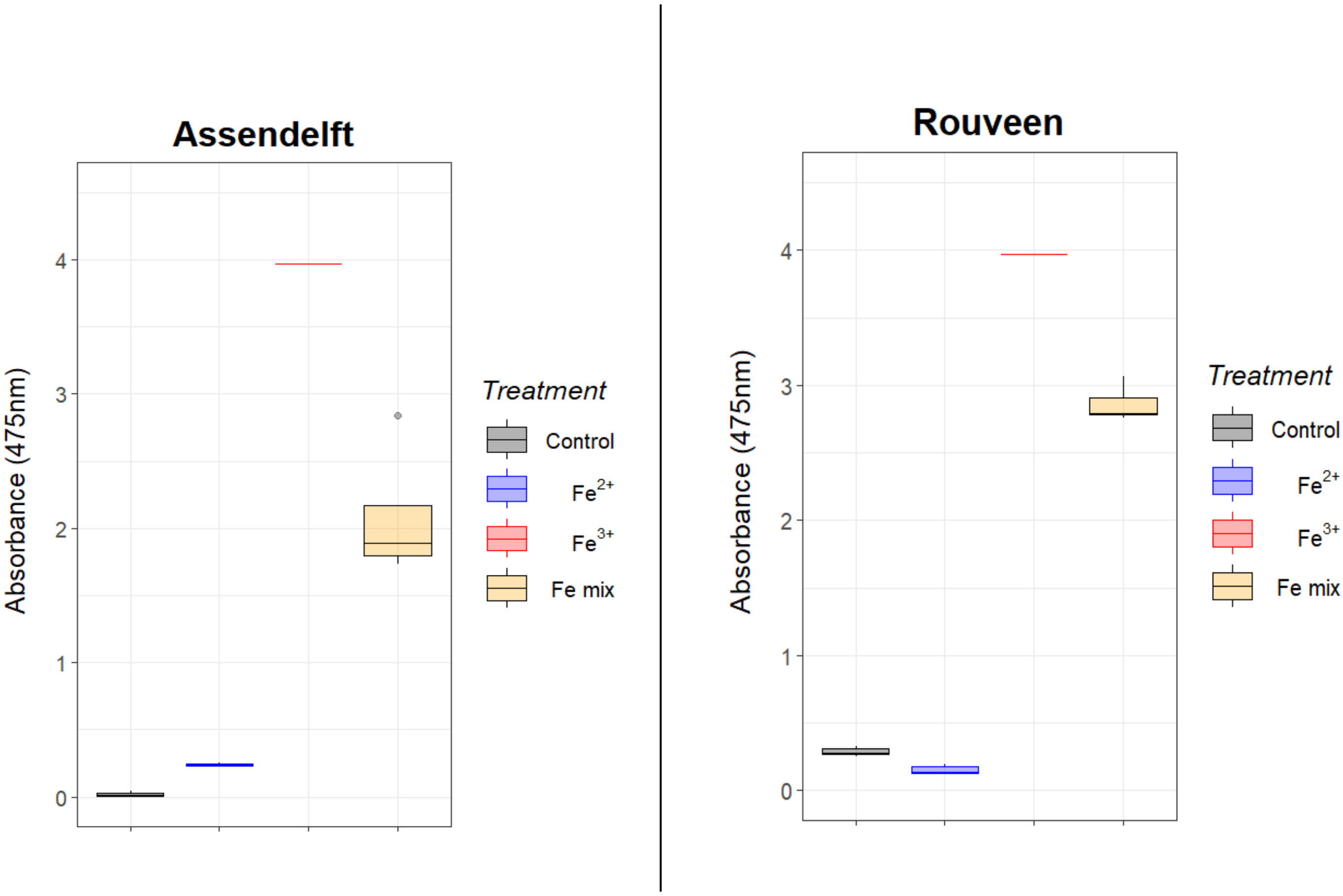


Figure 4: Absorbance results (475nm) of the Potential POX experiment for Assendelft and Rouveen peat. Treatments (left to right) are respectively control (in black), Fe^{2+}(in blue), Fe^{3+}(in red), Fe mix (in yellow)



