Comparison of the occurence of BNI activity of ribwort plantain (Plantago lanceolata L.) and a common cover crop mixture (phacelia and white mustard) to a bare soil

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Abstract

Nitrogen (N) management in agriculture plays a critical role in addressing environmental challenges such as nitrate leaching and greenhouse gas emissions. Sustainable practices, including the use of cover crops, and Biological Nitrification Inhibition (BNI) offer a promising approach to mitigate these issues. This study investigated the potential of three cover crops—plantain (*Plantago lanceolata)* and white mustard (*Sinapis alba*) + phacelia (*Phacelia tanacetifolia*), a common cover crop mixture—to influence soil nitrogen dynamics and emissions across a crop rotation cycle. The objective is to assess whether these crops could reduce nitrification rates and contribute to more sustainable nitrogen management.

Field experiments were conducted in a randomized block design, with cover crops sown as intercultures according to two main comparisons: late ploughing (Lp) versus early ploughing (Ep) and control (C) versus cover crops (M). Soil samples were collected periodically to measure nitrate (NO_3^-), nitrite (NO_2^-), and ammonium (NH_4^+) concentrations, while gaseous emissions of nitrous oxide (N_2O) were monitored using static chamber techniques. Laboratory analyses were performed to determine potential nitrification rates. Statistical analyses included a univariate analysis of variance (ANOVA) and multivariate approaches to explore the influence of treatments on nitrogen dynamics.

The results showed that white mustard had a highest biomass production, which was linked to more pronounced interactions with soil nitrogen dynamics compared to phacelia. Nitrate concentrations were significantly reduced by cover crop mixture. N_2O emissions were principally influenced by soil moisture levels, with wetter conditions leading to increased denitrification. The study highlighted variability in nitrogen dynamics impacted by weather conditions, emphasizing the complexity of predicting the impact of the type cover crop on nitrogen dynamics. The termination treatment also showed effects on nitrogen dynamics.

In conclusion, the results partially met the initial objectives by demonstrating the influence of cover crops on nitrogen management. White mustard emerged as the most effective in reducing nitrate leaching risks. For producers, the findings underscore the importance of selecting cover crops adapted to local conditions to optimize their environmental benefits. Future research should focus on long-term studies to confirm the cumulative effects of BNI-capable crops and explore their interactions with other management practices, such as timing of sowing and incorporation into the soil.

Summary

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AOB = ammonia-oxidising bacteria

NOB = nitrite-oxidising bacteria

AOA = ammonia-oxidising archaea

AMO = ammonia monooxygenase

HAO = hydroxylamine-oxidoreductase

NOR = nitric oxide reductase

Nr = reactive nitrogen

NUE = nitrogen use efficiency

SOM = soil organic matter

SNI = synthetic nitrification inhibitor

BNF = biological nitrogen fixation

NH_4^+ = ammonium

NO_2^- = nitrites

NO_3^- = nitrates

N_2O = nitrous oxide

PCA = principal components analysis

WM = white mustard

Ph = phacelia

WC = water content

I. Introduction

Agricultural practices, particularly the use of synthetic fertilisers and nitrogen (N) management, have significantly contributed to environmental issues such as nitrate leaching, greenhouse gas emissions, and soil degradation. Addressing these challenges is crucial for improving the sustainability of farming systems while maintaining crop productivity. One possible solution lies in the incorporation of plants with Biological Nitrification Inhibition (BNI) potential into crop rotations, particularly during intercrop periods. BNI plants release compounds through their root systems that can suppress soil nitrification, thereby reducing nitrogen losses and improving nitrogen use efficiency. However, the impact of integrating BNI plants on a rotational scale remains largely unexplored.

This study focuses on assessing the BNI capacities of three plant species commonly used as cover crops: plantain (*Plantago lanceolata*), phacelia (*Phacelia tanacetifolia*), and mustard (*Sinapis alba*). By examining their ability to inhibit nitrification and their potential effects on nitrogen dynamics, this work aims to provide insights into the environmental benefits and practical implications of using BNI plants in sustainable agricultural systems. Such findings could offer valuable guidance to producers seeking to reduce input costs, improve soil health, and meet the growing demand for environmentally responsible farming practices.

II. Literature review

1. Context

Over the 20th century, global production of reactive nitrogen (Nr) rose dramatically from 25 teragrams (Tg) N to 156 Tg N until 1995, driven primarily by the advancement of agricultural practices during the Green Revolution and increased reliance on fossil fuels (Galloway et al., 2008; Wang et al., 2021). According to the FAO, this production continued to climb, reaching 187 Tg N in 2005, mainly due to a 20% increase in cereal production and a 26% rise in meat production (cited by Galloway et al., 2008). Today, approximately 80% of Nr produced is used in fertiliser manufacturing (Galloway et al., 2008).

Modern agricultural systems rely heavily on various nitrogen (N) fertilisers, the most common being ammonium-based fertilisers like urea, anhydrous ammonia (NH_3), ammonium sulfate ($(NH_4)2SO_4$), and ammonium nitrate ($NH_4NO_3^-$) (Coskun et al., 2017a). The Haber-Bosch process has also played a pivotal role in transforming global nitrogen cycles by converting nitrogen gas (N_2) into biologically available ammonia (NH_3) (Coskun et al., 2017b). Those fertilisers supply nitrogen mainly in mineral forms (e.g. ammonium and nitrate), which are essential for plant uptake (Beeckman et al., 2018). Mineral fertilisers also increase the amount of mineral nitrogen in soils, contributing to environmental nitrogen losses due to ammonia volatilisation and subsequent oxidation (Beeckman et al., 2018; Pijlman et al.,

2019). Alongside mineral fertilisers, organic options like green manure, compost, and poultry manure remain significant (Anas et al., 2020).

However, agriculture and biofuel combustion significantly contribute to atmospheric nitrogen losses (Tilman et al., 2015). Notably, agriculture accounts for around 50% of all ammonia volatilisation worldwide (Sommer et al., 2004, as cited by Cameron et al., 2013). Indeed, both the production and application of nitrogen fertilisers generate greenhouse gas emissions, amplifying agriculture's environmental footprint (Erisman et al., 2011). This introduction outlines the environmental challenges linked to nitrogen losses in modern agricultural systems, emphasizing the crucial role of nitrogen fertilisers in these processes.

2. Nitrogen cycling

The nitrogen cycle is an oxido-reduction network governed and catalysed by different bacteria, archaea, fungi and plants (Dietz et al., 2012; Coskun et al., 2017b; Beeckman et al., 2018). In natural ecosystems, the largest pool of nitrogen is dinitrogen (N_2) that plants are not able uptake and assimilate (Coskun et al., 2017a). By biological N_2 fixation (BNF), diazotrophic microbes as bacteria and archaea can reduce N_2 into ammonia (NH_3), and thus making nitrogen more easily available to plants (Coskun et al., 2017b). Likewise, organic nitrogen from soil organic matter (SOM) can also made available for plants by reduction (Dietz et al., 2012). The two main forms of nitrogen efficiently taken up by plants are NH_4^+ and NH_3 (Beeckman et al., 2018). Soils of natural ecosystems can retain much higher NH_4^+ and contain less NO3- than agricultural soil. Ammonium and ammonia are in a pH- and water-dependant equilibrium in which NH_4^+ can be transformed to NH_3 by deprotonation (Coskun et al., 2017a; Beeckman et al., 2018).

Concerning ammonia, it can be rapidly oxidised to nitrate ($NO3^-$) by microorganisms during an aerobic process called nitrification (O'Sullivan et al., 2016; Coskun et al., 2017a; Beeckman et al., 2018). Nitrification is governed by ammonia-oxidising bacteria (AOB), nitrite-oxidising bacteria (NOB) and ammonia-oxidising archaea (AOA), which outnumber the other kind of microorganisms, as well as comammox bacteria (Coskun et al., 2017b; Beeckman et al., 2018). The first step of nitrification is ammonia oxidation, where NH_3 is converted to nitrate (NO_2^-) via an intermediate from, the hydroxylamine (NH_2OH) (Dietz et al., 2012). The preferred form of nitrogen during nitrification is NH_3 regarding with its higher mobility in soil, unlike NH_4^+ (O'Sullivan et al., 2016). This first step of ammonia oxidation is the rate-limiting step in most environment and is catalysed by ammonia monooxygenase (AMO) through an action on AOB and AOA and comammox bacteria, while the second step is catalysed by hydroxylamine-oxidoreductase (HAO) (Dietz et al., 2012; O'Sullivan et al., 2016; Coskun et al., 2017b; Beeckman et al., 2018). Finaly, NO_2^- are converted to NO3-$^-$ through nitrite oxidation managed by Nitrobacter by means of the nitric oxide reductase (NOR) (Dietz et al., 2012; Ruser et al., 2015).

Denitrification is the following step while NO_3^- is reduced to N_2 by going through several intermediates in anaerobic conditions (Beeckman et al., 2018). Those intermediates are NO_2^-, NO^-, N_2O and N_2 (Coskun et al., 2017b; Beeckman et al., 2018). The back conversion of NO_3^- to NO_2^- is called dissimilatory/assimilatory nitrate reduction (D/A NR) (Beeckman et al., 2018). As nitrification, denitrification is also governed by bacteria, archaea and fungi (Coskun et al., 2017b). It finally exists alternative processes to reduced nitrogen. The first one is the dissimilatory nitrate reduction to ammonia (DNRA) which is the reduction of NO_3^- to NH_3 via NO_2^-, by several bacteria and fungi and happened in anaerobic conditions. On the other hand, anaerobic ammonium oxidation (anammox) is another important process in oxygen-poor environments, where nitrite (via NO and N2H4*)* and ammonium (via N2H4*)* are converted into dinitrogen (N_2) (Coskun et al., 2017b; Beeckman et al., 2018).