Respiration rates

Average CO_2 production rates measured in the peat samples ranged between 0.009 and 1.980 µmol gDW^{-1} h^{-1} in Assendelft (Figure 5) and between 0.008 and 0.527 µmol gDW^{-1} h^{-1} in Rouveen (Figure 6). As shown in Figure 5A,5B and 6A,6B, basal respiration rates did not exceed 0.4 µmol gDW^{-1} h^{-1} across all treatments in both study sties and were not significantly affected by addition of iron ($W = 1$, $p = 0.25$ for Assendelft and $n=4$; df 3; $t= 0.13313$; $p = 0.9005$ for Rouveen) or sulfate ($W = 0$, $p = 0.125$ for Assendelft and $n=5$; df 4; $t= 0.40818$; $p = 0.704$ for Rouveen).

Substrate-induced respiration rates were higher in Assendelft (figure 5C and 5D) than in Rouveen (figure 6C) and responded differently to the addition of iron or sulfate. Rouveen SIR rates in both iron-treated and control samples were very low (< 0.2 µmol gDW^{-1} h^{-1}) and no significant effect on

SIR rates was observed with iron addition (W = 6, p = 0.8125). In contrast, Assendelft peat had higher SIR rates (0.42-1.98 µmol gDW^{-1} h^{-1}) compared to samples without lactate addition. Sulfate addition resulted in an increase in SIR rates (W = 3, p = 0.625) in Assendelft samples. In contrast, iron addition to Assendeflt peat (figure 5C) resulted in a significant decrease in SIR rates (n=4, df 3; t= 3.6865; p = 0.0346). In Assendelft, differences in BR rates and SIR in control samples were also observed. Control samples used for the sulfate addition experiment showed higher SIR rates than control samples used in the iron addition experiment. A similar trend can be seen in control samples used to measure BR rates.

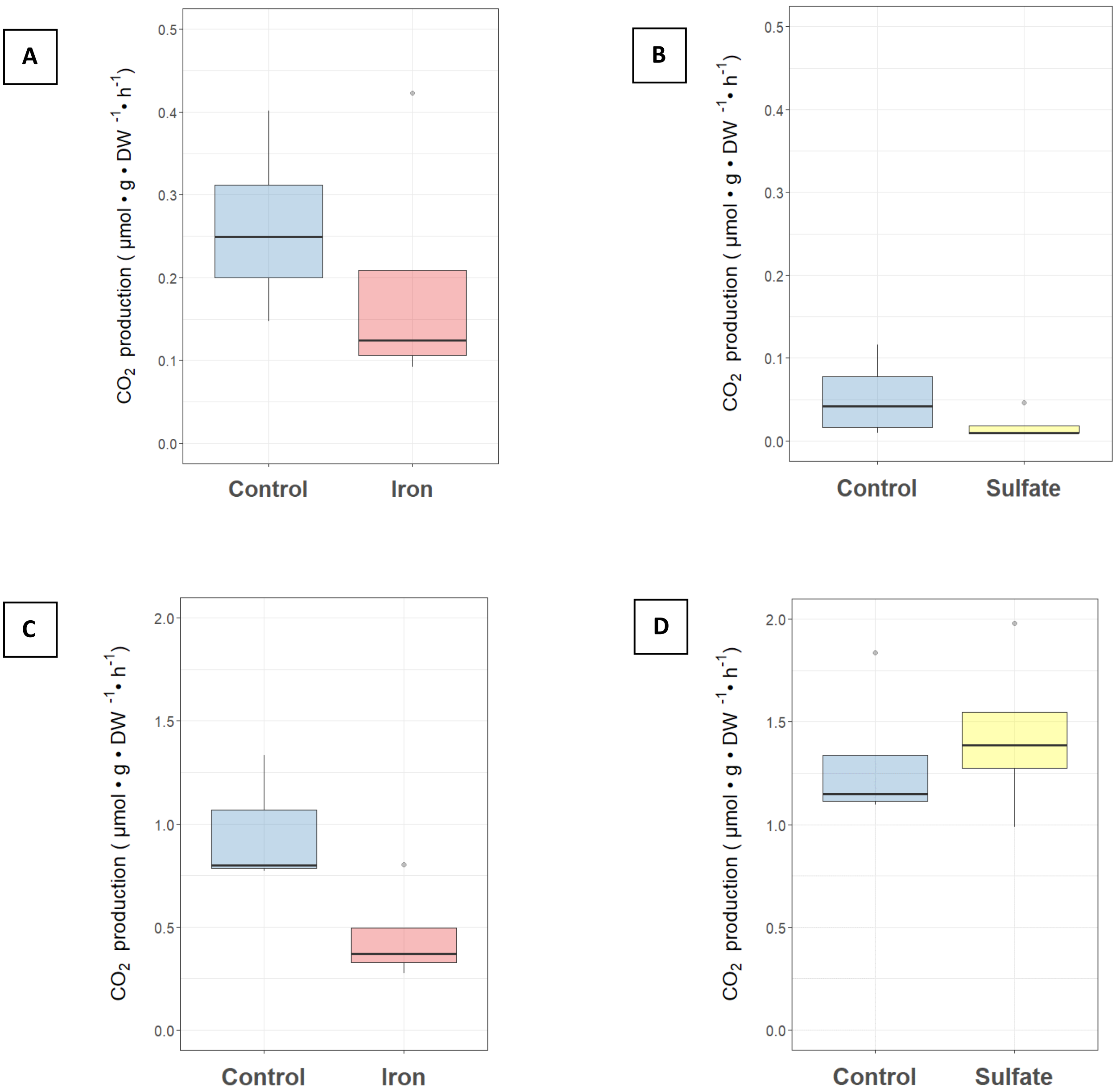
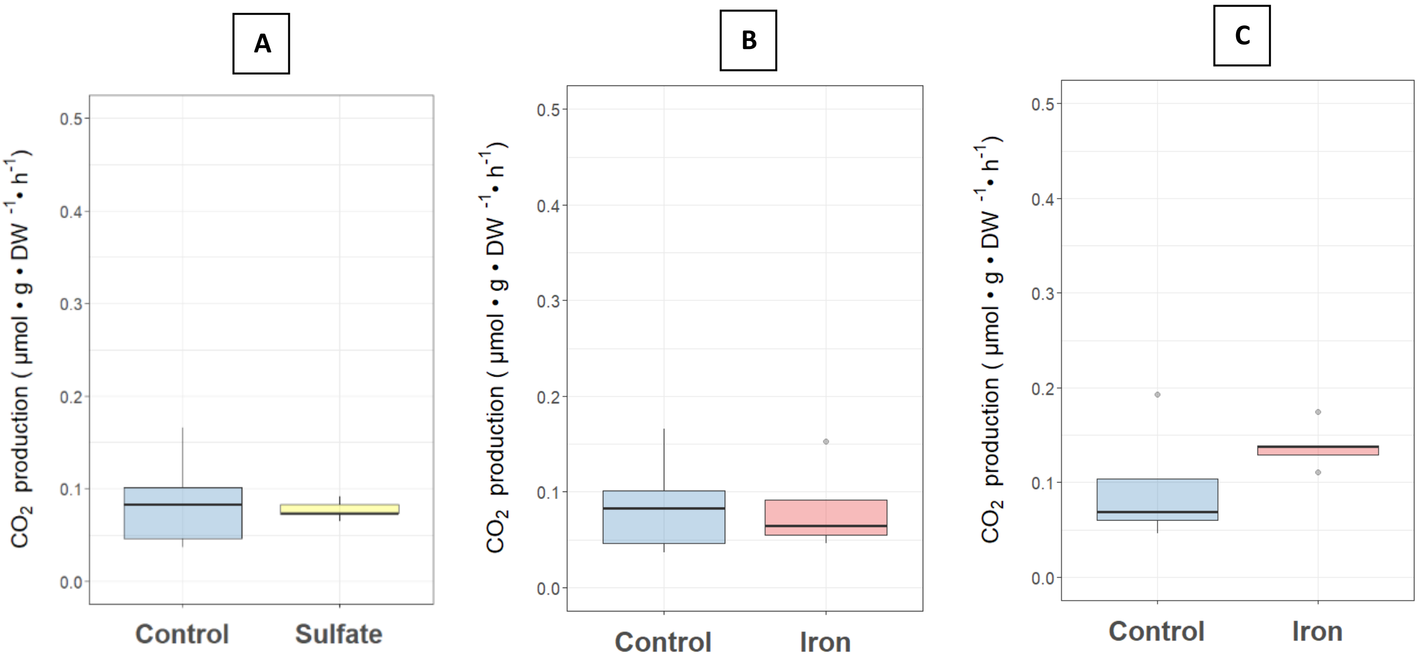