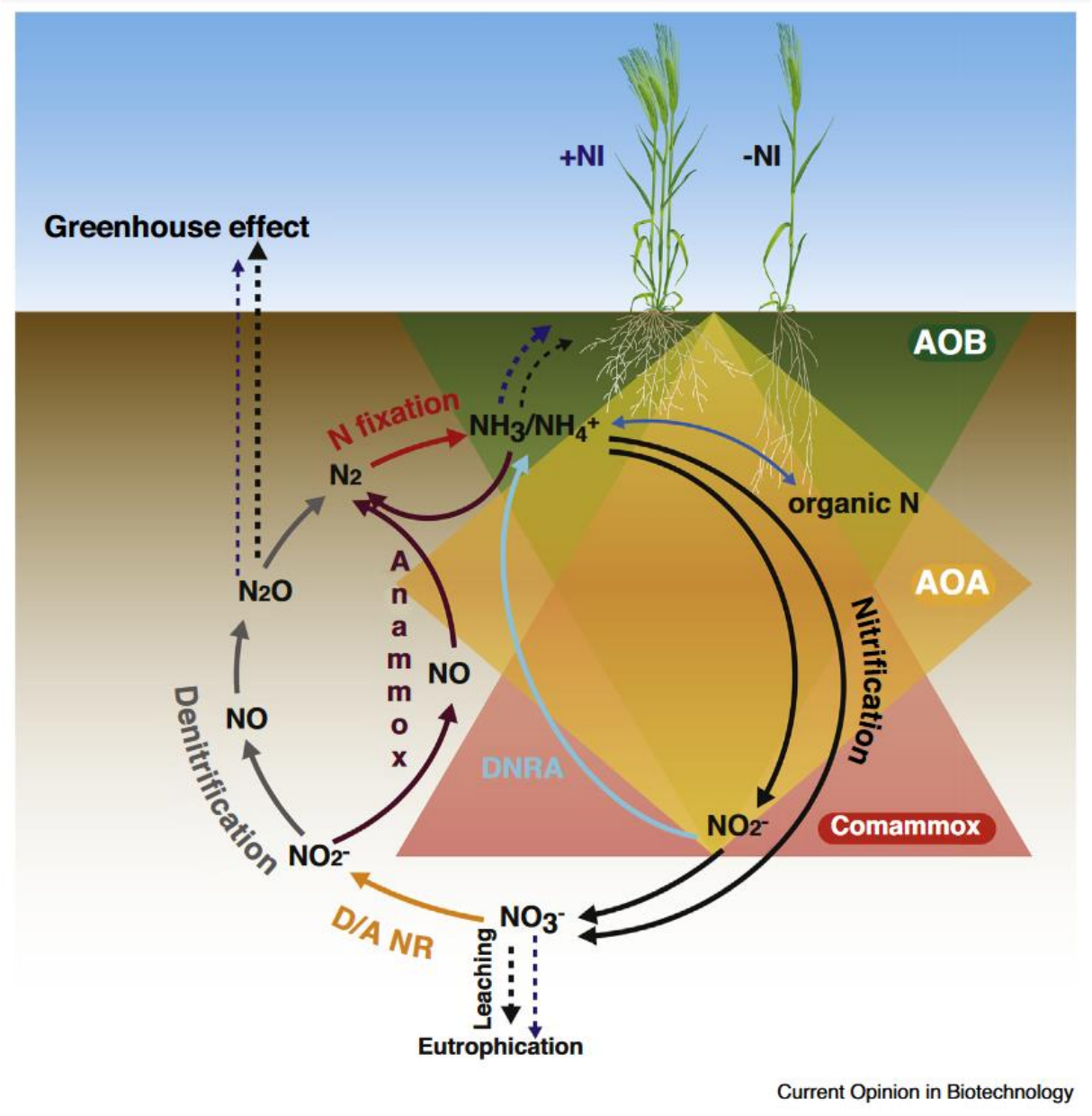


Figure 1: nitrogen cycling (Beeckman et al., 2018).

3. Sources of nitrogen losses in agricultural systems

In modern agricultural systems, nitrogen use efficiency (NUE) is alarmingly low, with only 30% to 50% of applied nitrogen being effectively used by crops, the rest being lost to the environment in various forms (Cassman et al., 2002; Lahda et al., 2005 cited by Wang et al., 2021). The two primary pathways for nitrogen losses are through $NO3^-$ leaching or runoff, and gaseous emissions (Anas et al., 2020). It has been estimated that nitrification process, accounts for about 19% of synthetic nitrogen losses through runoff and leaching (Di et al., 2017). Due to its negative charge and poor soil retention, nitrate is highly mobile, making it susceptible to leaching, which further exacerbates nutrient losses and reduces NUE (Huber et al., 1977; Qiao et al., 2015; Beeckman et al., 2018).

Gaseous nitrogen losses occur through processes such as ammonia volatilisation (NH_3) and nitrous oxide emissions (N_2O) via nitrification and denitrification processes (Qiao et al., 2015; Coskun et al., 2017a). Notably, N_2O , with a global warming potential 298 times that of carbon dioxide, is a major contributor to climate change and the most important ozone-depleting substance in the atmosphere (Forster et al., 2007; Coskun et al., 2017a). These emissions contribute to 60% to 80% of global anthropogenic N_2O emissions (Liquist et al., 2012 cited by Coskun et al., 2017a). N_2O emissions are particularly prevalent when soil conditions promote high mineralisation rates and contain substantial amount of SOM (Pijlman et al., 2019). Both nitrification and denitrification may produce nitrous oxide, with nitrification—primarily driven by AOB, AOA, and comammox bacteria—playing a significant role under anaerobic conditions (Beeckman et al., 2018; de Klein et al., 2022). Nitric oxide (NO) and nitrogen gases (N_2, NO_X) are also released during these processes, with NO typically associated with nitrification and N_2O mainly produced during denitrification (Qiao et al., 2015; Wu et al., 2017).

Both direct and indirect N_2O emissions have to be considered (Qiao et al., 2015; Coskun et al., 2017a). Direct N_2O emissions are attributed to nitrification and denitrification processes while indirect emissions occur as a result of NH3 volatilisation. Generally, these losses, both direct and indirect, by leaching or gaseous emissions, are influenced by multiple factors, including the type of fertiliser used, soil properties like pH, moisture, temperature, and cation exchange capacity, and the presence of soil microorganisms that drive nitrification and denitrification reactions (Cameron et al., 2013; Coskun et al., 2017a). Abiotic factors such as wind speed and rainfall also play roles in determining gaseous emissions (Coskun et al., 2017a).

Ammonia volatilisation accounts for approximately 18% of synthetic nitrogen losses and occurs when NH_4^+ is deprotonated. This process can be intensified in soils, with up to 65% of nitrogen from urea-based fertilisers potentially volatilizing. (Coskun et al., 2017a; Sutten and Grisven, 2011 cited by Tei et al., 2020). Erosion can lead to further nitrogen depletion, and soil nitrogen can also be mobilized by

organisms living within the ecosystem (Pijlman et al., 2019). Understanding these dynamics is crucial for developing strategies to mitigate nitrogen losses and improve nutrient use efficiency in agriculture.

4. Consequences of nitrogen losses

Nitrogen losses cited in the previous point are responsible of divers consequences on the environment, climate, human health and economy at local, regional and global scales (Galloway et al., 2008; Anas et al., 2020; de Klein et al., 2022). Firstly, nitrate leaching leads to a contamination and acidification of soil, ground water and surface water as lakes, rivers and oceans that can affect water quality responsible of health risks like cancers and or reproductive issues and causes eutrophication (Vitousek et al., 1997; Cameron et al., 2013). Human health can also be impacted by contaminated food directly and human welfare is also affected by following environmental perturbations. (Galloway et al., 2008; Cameron et al., 2013). Indeed, the occurred eutrophication of surface water results in increased in algae and water weeds production that can be toxic affecting fish populations (Cameron et al., 2013; Beeckman et al., 2018). Nitrogen losses are responsible of biodiversity and ecosystemic services losses for air, water and soils (Cameron et al., 2013; Qiao et al., 2015; Tilman et al., 2015; Coskun et al., 2017a). Indeed, as explain in a study realised by Tilman and Isbell, the authors saw a decrease of 30% of plant diversity after 135 years of fertilisation compared to the unfertilised control (Tilman et al., 2015).

Secondly, greenhouse gases emissions are responsible of air pollution as well as depletion of ozone layer by modification of atmosphere chemistry. They both lead to the degradation of air quality (Galloway et al., 2008; Cameron et al., 2013; Wang et al., 2021). They also disrupt both global nitrogen cycle and carbon cycling, increasing organic carbon stored in terrestrial soils because of the alteration of carbon uptake (Vitousek et al., 1997; Galloway et al., 2008; Erisman et al., 2011). Then, NO_x gas emission can promote the formation of O_3 which is the third most important greenhouse gas (Erisman et al., 2011). All those consequences are finally responsible of global climate changes affecting human health (Cameron et al., 2013).

Finally, the reduce NUE and depletion of soil nutrient solution due to nitrogen losses increase production costs as a low NUE means that much part of nitrogen is lost and not taken up by the crop (Coskun et al., 2017a; Nuñez et al., 2018; Anas et al., 2020). By using fertiliser with a higher NUE, producers and farmers would be able to reduce fertiliser uses and their production costs. Moreover, modification of N cycling can also lead to nutrient losses reducing plant growth (Coskun et al., 2017a). Finally, reducing N losses, and thus increasing the NUE, can also improve yields and farmers remuneration (Ghadirnezhad Shiade et al., 2024).

5. Nitrogen losses management

In addressing global changes, managing nitrogen losses is critical to reducing their environmental impact and safeguarding both human health and biodiversity. A main objective is to increase NUE by managing factors influencing soil emissions and nitrogen leaching, as well as optimising plant-microbe interactions. Indeed, improving crops' nitrogen-uptake efficiency could reduce (Nr) creation by approximately 15 Tg N per year (Cassman et al., 2002; Galloway et al., 2008; Bowatte et al., 2018). Management of nitrogen levels can also be a good asset to control plant growth and development, photosynthetic efficiency, quantitative and qualitative yield and water availability (Ghadirnezhad Shiade et al., 2024).

One promising approach involves the use of slow-release fertilisers, which regulate fertiliser availability through specific physical or chemical modifications, such as urea encapsulation, ensuring nitrogen synchronises with plant growth demands (Subbarao et al., 2012; Naz et al., 2014). Similarly, urease inhibitors, applied with urea fertilisers, reduce hydrolysis rates, thus curtailing nitrogen losses (Abalos et al., 2014; Yang et al., 2022). Polymer-coated fertilisers (PCFs) also represent a valuable tool in nitrogen management, helping to control nutrient release over time (Akiyama et al., 2010). Additionally, nitrification and denitrification inhibitors —either biological or synthetic— play a significant role in mitigating nitrogen losses, with synthetic inhibitors often classified as "enhanced-efficiency fertilisers" (Coskun et al., 2017b; Nuñez et al., 2018; Wang et al., 2021). Fertiliser application timing that coincides with plant growth stages can further improve NUE and reduce environmental impact (Coskun et al., 2017a).