Figure 5 (above): Average CO_2 production of Assendelft peat A) Basal respiration with iron addition B) Basal respiration with sulfate addition C) Substrate-induced respiration with iron addition D) Substrate-induced respiration with sulfate addition

Figure 6 (below): Average CO_2 production of Rouveen peat A) Basal respiration with sulfate addition B) Basal respiration with iron addition C) Substrate-induced respiration with iron addition



Discussion

Unreliable measurements of POX activity

When measuring potential POX activity on iron-treated peat, this study found a deviating pattern between raw absorbance data at 475nm and the potential POX activity data expressed in μmol $gDW^{-1}min^{-1}$. Analyzing the raw absorbance data without correcting for dry bulk density revealed that absorbance rates of Fe^{3+} treated peat samples was surpassing the detection limit of the plate reader, resulting in identical absorbance (3.968) across all replicates and both study sites. Therefore, the potential POX activity in μmol $gDW^{-1}min^{-1}$ for this treatment is considered to be unreliable.

Ferric iron able to bind L-DOPA

Potential POX concentrations are estimated by converting absorbance data for 1g of peat into enzymic activity following the Beer-Lambert law (Dunn et al., 2014). This conversion uses the dry bulk density (DW) of the soil to estimate POX activity, as DW and POX activity have an inverse relationship (Dunn et al., 2014, Sainju et al., 2022). In this research, Assendelft peat was found to have a higher DW than Rouveen due to higher soil compaction and structure of the peat. Thus, the higher POX activity shown with Fe^{3+} and Fe mix addition in Rouveen is likely a result of this conversion and does not indicate higher POX activity occurring in Rouveen peat. Additionally, it is unusual for all Fe^{3+} samples to show identical absorbance, as peat soil is heterogenous (Rezanezhad et al., 2016) and hardly results in similar absorbance rate. Although an increase in POX activity with Fe^{3+} addition was hypothesized, the large difference in absorbance between Fe^{3+} containing treatments (Fe^{3+} and Fe mix) and treatments without Fe^{3+} is highly unusual and hard to attribute to the addition of iron that POX can use. While this could initially indicate Fe^{3+} being used as a TEA, these results are likely an artifact caused by a measuring error and by an unforeseen interaction between ferric iron and L-DOPA.

In this study, potential POX activity was measured by using l-3,4-Dihydroxyphenylalanine (also known as L-DOPA or levodopa) as a substrate for POX enzymes to bind. L-DOPA has applications in multiple field, such as being a substrate to measure the activity of oxidative enzymes as well as a component of medications for Parkinson's disease (Nagatsu and Sawada, 2009) and dementia (Molloy, 2005), since L-DOPA is also a dopamine precursor (Stansley and Yamamoto, 2015). Several studies in the medical field have indicated the formation of L-DOPA:Fe complexes when Fe^{3+} is present (Alhassen et al., 2021; Zaidi and Fatima, 2017; Greene et al., 1990). This binding capacity of L-DOPA allows the compound to bind iron via chelation through its catechol group, forming a stable complex (Alhassen et al., 2021). While this interaction has been object of recent pharmacology and neuroscientific studies, it is still undocumented in soil enzymology, soil ecology and environmental biology literature. By taking this interaction into consideration, the high absorbance obtained whenever Fe^{3+} was provided to peat, could then represent: potential POX, L-DOPA:Fe complexes or, most probably, a combination of the two. Moreover, the absorbance recorded in the Fe^{3+} treatment does not represent the real absorbance, but a partly-detected much higher absorbance value due to a combination of both L-DOPA:Fe and L-DOPA:POX complexes.

One explanation on the high absorbance rates is that excessive iron (16.1g/L) was provided to the peat and oversaturated the L-DOPA. This was suggested by a follow-up study on the Fe – L-DOPA interaction conducted by a BSc student at Utrecht University in December 2022 (Appendix 1), where much lower Fe^{3+} concentrations (0-5g/L) than the ones used in this study led to a major increase in absorbance (0-1.2). Additional evidence to support the L-DOPA:Fe interaction can be found in the absorbance rates of the Fe mix treatments, which were 25 to 40% higher in Rouveen than in Assendelft. Since the Fe mix treatment contained a 1:1 mix of Fe^{2+} and Fe^{3+} (halved amount of Fe^{3+} compared to the only Fe^{3+} treatment), resulting absorbance rates from Fe mix addition should have a range of about half of the absorbance recorded for the Fe^{3+} treatment, if no real POX activity is being measured. While this is the case in Assendelft, Fe mix samples in Rouveen revealed higher absorbance rates. This difference between the two sites is likely an indicator of an iron effect with L-DOPA which is visible in both sites, and a partial potential POX activity being measured in Rouveen.

Rouveen peat is also known to contain higher concentrations of Fe^{2+} in the reduced zone, where peat samples for this study were collected. Other studies have also reported an inverse relationship between Fe^{2+} concentrations and POX activity. According to the iron-gate theory, Fe^{2+} has a protective effect on peat from decomposition shortly after oxygenation. Therefore, it is also possible that the absorbance rates measured in this study only represent L-DOPA:Fe complexes and not POX activity at all. In this scenario, the high concentration of Fe^{2+} in Rouveen peat samples would be completely blocking POX from being active, resulting in L-DOPA only binding leftover Fe^{3+}. However, this is unlikely as peat samples in this study were kept anoxic throughout the entire duration of the project.

Implications of the L-DOPA:Fe interaction for peat enzymology and peat loss models

Unfortunately, with the current data from this study it difficult to make an estimate on potential POX activity in the samples and further analyze the data. Nevertheless, while this experiment shows important insight on the role of Fe^{2+} and Fe^{3+} in peat, it also highlights how the lack of documentation on L-DOPA:Fe interaction has the potential to create large bias in studies utilizing the L-DOPA assay. Still today, the L-DOPA assay, together with ABTS and pyrogallol, is among the most used assay in soil enzymology to measure activity of oxidative enzymes (Bach et al., 2013; Wiedermann et al., 2017). Since most studies working on phenol oxidase also use L-DOPA as a substrate (Bach et al., 2013), this finding opens many questions on the validity of results when measuring in POX in soils with high-iron concentration. For instance, a study using L-DOPA on Asian mangrove wetlands (Kim et al. 2020) reports high POX activity under anaerobic conditions in Okinawa, Japan, followed by an atypical 95% decrease in enzymic activity after oxygenation of the soil (Kim et al. 2020). However, the study area of the research also presents high concentrations of iron in the soil, suggesting that iron probably interfered with the measurements of POX activity. Similarly, previous studies have indicated high phenol oxidase activity in soils rich in both Fe^{2+} (Van Bodegom et al., 2005) and Fe^{3+} (Zhao et al., 2021). Another example is studies on *Sphagnum*-dominated peatlands, where runoff rich in ferric iron (350–470 μg) has recently been observed (Krachler et al., 2016). Many publications on *Sphagnum* peat enzymology report high phenol oxidase activity in the saturated layers without considering the L-DOPA:Fe interaction. For instance, Williams et al. (2000) reports high POX activity in both oxic and anoxic *Sphagnum* peat, concluding with an exponential increase of enzymic activity with soil pH. Xu et. al (2021) also reports POX activity occurring in both saturated and unsaturated *Spagnum* peat using L-DOPA and suggest a revision of the "enzymic latch theory" based on the data. It is possible that results from several studies on POX activity are simple biases causes by an accidental overlook of the L-DOPA:Fe interaction, and do not represent reliable estimates of POX activity in anoxic conditions. Major publications such as Wang et al, 2017, where the iron gate theory originated, have used the L-DOPA assay to measure an increase in POX activity during water-table decline. Therefore, it may be possible that the entire iron gate theory, as well as contrasting results and models comparing the iron gate theory with the Enzymic Latch, are based on this bias and do not consider the interaction of ferric iron with L-DOPA. Moreover, several studies also use L-DOPA as a substrate for measuring the activity of other oxidative enzymes (Bach et al., 2013). This could lead to a severe overestimation of, for instance, peroxides in iron-rich soils in both oxic and anoxic conditions, which together with POX overestimation could highly affect estimates of peat loss. Overall, different biogeochemical pathways regulating OM decomposition in peat have been proposed over the years. This study suggests that the L-DOPA:Fe interaction constitute a main bias in existing literature on peatland enzymology, which is probably contributing to the on-going debate about molecular mechanisms regulating peat loss and contrasting results on POX activity and other oxidases in peat.