Modifying agricultural practices is another pathway to enhance nitrogen management. Adjustments in the rate, source, timing, and placement of fertilisers are shown to optimise nitrogen use, reducing losses (Cameron et al., 2013; Coskun et al., 2017b). Chanes in crop rotation changes and soil management—altering SOM, moisture, aeration, and pH— significantly impact nitrogen dynamics (Basso et al., 2020). For example, increasing soil pH by applying lime can reduce denitrification rates (Thomson et al., 2012). Improved irrigation and soil drainage prevent anaerobic conditions, supporting optimal nitrogen retention and uptake, while reduced soil compaction enhances aeration (Thomson et al., 2012; Cameron et al., 2013). Avoiding intensive practices and adjusting fertiliser levels to align with plant needs can also support sustainable farming (Thomson et al., 2012; Cameron et al., 2013). Furthermore, cover crop and catch crops offer another strategy to capture residual soil nitrogen, reducing leaching and supporting long-term soil fertility (Kaye et al., 2019; Fernandez Pulido et al., 2023).

5.1. Catch crop

Catch crops are a strong asset in management of nitrogen dynamics and overall soil health, pest control and land preservation (Daryanto et al., 2018 cited by Fernandez Pulido et al., 2023). They can impact N_2O emissions significantly, reducing them by 12-36% compared to bare soil (Maljanen et al., 2004 cited by De Klein et al., 2019). This effect is caused by competition for matter, O_2 consumption, root exudates, changes of pH and C availability altering nitrification and denitrification rates (De Klein et al., 2019). Catch crops, especially non-leguminous, are known to reduce nitrate (Abdalla et al., 2019; Fernandez Pulido et al., 2023). According to Notaris et al. (2018), they reduce nitrogen leaching by an average of 23 kg N ha^{-1} $year^{-1}$ (60%) over a four years rotation in organic and conventional systems, making them a crucial factor in limiting nitrogen loss. Their effectiveness depends on crop type with grasses being more effective in reducing nitrogen leaching, while legumes may enhance soil fertility and yield of the subsequent crop (De Notaris et al., 2018; Abdalla et al., 2019; Fernandez Pulido et al., 2023). Common mixtures of non-legume cover crops may effectively reduce nitrate concentrations, particularly vulnerable to leaching during autumn and winter, and subsequently make nitrogen more available for following crops (Sapkota et al., 2012; Abdalla et al., 2019)

In addition, to improve nitrogen management, catch crops contribute to increase SOM and enhance carbon sequestration in soils. The decomposition of dead roots and biomass from cover crops improves both the quantity and quality of SOM (Abdalla et al., 2019). With regards to N_2O emissions, studies have showed that direct N_2O emissions can be either decreased or increased. Indeed, N_2O production depends on many factors and conditions of studies affecting results. An increase of N_2O emissions would also be explained by a higher root production stimulated by an increase of carbon supply (Abdalla et al., 2019; Fernandez Pulido et al., 2023). However, it seems that catch crops are able to reduce indirect N_2O emissions by limiting nitrate availability which is the substrate of nitrification. Overall, catch crops can mitigate net greenhouse gas balance by 2.06 ± 2.10 Mg CO_2 equivalent per ha per year (Abdalla et al., 2019).

5.2. Inhibiting nitrification

5.2.1. Synthetic nitrification inhibitors (SNI's)

Synthetic Nitrification inhibitors (SNI's) can improve fertilisers efficiency by inhibiting bacterial oxidation of NH_4^+ to NO_3^- and reducing competition for nitrogen between plants and soil microorganisms (Beeckman et al., 2018; Cooledge et al., 2022). This decrease in microbial competition helps mitigation of N_2O emissions and nitrate leaching (Beeckman et al., 2018). Synthetic nitrification inhibitors decrease direct N_2O emission by limitation of denitrification substrate (NO_3^-) by 8–57% and nitrate leaching by 38-56% (Coskun et al., 2017a; Lam et al., 2017; Wu et al., 2017). Furthermore, SNI's maintain more ammonium (NH_4^+) available for plant uptake by prolonging its retention time in soils

(Beeckman et al., 2018; Wang et al., 2021). The sustained presence of NH_4^+ can also stimulate lateral root branching, enhancing nutrient access for plants embodying a dual strategy for improving fertiliser efficiency and promoting sustainable agriculture (Beeckman et al., 2018). There are three types of inhibition mechanisms depending on their chemical structure (Dietz et al., 2012; Ruser et al., 2015):

- Direct Binding and Enzyme Interaction: NIs such as nitrapyrin and other compounds either bind directly to the active site of the enzyme ammonia monooxygenase (AMO) or attach to secondary binding sites that are not involved in ammonia oxidation. This type of interaction, involving non-polar inhibitors, is found to obstruct enzyme activity due to increased steric hindrance (Keener et al., 1993).
- Chelation of Copper Cofactors: Chelators such as dicyandiamide (DCD) and 3,4-dimethylpyrazole phosphate (DMPP) reduce copper availability, a key cofactor for AMO, thereby inhibiting NH_4^+ oxidation. This action disrupts AMO's ability to facilitate nitrification (Subbarao et al., 2006).
- AMO Inhibition: Some NIs lead to irreversible enzyme inactivation by modifying AMO structure by oxidizing substrates or the AMO enzyme itself, producing highly reactive compounds that irreversibly inactivate AMO or other associated enzymes (Subbarao et al., 2006; Dietz et al., 2012). Its reactivation requires synthesis of new enzymes (Ruser et al., 2015).

However, SNI's cause several drawbacks. They are difficult to use, especially because of difficulties of in application and they are costly for farmers (Coskun et al., 2017a, 2017b; Wang et al., 2021). Moreover, their production is a source of pollution and a risk for the environment because of leaching in soils contaminating water (Coskun et al., 2017b; Cooledge et al., 2022). They are also responsible to an increase of NH3 volatilisation by 3-65% responsible of indirect N_2O emissions (Qiao et al., 2015; Lam et al., 2017). The effectiveness varies according the SNI and is relatively short. It also depends on soil texture, structure, pH, crop type and management factors such as N fertiliser rate, irrigation, rainfed crops (Abalos et al., 2014; Wu et al., 2017; Beeckman et al., 2018). It have also been discovered that they could represent a risk for human health by an entry in food system (O'Sullivan et al., 2016; Cooledge et al., 2022).

5.2.2. Biological nitrification inhibition (BNI)

Biological nitrification inhibition (BNI) represents an innovative and sustainable strategy for managing soil nitrogen by making use of the production and release of compounds that inhibit nitrification. This process was first defined in 2003, with the tropical grass *Brachiaria humidicola* noted for its ability to suppress ammonia oxidation in soils (O'Sullivan et al., 2016). Biological nitrification inhibitor involves either the release of secondary metabolites or allelochemicals from plant roots, or decomposing plant tissues that act as nitrification inhibitors suppressing the activity of soil-nitrifying microorganisms. This

suppression slows the conversion of ammonium to nitrate (Dietz et al., 2012; de Klein et al., 2022). Studies highlighted significant biological nitrification inhibition influence on the nitrogen cycle, particularly on enzymes such as AMO and HAO, which are directly involved in nitrification processes (Subbarao et al., 2009; Coskun et al., 2017a).

The ecological and agronomic benefits of BNI are numerous. This process helps to reduce nitrogen losses through N_2O emissions and nitrate leaching, thus improving NUE by and allowing to fertiliser overuse (Subbarao et al., 2015a; Coskun et al., 2017a; Nuñez et al., 2018; de Klein et al., 2022). Including high-BNI plants in rotations with low-BNI plants can enhance nitrogen retention and boost yields in nitrogen-limited environments (Coskun et al., 2017a; Subbarao et al., 2013). Evidence of BNI's role in reducing N_2O emissions has been observed in field studies, underlying its potential for greenhouse gas mitigation in agriculture (De Klein et al., 2019).

Biological nitrification inhibition operates similarly to SNI's but with natural and continuous supply of nitrification inhibitors throughout the plant's growth. Unlike synthetic options, BNI does not require external application or costly machinery, making it an accessible, making BNI low-cost alternative with sustained effects (O'Sullivan et al., 2016; Nuñez et al., 2018; De Klein et al., 2019). In addition, BNI compounds remain in the soil's upper layers where fertilisers are applied reaching nitrifying sites in the soils through the root network, further enhancing efficiency (O'Sullivan et al., 2016; De Klein et al., 2019). Finally, it does not requires any synthetic production reducing the impact on the environment and leading to a greater public acceptance (De Klein et al., 2019). Henceforth, BNI could be selectively cultivated or enhanced in crop rotations, intercropping, or through breeding programs to maximize nitrogen conservation across different cropping systems (Coskun et al., 2017b; Beeckman et al., 2018).

Research has shown that the release of BNI compounds is a dynamic process regulated by environmental stimuli such as soil pH, moisture and aeration as well as nematode activity or ammonium concentration (Coskun et al., 2017b; De Klein et al., 2019). For example, high NH_4^+ levels near plant roots can stimulate BNI activity, particularly in plants adapted to nitrogen-limited environments. This adaptive mechanism is observed in species like *B. humidicola*, where BNI activity is triggered when roots come into direct contact with ammonium-rich soils (Subbarao, Wang, et al., 2007). Concerning *B. humidicola*, it is able to release BNI when roots are directly in contact with NH_4^+ (Coskun et al., 2017b). The presence of NH_4^+ and its uptake enhance BNI activity, with a synergetic effect observed in acidic soils with low NH_4^+ concentrations (Subbarao, Wang, et al., 2007; Vega-Mas et al., 2023). An environment or a rhizosphere rich in ammonium promote synthesis of BNI by plants while a greater nitrate concentration slows BNI synthesis (Zhang et al., 2019). Furthermore, synthesis and release of BNI is regulated by available nitrogen as shown with *Sorghum bicolor* (Zeng et al., 2016

cited by Wang et al., 2021). BNI compounds can be hydrophobic, maintaining activity close to root surfaces, or hydrophilic which are more mobile, allowing them to spread farther into the soil, affecting nitrification sites at greater distances (Subbarao et al., 2015b) the proportion of hydrophilic and hydrophobic BNI depends on plant species (Subbarao et al., 2013).

The effectiveness of BNI depends on factors such as plant species, developmental stage, and soil conditions while BNI potential depends on plant development or accumulation of BNI compounds in soils (Sullivan et al., 2017; Nuñez et al., 2018). When incorporated into farming systems, BNI can offer precision in nitrogen management with minimal external inputs, representing a long-term, cost-effective solution compared to SNI's (Beeckman et al., 2018). The gradual accumulation of BNI compounds in soils through root turnover and plant residues decomposition further enhances its effect over time, offering a sustainable alternative for improving nitrogen recovery and reducing nitrogen pollution in agricultural systems (Nuñez et al., 2018; Subbarao et al., 2006).