Respiration rates: differences between BR and SIR

Measuring average CO_2 emission rates from samples indicated no effect of iron addition or sulfate addition on basal respiration (BR) rates in both sites. In contrast, substrate-induced respiration (SIR) rates significantly decreased with iron addition (n=4, df 3; t= 3.6865; p = 0.0346) and increased with sulfate addition in Assendelft samples. Strangely, Rouveen samples did not respond to SIR with iron addition and showed similar CO_2 emission rates as the BR samples.

Iron addition may protect OM from decomposition under anoxic condition

While addition of iron did not show any significant effects on anoxic BR rates in both study areas, results on SIR rates on Assendelft peat showed a significant decrease in CO_2 production rates in comparison to the control samples. Also, a slight decrease in BR was observed when iron was added to peat. Since this is a similar pattern to what observed with SIR respiration with iron, it could represent a decreasing trend that does not show significance due to a low number of replicates (n =4). Therefore, Assendelft results indicate that iron addition may have a protective effect on peat under anoxic conditions. Previous studies have indicated a protective effect of iron on peat with high concentrations of Fe^{2+}, mostly after short-term oxygenation or in fully oxic-conditions (Wang et al., 2017). Therefore, it is possible that Fe^{2+}, commonly present under anoxic conditions, is responsible for the protective effect. However, since both Fe^{3+} and Fe^{2+} were added to peat, it is difficult to separate the effects of the two ions from the data available from this study.

In contrast to Assendelft, Rouveen SIR rates with iron addition did not show a similar decrease on decomposition rates. Therefore, it is important to consider the differences in TEAs concentrations among the sites. In other words, it is possible that the high concentrations of Fe^{2+} already present in Rouveen peat nullified the effect of added iron, resulting is no differences in decomposition rates with control samples. However, this is not in accordance with previous measurements on samples for the same site (missing data) which indicate anoxic emission rates of 0.4 µg CO_2 DW^{-1} h^{-1}. Also, it does not explain a lack in increase in respiration rates when lactate was added to peat. Addition of a substrate to measure SIR is expected to produce higher respiration rates (Swaina et al., 2014), as soil microbes are provided with easily accessible material to decompose. Additionally, lactate is known to be a good carbon source in anoxic condition, in contrast with glucose which is often used to measure SIR in oxic experiments. Therefore, a possible explanation for these results is that the microbial community in Rouveen samples was mostly inactive because of other environmental conditions, which could range from pH changes in the soil, thermal shock due to temperature changes between transportation of the samples, or simply the presence of a very small microbial community that was unable to decompose lactate. Another explanation could be the long-term storage (3-4 months) of Rouveen peat samples, which could have resulted in a destabilization of the microbial community in the samples, which could have died due to lack of resources for survival once the easily-degradable material was used.

Differences in emission rates in control samples

When measuring both BR and SIR rates in Assendelft peat, it is possible to see differences in emission rates of control samples used for the iron addition experiment and the sulfate addition experiment. This could have been due to several reason. Peat is a heterogenous material, so it is possible that some samples had a higher concentration of easily degradable OM, which ultimately results in higher emission rates. However, since BR rates are higher across all samples in the iron series compared to the sulfate series, a more probable explanation is that the iron series was measured and incubated for a longer time, allowing higher respiration rates to occur.