In contrast, although BNI capability extends to a large range of plant species, including grasses, weeds, and agricultural crops, plants that primarily absorb NO3, such as certain high-yield cereal cultivars, such as wheat, maize and rice, display little to no BNI activity (Coskun et al., 2017a; Peterson et al., 2024). It is important to note that recent research indicates that BNI capacity may depend on the specific cultivar and results of influenced by its conditions of experiment (Subbarao, Rondon, et al., 2007; Zakir et al., 2008; Coskun et al., 2017a). Moreover, studies reveal that BNI activity is not limited to root exudates but also occurs within decomposing plant tissues, potentially prolonging the inhibition effects after crop rotation or tillage (Coskun et al., 2017b).

The diversity of BNI compounds across plant species highlights their adaptation to specific environmental pressures, illustrating an evolutionary mechanism for nitrogen conservation in ecosystems where this element is limiting (De Klein et al., 2019). For example, tropical grasses like *Brachiaria spp.* show high BNI activity, possibly as a strategy evolved in low-nitrogen soils, whereas crops like wheat and maize, typically grown in nitrogen-rich systems, display minimal BNI (Gopalakrishnan et al., 2007; Subbarao et al., 2015a). The process influences the balance among ammonia-oxidizing archaea, ammonia-oxidizing bacteria, and nitrite-oxidizing bacteria, which compete with plants for NH_4^+, thus disrupting the NH_4^+/NO_3^- balance (Beeckman et al., 2018). Henceforth, BNI provides an advantage to weeds in competition for nitrogen access, as cultivated crops generally prefer NO_3^- while weeds prefer NH_4^+ (Blanck and Morgan, 2012 cited by Sullivan et al., 2017).

5.2.3. Potential BNI activity of ribwort plantain

Ribwort plantain (*Plantago lanceolata*) has been widely studied for its potential impact on nitrogen (N) cycling, particularly in relation to N_2O emissions and nitrate leaching, two critical concerns for sustainable agriculture. Research highlights a range of outcomes, influenced by plantain's bioactive compounds and morphological traits, but also indicates substantial variability and inconsistencies across different experimental conditions.

Plantain's influence on N_2O emissions has shown promises in several studies. Coskun et al. (2017b) and Carlton et al. (2019) report that applying identical livestock urine patches to monocultures of plantain instead of perennial ryegrass led to a 28% reduction in N_2O emissions, suggesting a plant-induced mechanism (Luo et al., 2018). Furthermore, Luo et al. (2018) emphasized that plantain had lower N_2O emissions across most seasons compared to ryegrass, a significant contributor to N_2O emissions from pasture soils. Yet, de Klein et al. (2022) point out that the relationship between plantain's BNI activity and N_2O emissions remain unclear, and evidence of a direct link is still lacking. Indeed, results are inconsistent, with some studies showing both reductions and increases in N_2O emissions (Simon et al., 2019; Pijlman et al., 2020; Bracken et al., 2021).

Nitrate leaching is another key area of interest. Carlton et al. (2019) found that adding plantain to a mix of perennial ryegrass and white clover reduced nitrate leaching, likely due to BNI compounds from plantain root exudates and lower soil drainage rates. In these studies, AOB abundance decreased by 33% after 30 days, while NH_4^+ concentrations were 57% higher, and nitrate concentrations were reduced by 8%, although AOA concentrations increased. Similarly, Massaccesi et al. (2015) demonstrated that plantain-dominated plots had lower NO_3^- concentrations, mineralisation, and nitrification rates, with higher NH_4^+ levels due to reduced nitrification. Interestingly, incorporating plantain leaves also reduced NO_3^- concentrations and improved N use efficiency, as shown by Dietz et al. (2012).

Bioactive compounds play a pivotal role in plantain's effects on N cycling. Aucubin, one of plantain's iridoid glucosides, increases during plantain growth (Navarette et al., 2016, cited by Cooledge et al., 2022). Dietz et al. (2012) and Gardiner et al. (2018) have identified catalpol, aucubin, and octeosides as potential BNI compounds. Both of them found that aucubigenin, a breakdown product of catalpol, could inhibit nitrification by affecting ammonia monooxygenase (AMO), a key enzyme in the process. Nonetheless, Dietz et al. (2012) noted that aucubin did not consistently inhibit nitrification in their soil incubation studies. Similarly, Gardiner (2018) observed that while plantain leaf extracts and aucubin solutions could reduce N_2O emissions significantly, they did not consistently affect inorganic nitrogen concentrations. This highlights the need for further long-term research on the persistence and

effectiveness of these secondary metabolites, as also suggested by Bracken et al. (2021) and Cooledge et al. (2022).

Peterson et al. (2024) illustrated that root exudates from plantain and urine from plantain-fed sheep could inhibit nitrification rates, but the effect varied with the plantain cultivar used. Rodriguez et al. (2023) showed that nitrification was reduced by 50% in plantain swards and 36% compared to ryegrass-white clover swards. Their findings also revealed that aucubin was predominantly found in leaves, reproductive stems, and roots, but its presence in root exudates was not confirmed. A comparison of gene abundance conducted by Simon et al. (2021) showed that plantain could inhibit the amoA gene, a marker for nitrification, suggesting potential BNI activity. However, Dietz et al. (2012) caution that aucubin's nitrification inhibition effects were short-term, and no studies have thoroughly examined the long-term persistence of these metabolites in soil systems.

Soil N processes were also examined in cattle grazing studies. Judson et al. (2019) reported that the urine from cattle fed plantain delayed the transformation of ammonium to nitrate, consistent with the BNI hypothesis, and similar outcomes were echoed in related studies. Nevertheless, Bowatte et al. (2018) found a negative relationship between soil nitrification potential (SNP) and relative N_2O emissions. High SNP correlated with increased nitrifier activity in the absence of plantain but decreased significantly when plantain was present, indicating a transient BNI effect when NH_4^+ concentrations were high. Moreover, no residual BNI activity was observed post-experiment, highlighting plantain's short-lived influence on nitrification.

Despite promising results, evidence remains mixed. Fernandez Pulido et al. (2023) found no significant effects of plantain on nitrification potential, NH_4^+ concentrations, or N_2O emissions. Bracken et al. (2021) observed a decrease in N_2O emissions by 31% to 75% water-filled pore space under drier conditions when plantain was added, but this effect was not evident under wetter conditions. Finally, while Pijlman et al. (2019) noted a 40% reduction in N_2O fluxes with plantain, the results did not correlate with plantain's proportion, suggesting an indirect influence on soil mineral nitrogen dynamics.

In conclusion, while ribwort plantain demonstrates potential to modulate N cycling and reduce N_2O emissions, the mechanisms remain elusive and effects variable. Studies by Dietz, Gardiner, Luo, Simon, and Rodriguez suggest that plantain may inhibit nitrification through compounds like aucubin, affecting AMO or by altering soil properties. However, variations in outcomes and the short-term nature of observed effects emphasize the need for further research to understand plantain's long-term impact and optimize its use in agricultural systems.

5.2.4. Focus on phacelia and mustard

According to a study aimed to screening BNI capacity of catch crops, there were no results concerning potential BNI of Phacelia and white mustard (Leemans, 2021). No information about it have been find in literature too concerning phacelia. However, a study realised by Brown and Morra in 2009 highlighted white mustard contains glucosinolates that produce isothiocyanates, which inhibit nitrification. The study found that soils amended with white mustard showed significant accumulation of NH_4^+ and NO_2^-, indicating potential nitrification inhibition linked to glucosinolate concentrations (Brown et al., 2009).

III. Objectives and question research

1. Initial objectives

The initial main objective is to compare the occurrence of BNI from two types of cover crops, White mustard and Phacelia (common cover crop mixture) and Ribwort plantain compared to a bare soil. Variables of interest are mineral nitrogen pools in soils, N_2O emissions, as well as nitrification rate in soils.

As a second objective, a comparison between terminated and non-terminated crops were supposed to be made, to understand nitrogen dynamics and BNI dynamics after termination of the two cover crops. It was supposed to be achieved by measuring N_2O emissions and mineral nitrogen one week after early termination of cover crop. Early ploughing is the termination of cover crop at the end of autumn while late ploughing is the termination of cover crop in spring.

Research questions:

- What does cover crops (ribwort plantain and White mustard + Phacelia) on mineral nitrogen pools in soils, N_2O emissions and nitrification rates compared to each other and to bare soil? Is there a long-term effect of cover crop termination time?
- Is there a presence of BNI for those two cover crops compared to a bare soil?
- There is still an effect of a plantain cover crop and/or the common cover crop mixture one week after termination?

2. Redirecting of objectives

Unfortunately, due to a delay of sowing and poor quality of the seeds of ribwort plantain, the germination rate was low and slow, resulting in few and small plants during the whole experimental phase. Therefore, the study only focusses on the comparison between bare soil and white mustard + Phacelia crop. Furthermore, the delay in sowing also delayed the early termination and did not allow to test the effects of cover crop one week after termination

New research questions:

- What are consequences of phacelia and white mustard mixture on mineral nitrogen concentrations, N_2O emissions and nitrification rates for each cover crop? Is there a long-term effect of cover crop termination time?
- Does White mustard or Phacelia show indication of BNI potential compared to bare soil?

IV. Materials and methods

1. Site description

The experimental field where data were collected is located in Gembloux (50.564443, 4.712369), within the Walloon region of Belgium. It is part of the experimental plots managed by Gembloux Agro-Biotech, a faculty at University of Liège (ULiège). It is situated in the loam agricultural region well known for its fertile arable lands where cereals, sugar beets and potatoes are commonly cultivated (SPW, October-15-2024). According to the Geoportal of the Walloon Region, the soils at this site are predominantly silty and benefit from good natural drainage (Figure 3). The research area encompasses several soil types described in the following table.

Table 1: type of soils according to "Carte numérique des soils de Wallonie".

Soil type	**Definition**
Aba(b)0	loamy soil with a textural B horizon spotted and a thick A horizon, with a good drainage capacity (non-gley soils)
Aba1	loamy soil with a textural or structural B horizon and a thick A horizon, with a good drainage capacity (non-gley soils).
AbB	loamy soil with a textural B horizon and a thin A horizon, with a good drainage capacity (non-gley soils)

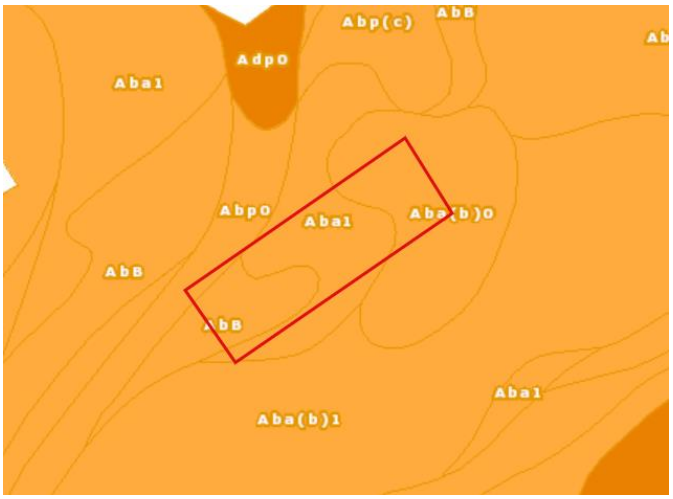


Figure 3: carte numérique des sols de Wallonie" of the experimental field ("WalOnMap," November-21-2024)

Figure 2: aerial view of experimental field

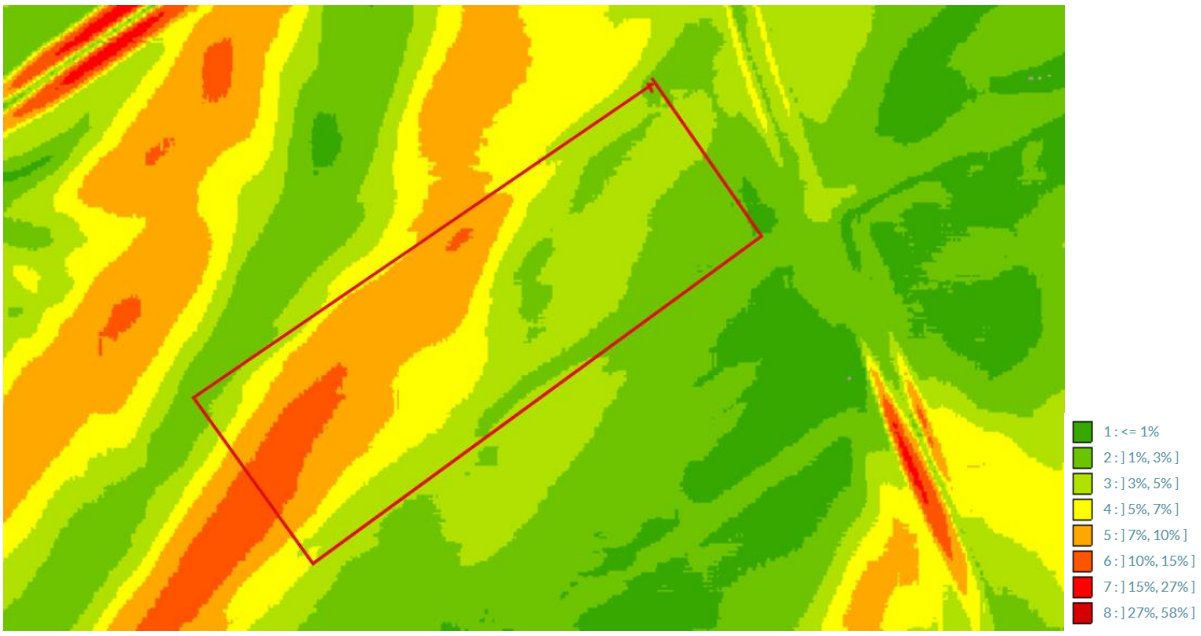


Figure 4: gradient of slope on the experimental field

Additionally, the field area is about 16 800 m² (224m of long and 75m of large) and is situated on a plateau at on average of 162 m of altitude ("Carte topographique Belgique, altitude, relief," November-21-2024). A slight slope is also observable in the field (Figure 4), leading to some differences in properties. This affects the thickness of the soil and soil moisture due to erosion and drainage capacity. Furthermore, the bottom of the slopes contains more residues (Denis, 2023). During 2024, winter wheat was grown on the field.

2. Weather conditions

2.1. Climate

The site is charactered by an oceanic temperate climate. With relatively warm summers on average 19°C on July (the warmest month of the year), while the average temperature in Winter is 10,2°C, higher than Belgium average. The area normally has 56.3 days of freeze per year with 1.8 days with maximal temperature below 0°C. Concerning rainfall, there are about 130.3 rainy days with a total 793.4 mm of rain per year on average. The most wet season starts at the beginning of February until May with a probability of rainfall higher than 29% (KMI between 1991 and 2020).

This year, 2024, was an exceptionally wet year with in total of 951.3 mm from the beginning of the year until the end of the experiment on December 17. However, the weather was relatively mild with a mean temperature of 11.5°C.

2.2. Rainfall

Following data concerning weather conditions during the experiment come from Agro-met.be. This website contains weather measurement from Gembloux station of CRA-W. The weather station is located just beside the experimental field.

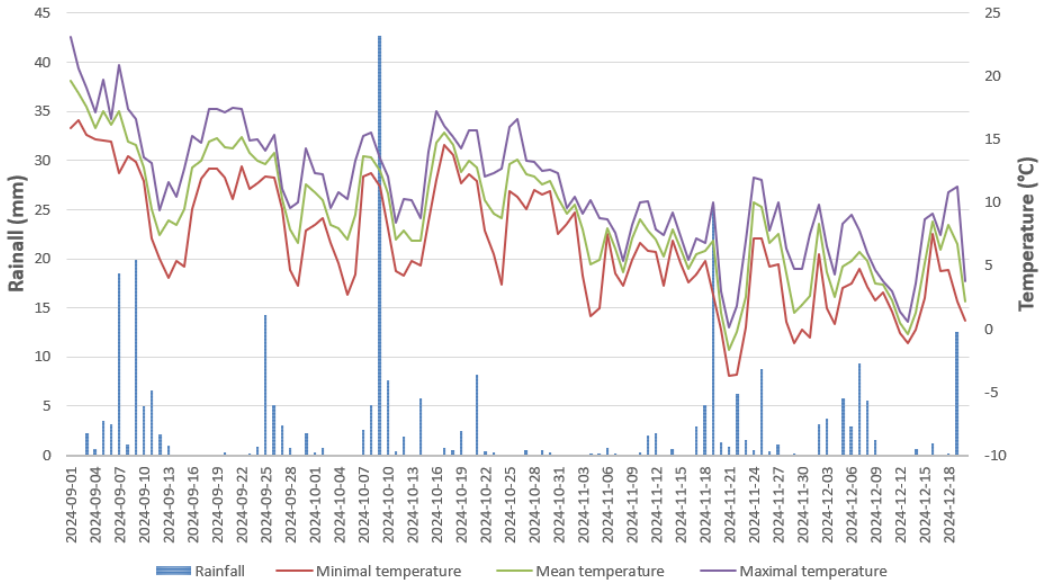


Figure 5: rainfall per day from September 1 until December 17

The cover crop should have been seeded before the September 15 to respect the PGDA legislation in Belgium. As showed on Figure 5, rainfall was relatively high at the period, making seeding impossible. Significant amounts of rainfall had also come before September (748.6 mm during the year, much in summer), and soils were very wet. A derogation was approved, allowing a seeding until September 30. This postponed all coming data collection After that, several rainy periods appeared with nearly 300 mm of precipitation influencing data collection in field too. We can note an important rainfall event on the October 9 with 42,7 mm of rain on figure 6.

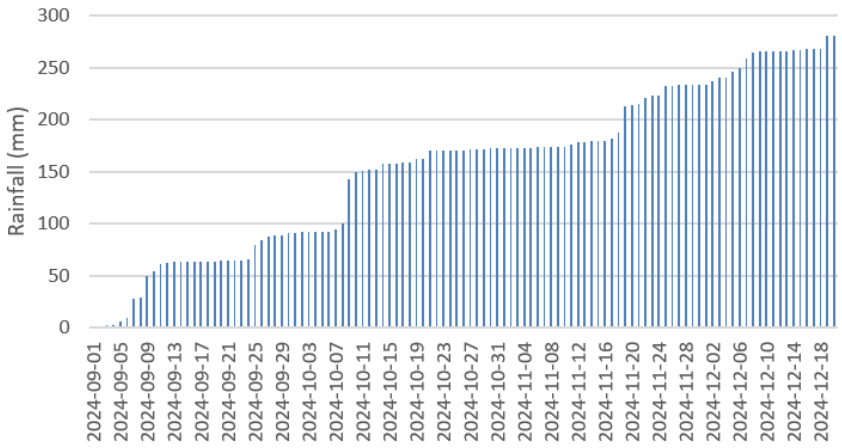


Figure 6: cumulative rainfall from September 1 until December 17

2.3. Temperature

Temperature decreased during the season and varied much over time. Minimal temperature was below 0°C with three times during experimental period. On November 21, the soil got covered in snow that stayed for a couple of days. The average temperature was below 0°C on that day leading to a frost episode.

The number of Growing Degree-Day (GDD) during the experimental period of growing (from seeding to the end of the experiment) is 192.1.

3. Description of cover crops

3.1. Ribwort plantain L.

Ribwort plantain, from Plantaginaceae familia, is a pasture species that thrives in temperate climates and is capable of adapting to a wide variety of soils. This plant is highly effective in reducing nitrogen losses in the soil (Stewart, 1996).

3.2. Phacelia tanatetifolia Benth.

Phacelia Tanacetifolia, from Boraginaceae familia germinates in 10 days and completes its growth cycle in 98 days, with optimal yields when sown in mid-April. It is frost-resistant, with young plants tolerating temperatures as low as -8°C. It requires about 480 degree-days to achieve 50% soil coverage. This species thrives in aerated, moderately moist, and slightly basic soils. Highly adaptable, it is resistant to diseases and pests, and effectively controls weeds ((Kubíková et al., 2022).

3.3. *Sinapis alba* L.

White mustard, from Brassicaceae familia, germinates within 7 to 14 days and completes its growth cycle in 85-90 days. This plant can be sown from March to October. it is sensitive to frost, as temperatures below 0°C can be detrimental to its development. White mustard is a versatile plant, protecting crops from diseases, insects, and weeds while preventing soil leaching by rain (“Moutarde blanche", Semaille).