Sulfate probable to be used as a TEA in anoxic conditions

Another important results from this experiment can be seen with sulfate addition. While BR rates were not affected, SIR rates in Assendelft seem to increase when sulfate is added. Although no significant difference was observed when analyzing data for the sulfate addition treatment, increases in CO_2 production rates could indicate a trend of sulfate being used as a TEA by soil microorganisms. Unfortunately, the lack of data of SIR rates with sulfate addition in Rouveen cannot confirm this hypothesis, as a similar pattern in Rouveen sulfate-treated peat could have resulted in supporting evidence for this hypothesis. In contrast, if sulfate addition would have reduced SIR in Rouveen, then a different hypothesis involving Fe-S-OM interactions could be generated, as the high Fe^{2+} concentrations in Rouveen peat could have led to stabilization of OM. Nevertheless, these results highlight how biogeochemical interactions between iron oxidation states, sulfate and OM are possibly behind the regulation process of peat decomposition under anoxic conditions. Sulfate and other sulfur compounds are known to be highly reactive with iron (Karimian et al., 2018), especially under anoxic conditions where sulfur binds iron more strongly than other elements such as phosphorus (Karimian et al., 2018). Although it is estimated that sulfate presence in the soil only contributes to 10% of peat oxidation rates (Vermaat et al., 2016), with aerobic decomposition covering the other 90%, sulfate-iron interaction could still represent the main regulatory mechanism for OM decomposition under the water table. Recent studies have shown that Fe(II)-oxidizing bacteria require an organic co-substrate to recurrently oxidize Fe^{2+} to Fe^{3+} (Muehe et al., 2009). Studies on coastal peatlands, where sulfate is also abundant due to salt water intrusion, have shown increased GHGs emissions in acetate is naturally present in the peat soil (Gosch, 2019; Muehe et al., 200; Weston et al., 2011). Therefore, it is possible that acetate and soil pH changes may also have a major effect on decomposition. If acetate has a central role on the process, this would also explain why much higher respiration rates were observed with lactate addition, as acetate is a byproduct of lactate degradation. Also, both phosphorus (P) and ammonium (NH_4^+) have been reported to have direct links with the iron redox cycling and water table changes in peatlands. Phosphorous has strong affinity with iron oxides (Kraal et. al, 2022) and could help the stabilization of OM by interacting with Fe^{2+} in the anoxic layers of the peat. Additionally, Riedel et al. (2013) suggest that the formation of Fe^{3+}- OM complexes may lead to increased immobilization of DOC and P concentrations, thereby restricting substrate availability for POX, as well as C and P required for enzyme production.

Similarly, high ammonium concentrations, which have also been recorded in Assendelft (Erkens et al., 2021), also contribute to the decomposition process, as ammonium can function both as an electron donor for iron oxidation and as a product of bacteria metabolism to be used in iron reduction (Gosch, 2019). Ammonium can be used by soil microorganisms as a source of nitrogen to synthesize amino acids and other molecules that support their growth and metabolism (Trovato et al., 2021, Gosch, 2019). As such, the availability of ammonium can influence the composition and function of microbial communities in peatlands, which in turn can affect the accumulation, stabilization and decomposition rates of peat.

Overall, this experiment highlights the complexity of the process of peat loss under anoxic conditions. It is suggested that OM decomposition is the result of a complex biogeochemical balance between different elements in the peat, with Fe-S interactions having a central role on the mechanisms. Drainage of peat, together with climate change effects, majorly threaten this balance, as changes in the water table seem to be directly linked to changes in Fe, S, P and NH_4^+ concentrations in the soil, making peat loss unpredictable if decomposition pathways are not fully understood.

Project limitations and suggestions for future studies

This project presented several limitations. As discussed above the L-DOPA assay severely hampered measurements of POX activity and a new assay is required to complete this analysis. Additionally, due to problems in the data collecting phase, respirometer samples were not always measured long enough to ensure stable BR measurements. Therefore, the increase in both BR and SIR presented in this report may not represent rates the steady respiration of the soil, but on-going growth of the microbial community and leftover decomposition of OM matter, which could have resulted in increases in CO_2 emissions. Future experiments and follow-up project should focus on separating the effects of SO_4^{2-}, Fe^{2+} and Fe^{3+} on anoxic peat decomposition. This is a necessary step before testing interactions with other abundant elements in peat such as nitrate, phosphorous or ammonium. For the respiration rate experiment performed in this study, a 1:1 Fe^{2+}/Fe^{3+} solution as used. This made it challenging to separate the effects of the two ions on CO_2 production rates. It is suggested to repeat the experiment by measuring Fe^{2+}/Fe^{3+} concentrations in the soil and perform addition on peat of one iron ion at the time to confirm the trends observed in this study. Regarding sulfate instead, it is important to test sulfate addition on Rouveen (complete the dataset presented in this paper) as results could show important insights on sulfate being used as a TEA in Dutch peatlands. Additionally, while this project focused on CO_2 emissions, pathways leading to methane (CH_4) production involving iron and sulfate are also known to occur in peat and should not be overlooked.

Conclusion

This project shows new insights on TEAs use in peat in anoxic conditions. Results from the respiration rate experiment suggest that sulfate could be used as TEA for OM decomposition. Instead, iron addition seems to have a protective effect on the peat and interactions between sulfate and iron are probably responsible for regulating peat decomposition under anoxic conditions. Lastly, this project found an unknown limitation of the L-DOPA assay when measuring oxidative enzymes in iron-rich soils. This finding opens new questions and could explain existing inconsistences in literature regarding anoxic POX activity across different peatlands.

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Appendix 1

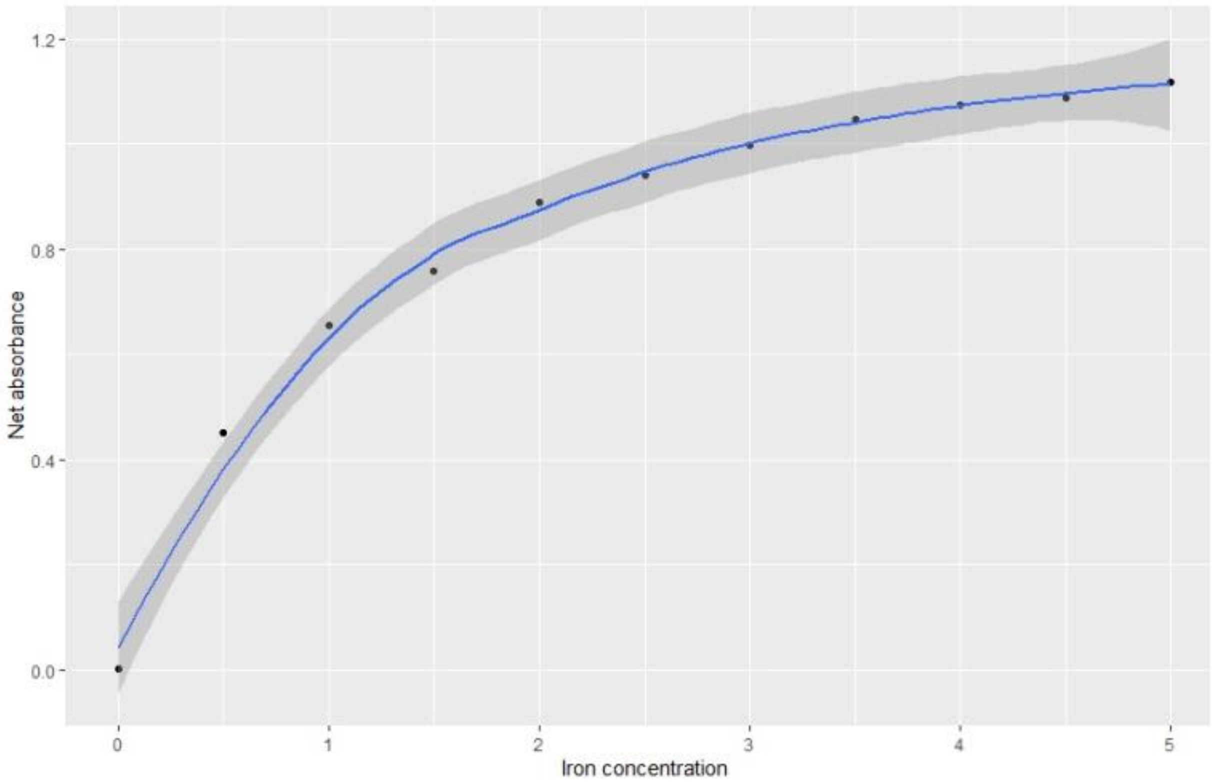

Figure A.1: Increase in net absorbance (ABS) value for increasing iron concentrations (g/L) with L-DOPA. Adapted from a soil-free experiment conducted by Michiel Renkema, BSc, at Utrecht University in December 2022.