4. Experimental design

Each plot has been delimited respecting the design on Figure 7. It is composed by four treatments: Late harvest (**Lh**), Late Ploughing (**Lp**), Early Ploughing (**Ep**) and strip-till taking part of a log-term experiment from 2008. The experimental design is a randomised block design composed of four blocks disposed according slope gradient, horizontally on figure 7. Each plot is divided in two zone. The right side is used for estimating yields and cannot be used for any other measurements while the left side can be used for both destructive sampling and manipulation of the experiment.

This study focuses exclusively on late ploughing (**Lp**) and early ploughing (**Ep**). No data were collected for the strip till treatment. This explains why those subplots were not assigned any identification

number. Data regarding the three other treatments were collected, even though they were not analysed as part of this work.

Finally, subplots (quadrats) have also been delimited according three different cover crops: bare soil (**C**), plantain (**P**) and the common cover crop mixture composed by phacelia and white mustard (**M**). There are three repetitions per type of cover crop in each plot (except for strip-till) respecting the following design. When sampling, samples were collected from two or three of these subplots and merged (biomass), pooled (soil samples), or taken an average of (N_2O measurements) for further analysis. As plantain did not grow well in the experiment, those plots have not been included in the analysis of this thesis.

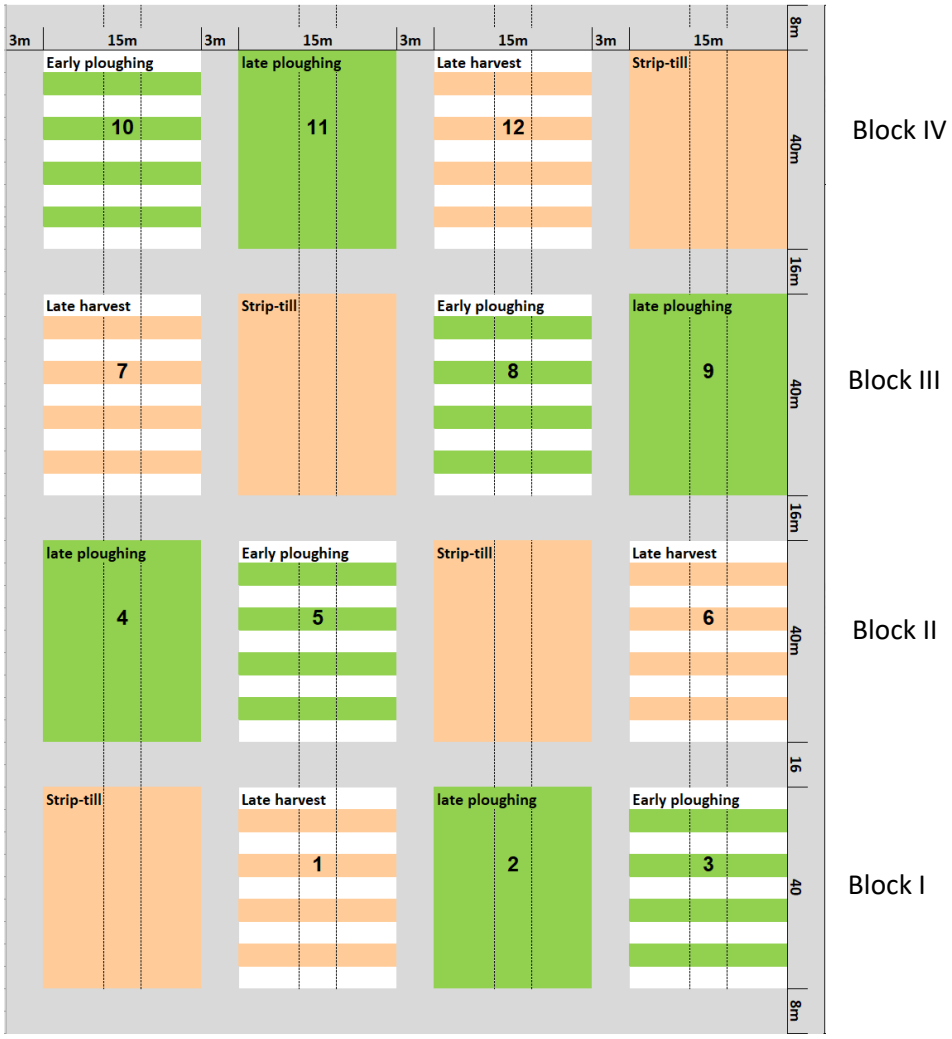


Figure 7: experimental design

5. Description of the experiment

5.1. Preparation of the field experiment

The first step of the experiment is sowing of the common cover crop composed by phacelia and mustard. It was done mechanically by Cepicop's staff on the September 24. After a few days of growing, the different big plots were traced with the help of a GPS of a tractor and subplots were delimited by hand with a measuring tape as shown on the following Figure (Figure 11). Each subplot is a 2-meter by 2-meter square (4m²). The delimitation of plots was done on September 30.

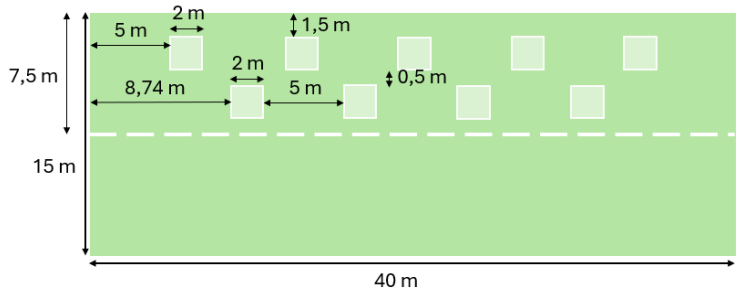


Figure 8: experimental design, subplot delimitation

The following day (October 1), quadrats designated for seeding with plantain, as well as bare soil plots, were cleared, taking advantage of the early development of the cover crop and avoiding to prevent the regrowth of the destroyed plants. The destruction of the cover was done manually with the help of a garden hoe. Finally, plantain was broadcast-seeded on October 2 with 5 grams of seeds spread in each subplot.

5.2. Data collection

On October 7, initial samples were taken down to a depth of 15 cm using a soil auger to analysis mineral nitrogen levels of each treatment. The rest of samples were taken on October 28, November 14 and November 26. It was decided to exclude the top 5 cm of soil and focus instead on the 5–15 cm depth range, where we think the BNI compounds could be more active. Sampling at greater depths would not accurately represent nitrogen content in the part of soil containing plant roots. One soil sample per subplot has been taken at a random place in it. Ultimately, sample from each subplot were merged and combined into a single composite sample corresponding to a modality (Cover*Treatment). Based on those soil samples, initial NO_2^-, NO_3^-, NH_4^+ concentrations and initial water content have been determined for all modalities. Just after sampling, 15 grams of soil from each sample were extracted on October 7 and 5 grams on the other sampling date using the method explained in the following point. All extracted samples were stored in a -20°C freezer until the analyses.

Nitrous oxide emissions were measured on November 14 and 16 and November 26 and 28 as measurements took more than a day. Nitrous oxide emissions were measured in two subplots with

white mustard and phacelia and with the bare soil control. The measurements were made during 17 minutes and only collected in block I, II and IV.

At the end of the experiment, potential nitrification rates just before termination of cover crop. Indeed, nitrification rates can be an interesting indicator of BNI activity (Nuñez et al., 2018). In each subplot, samples were collected along the roots of each type of plant, both white mustard and phacelia for the cover crop, and at random locations for the bare soil. For cover crop subplot, a random spot was chosen where plants were dogged up with sufficient soil attached. We meticulously selected soil that was in close contact with plant roots. Phacelia and white mustard were separated in two different bags. Samples of the three subplots of a same plant type were combined into one sample as for usual samples. Samples were collected the November 28 and analysed the following weeks the laboratory at ULB.

Biomass of the two cover crops were also collected on November 29. In each subplot, a 40 by 40 cm square has been randomly chosen to do biomass samples. Phacelia and white mustard have been cut just on the surface of soil. As for soil sampling, biomass of the three subplots of each modality have been combined into one common sample. Biomass of phacelia and white mustard have been separated. Finally, plants have been putted in plastic bag able to resist to high temperature and placed in a 35°C oven and dried for a few days. After a few days, temperature was increased until 70°C to finish drying. Finally, they have been weighed separately and biomass per square meter (g/m^2).

Early termination plots were terminated on the December 17 due to the PGDA legislation and weather conditions. This late termination did not allow for the planned comparison of soil conditions after termination.

5.3. Soil extraction

To get a representative subsample for mineral nitrogen determination, each sample was mixed and broken into smallest pieces to ensure the heterogeneity of samples and their representativity as they need to represent a great variability. Samples for initial soil mineral nitrogen were extracted with 15 g of soil and 20 ml of 0.5 K2SO4, after further testing it was decided to use KCl as extractant for the samples following the mineral nitrogen dynamics in the crop (Appendix 1). 5 g of soil was mixed with 25 ml of KCl and the tube is shaken for 1 hour at 165 rpm on an orbital shaker, placed horizontally. The soil is then allowed to settle for at least 1 hour. The supernatant is filtered through Whatman no. 42 filter paper using small funnels, yielding 12-15 ml of extract for further analysis.

5.4. Dry weight

From the homogenised soil samples, a 15 grams subsample were taken and put in 50 mL flasks. The samples were dried at 35°C about four days to determine the fresh/dry weight ratio. After drying, the

samples were weighed again using same scale. From those measurement, a “fresh/dry ratio” was calculated for each soil sample. This ratio was later used to calculate mineral nitrogen concentrations per g of dry weight soil.

6. Observations and measurements

Table 2: summary of experimental measurements. “Variable of interest” is the variables which has been measured. “Date of measurement” concerns all the moments where data were collected. “Collect of data” refers all data collected. “Data base” refers observations which have been conserved for statistical analysis.

Variables of interest	Date of measurement	Collect of data	Data base
N_2O emissions	/, /, 14th Nov, 26th Nov	PC2, PM2, PC3, PM3, PC4, PM4, PC5, PM5, PC10, PM10, PC11, PM11	PC2, PM2, PC3, PM3, PC4, PM4, PC5, PM5, PC10, PM10, PC11, PM11
Nitrite	7th Oct, 28th Oct, 14th Nov, 26th Nov	All subplots	PC2, PM2, PC3, PM3, PC4, PM4, PC5, PM5, PC8, PM8, PC9, PM9, PC10, PM10, PC11, PM11
Nitrate	7th Oct, 28th Oct, 14th Nov, 26th Nov	All subplots	PC2, PM2, PC3, PM3, PC4, PM4, PC5, PM5, PC8, PM8, PC9, PM9, PC10, PM10, PC11, PM11
Ammonia	7th Oct, 28th Oct, 14th Nov, 26th Nov	All subplots	PC2, PM2, PC3, PM3, PC4, PM4, PC5, PM5, PC8, PM8, PC9, PM9, PC10, PM10, PC11, PM11
Nitrification rates	/, /, /, 26th Nov	All subplots	PC2, PM2, PC3, PM3, PC4, PM4, PC5, PM5, PC8, PM8, PC9, PM9, PC10, PM10, PC11, PM11
Biomass	/, /, /, 26th Nov	All subplots	PC2, PM2, PC3, PM3, PC4, PM4, PC5, PM5, PC8, PM8, PC9, PM9, PC10, PM10, PC11, PM11
Water content	7th Oct, 28th Oct, 14th Nov, 26th Nov	All subplots	PC2, PM2, PC3, PM3, PC4, PM4, PC5, PM5, PC8, PM8, PC9, PM9, PC10, PM10, PC11, PM11

6.1. N_2O emissions

Nitrous oxide emissions were analysed using inox chambers with an area of 0.15 m^2 and approximately 8.5 cm higher were placed in each plot. These chambers were inserted 3.5 cm into the soil, leaving 5 cm of the chamber above the soil surface and total volume of about 0,008 m^3. Two chambers were installed per treatment modality (e.g., Lp*C), with one chamber placed in each subplot located at the edge of the plot in order to capture the greatest possible variability in the plot. Measurements were taken using analyser: “The LI-7820 N_2O/H_2O Trace Gas Analyzer” which directly draws air from the chamber and measure N_2O concentrations.

N_2O concentrations were recorded per second within the ventilated chambers. From these data, linear regressions of N_2O concentration over time were generated over 17 minutes, and the regression

coefficients of these lines were retained. Subsequently, the two coefficients from each treatment were averaged. This is the increase N_2O per minute (ppmv). Finally, N_2O emissions were calculated as follows:

$$\frac{CA*60*28*100*P}{8{,}314*(273{,}15+T)} * \frac{V}{A} * 24 * \frac{10000}{1000000} = N_2O \text{ emissions (g/ha/d)}$$

with,

CA being the increase N_2O per minute (ppmv),

A, the footing area (m^2),

V, the Chamber volume (m^3)

T, the mean temperature of air on measurement day (°C) and

P, the pressure (hPa).

If slopes were negative, they were not included in the average as they indicate a measurement error.

6.2. Mineral nitrogen determination

Concentrations of nitrite and nitrate were determined following the commonly used protocol by Miranda. Nitrite is measured using Griess reagents (Sulfanilamide 1% and N-naphthylethylenediamine dihydrochloride (NEDD) (0,1%)) which in contact with NO_2^- turns pink and the absorbance at wavelength 540 nm was measured after 15 minutes, using a spectrophotometer. The microwell plates sued for the absorbance measurements included two technical replicates for each sample, from which an average was later calculated, two blanks with technical replicates and two NO_2^- curves for latter calculation of NO_2^- concentrations in the samples (Figure 13).

	1	2	3	4	5	6	7	8	9	10	11	12
A	PM1		PM5		PM9		Blancks				C	C
B	PC1		PC5		PC9		Blancks				U	U
C	PM2		PM6		PM10						R	R
D	PC2		PC6		PC10						V	V
E	PM3		PM7		PM11						E	E
F	PC3		PC7		PC11						!	!
G	PM4		PM8		PM12						!	!
H	PC4		PC8		PC12						!	!

Figure 9: microplate design to determine nitrogen concentration

After that, absorbance of each replication was calculated by using a spectrophotometer. An average of the absorbance has been done for each sample. The absorbance value was corrected by subtracting the average absorbance of blanks. The NO_2^- concentration was calculated using linear regression of

NO_2^- from the reference curve with the highest r^2 has been chose to determine NO_2^- concentration. Finally, NO2 concentration per gram of dry soil (µM NO_2^- /g of dry soil) was determined by dividing the fresh weight with the fresh/dry weight ratio.

Before NO_3^- and NH_4^+ determinations, dilution of a couple of samples from the treatments were tested before diluting or analysing other treatments. After testing, it appeared that any dilution was required.

To determine NO_3^-, the soil samples extract was mixed with the reducing agent VCl3 and Griess reagents. Vanadium (III) chloride reduces NO_3^- to NO_2^- which then reacts with the Griess reagent. Absorbances at wavelength 540 nm was measured after 30 minutes. To determine the actual NO_3^-, the absorbance from the NO_2^- measurement is subtracted from that of the reduced NO_3^- sample. After this, the determination of NO_3^- per g of dry soil (µM NO_3^-/g of dry soil) was done as for NO_2^-.

Ammonium concentrations were determined by Batherlot method based on phenol hypochlorite assay (Patton et al., 1977). After reaction with NH_4^+ contained in samples, reagents (sodium phenolate 0.33M, Sodium nitroprusside 0,02% and Sodium hypochlorite 2%) turn blue. The absorbance at wavelength 635 nm was measured after 30 minutes, using the spectrophotometer. The determination of NH_4^+ per g of dry soil (µM NH_4^+ /g of dry soil) was done as for NO_2^- and NO_3^-.

6.3. Potential nitrification rates

The slurry test was conducted in the laboratory following the protocol in Appendix 3. For each sample, a fresh/dry weight" has also been calculated to determine NO_3^- concentration per gram of dry soil.

At the end of the slurry test, we obtained samples for each soil at six different time points: T0 = 0H00, T1 = 1H30, T2 = 3H00, T3 = 4H30, T4 = 22H30, T5 = 24H00 etc. For each collected sample, concentrations of NO_2^- and NO_3^- have been measured, following the same ah protocol above. Based on this, a linear regression was established for each soil over time. The linear coefficient was used to calculate the nitrification rate using the following formula:

$$\frac{Rate}{(mg\ N\ kg^{-1}h^{-1})} = Incr.[NO3] \times \frac{0.1\ L\ + volume\ of\ water\ in\ field}{weight\_dry\ soil\ in\ flask}$$

where volume of water in field is wet weight – dry weight of soil/density of water (=1000),

and

dry soil in flask is the extracted weight*dry weight/wet weight.

After that, to obtain a unique nitrification variable exploitable in data analysis, a weighed nitrification rate has been determined for each subplot of cover plot.

6.4. Water content

From the measurements made for dry matter, it was also possible to calculate the water content of each soil according to the following formula: $\left(\frac{Wet\ soil - Dry\ soil}{Dry\ soil}\right) * 100 = Water\ content\ (\%)$.

7. Data analysis

Data analysis was realised on R studio (Version 4.4.2). In the first instance, a univariate analysis has been realised for each variable in table X. The aim is to analyse the effect of each factor and their interaction on the collected data. Secondly, to observe interactions and influence of variables each other, a multivariate analyse has been realised by application of a PCA (principal components analysis).

7.1. Univariate analysis

For this statistical analysis, four factors have to be taken into count; the treatment, the type of cover crop, the Block and the date of the measurement.

Table 3: presentation of factors used in statistical analysis

Factor	Fix/Random	Quantitative/Qualitative	Number of modalities
Treatment	Fix	Qualitative	2: Lp (late ploughing), Ep (early ploughing)
Cover crop	Fix	Qualitative	2: M (mixture), C (control)
Block	Random	Qualitative	4: I, II, III, IV
Date	Fix	Qualitative	4: 7th oct, 28th Oct, 14th Nov, 26th Nov

Table 4: presentation of variables analysed in univariate analysis (ANOVA)

Variables	Number of repetitions
N_2O emissions	1
$[NO_3^-]$	1
$[NH_4^+]$	1
$[NO_2^-]$	1
Nitrification rates	1
Water content	1
Biomass of Phacelia	1
Biomass of White mustard	1

Data analysis was conducted using a generalized linear mixed model (GLMM) with the glmer() function from the lme4 package to account for the random factor (Block) variability. Model verification was performed with the Dharma package, followed by a three-factor ANOVA on the linear regression. As factor date was highly significant for each variable, two-factor ANOVAs per date were also conducted on linear models, with verification of application conditions with Levene's and Shapiro-Wilk tests applied to residuals. Post-hoc comparisons were performed using the emmeans() function for significant factors or interactions. Significant interactions were also explored by one-factor ANOVAs concerning treatment*Cover interactions.

Distribution normality was tested on the full dataset and per date. Non-normal data were transformed using inverse transformations, commonly applied for concentrations. Outliers were identified as values exceeding 1.5 times the interquartile range above the third quartile or below the first quartile ("Comment détecter les outliers avec R - DellaData," 2018).

Initial NO2− and NO_3^- concentrations were retained for trend comparisons but not directly compared with subsequent measurements due to differing extraction methods.

7.2. Multivariate analysis

Table 5: presentation of variables analysed in multivariate analysis (PCA and correlation)

Variables	Definition
WC.final	Water content at the end of the experiment
Delta.NH4gr	Variation of NH_4^+ concentration between October 28 and November 26
Delta.NO3gr	Variation of NO_2^- concentration between October 28 and November 26
Delta.NH4gr	Variation of NO_3^- concentration between October 28 and November 26
NO2gr.final	Final NO_2^- concentration per gram of dry soil (November 26)
NO3gr.final	Final NO_3^- concentration per gram of dry soil (November 26)
NH4gr.final	Final NH_4^+ concentration per gram of dry soil (November 26)
Ph.biomass	Biomass of phacelia on November 26 per m²
WM.Biomass	Biomass of white mustard on November 26 per m²
Mid-Time.N2O.emissions	N_2O emissions on November 14
Final.N2O.emissions	N_2O emissions on November 26
Nitrification rate	Potential nitrification rate

An initial analysis of the global dataset was conducted to understand interactions between variables. Subsequent analyses were performed with and without biomass, based on the results of the global analysis. Correlation analyses were conducted with Spearman method as some distributions was not normal. Finally Leave-One-Out (LOO) model was applied to assess the influence of explanatory variables on biomass for phacelia and white mustard.

V. Part 4: results and discussion

1. Univariate analysis

1.1. N_2O emissions

There was a highly significant impact of dates on N_2O emissions. To better understand this effect, additional analyses of variance were conducted separately for the two sampling dates. Analysing each date separately showed that on November 14 there was a significant difference between ploughing treatment (p=4.29e-05) as well as a high interaction between treatment and cover crop (p=2,54e-04).

*Table 6: results of variance analysis of N2O emissions for two factors. *<0.05, **<0,01, ***<0,001*

N2O emissions	14-nov	26-nov
Treatment	***	n.s
Cover type	n.s	n.s
Treatment*Cover	***	n.s

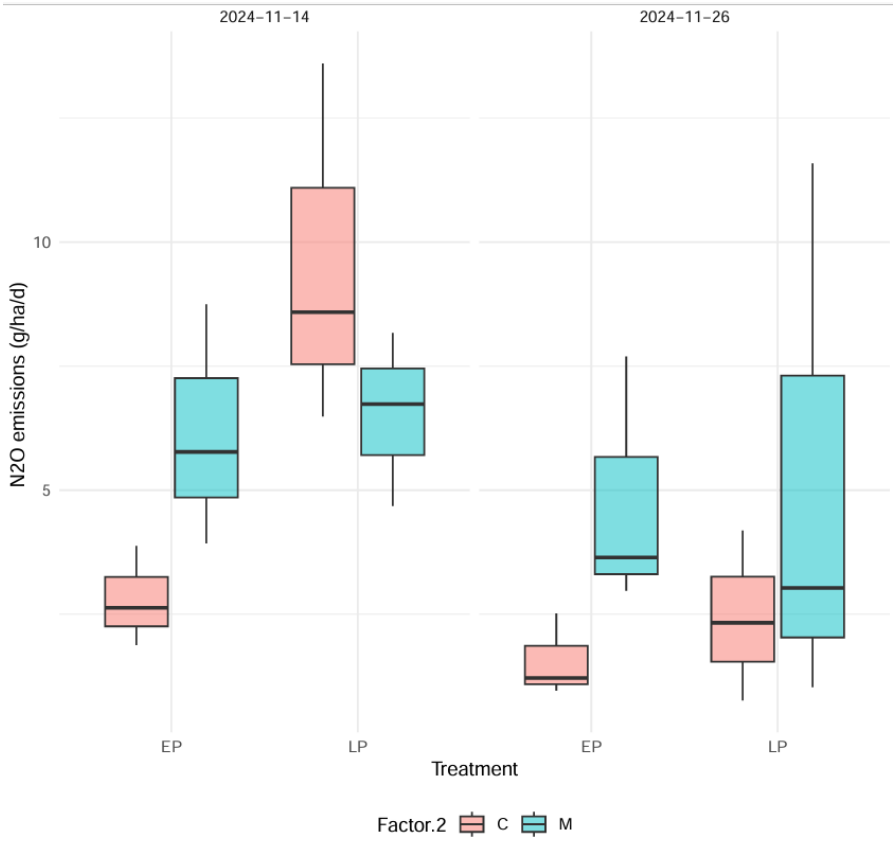


Figure 10: N_2O emissions (g/ha/d) in the different treatments and type of cover crop on November 14 and 26. Ep=early ploughing, Lp=late ploughing, M, crop mixture, C= control.

First analysis of this graph (Figure 14) suggests a temporal effect, as N_2O emissions are lower on the second date for each modality (Treatment*Cover). The difference between the two dates may be explained by weather events. Nitrous oxide emissions are positively correlated to the combination of soil water content and temperature. Temperatures has a greater impact when water content is higher

(Schindlbacher et al., 2004; Qiu et al., 2018). Those results correspond to the conditions of this study: a marked decrease of N_2O emissions with decreasing temperatures at a higher water content (diminution of 1,2°C on average between the two sampling dates).

An intensive frost happened between the two sampling dates and could be responsible of this decrease of N_2O emissions by a diminution of microbial activity caused by frost of the November 21It has already be demonstrated that microbial activity, particularly that of AOA, which are involved in the nitrification process), is positively correlated with temperature (Haizhou Li, et al., 2015). When nitrification rates slow down, so does the NO_3^- pool in the soil, which also reduces N_2O emissions. Furthermore, microorganisms responsible of denitrification rate would be similarly influenced, thereby explaining the observed reduction in N_2O emissions (Braker et al., 2010).

In early ploughing treatment, N_2O emissions were always higher and variable from cover crop mixture than from the bare soil control despite a decrease on November 26. However, emissions from late ploughing treatments had larger variability on both dates.

Moreover, late ploughing appears to result in higher overall N_2O emissions compared to early ploughing. On average, emissions were higher for Lp treatments, with an average N_2O emission of 5,93 compared to 3.82 for Ep treatments. This pattern could be attributed to the extended period during which the soil remains undisturbed in Lp treatments, allowing for the accumulation of SOM and nitrogen that are rapidly processed when the soil is eventually ploughed.

The presence of a cover crop generally resulted in higher emissions than bare soil in most treatments, except of LP treatment on November 14. Additionally, N_2O emissions appear to be more stable across both dates and treatments when a cover crop is present (Figure 11). It may be explained by the physical protection offered by the cover to the soil against weather events, as the frost period which happened during this experiment. This also explain why the decrease of N_2O emissions is less marked for LP.M modality than LP.C. It sees as the cover provides more stables conditions but does not prevent N_2O emissions.

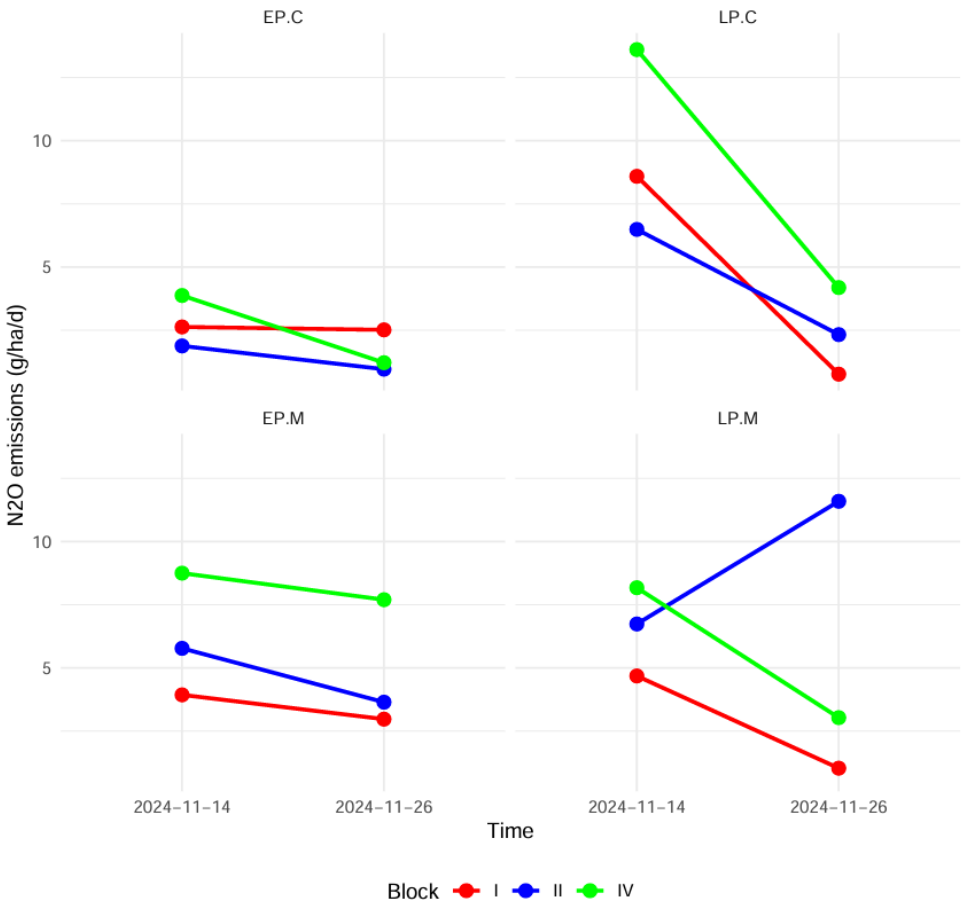


Figure 11: evolution of N_2O emissions over time

The evolution of emissions over time also seems more variable for Lp treatment, suggesting that it was more impacted by temporal factors. Furthermore, the variability in Lp.M treatment seems to come from plot P4M soil on Figure 11. This plot could be considered as an outlier as it is the only only with a contradictive behaviour.

The interaction between factors on 14th November provides further insights into dynamics of N_2O emissions. Treatments with cover crops maintained a more stable emission profile over time compared to bare soil, reinforcing the potential buffering effect of cover crops on N_2O emissions. Overall, these results emphasize the importance of both ploughing time and cover crop presence in determining N_2O emissions. Late ploughing and the absence of a cover crop (=Lp.C modality) are associated with higher emissions, while the use of cover crops appears to stabilize emissions across time and treatment types.

Table 7: emmeans values of the interaction between factors

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment*Cover	3	68.95	22.984	3.936	0.0538 .
Residuals	8	46.71	5.839		

Table 8: means of N_2O emissions per modality and per cover

N2O emissions	Modality	Cover
EP.C	2,797	6,177
LP.C	9,560	
EP.M	6,529	6,339
LP.M	6,151	

Concerning ploughing time, there are no significant short-term effect as explained in a study of Boecks et al., (2011) compared to long term effects which are more pronounced. Few direct links have been found in literature explaining differences between early and late ploughing. As showed by several studies, tillage practices (ploughing VS no-till) and ploughing timing have an impact on microbial activity and diversity by destroying interactions especially in the 0-20 first cm of the soils (Mo et al., 2024; Shi et al., 2024). Indeed, BNF and denitrification are higher in no till systems. If conditions following early ploughing were favourable to microbial activity (BNF and denitrification), such as warm temperatures and well-aerated soil provided by ploughing, higher NO_3^- levels and elevated emissions may occur early on influencing N_2O measurements. However, lack of N_2O emissions measurements do not allow to confirm it. Ploughing initially reduces total nitrogen and carbon in the soil as showed in a study of Laine with a gradual increase of these elements replenished by microbial activity over time (Laine et al., 2018). This delayed replenishment process could result in N_2O emissions occurring later in the season for late ploughing treatments which were possible to observe.

Concerning presence of a cover crop, means of cover crop and control are relatively the same with a little bit higher value for cover mean (Tab.4). As mentioned in the literature review, even if lower N_2O emissions would be expected with a cover crop, effect of catch crop on N_2O emissions depends on experimental conditions. Higher N_2O emissions could be explained by a higher root production stimulated by an increase of carbon supply provided by cover crop biomass (Abdalla et al., 2019; Fernandez Pulido et al., 2023). Concerning to stability of N_2O emissions, it can also be attributed to the stable environment provide by the cover (Wanic et al., 2019).

1.2. NO_2^- concentration

Neither significant interaction between nor significant factor have been highlighted by ANOVA test. From this, it can be concluded that the cover or the treatment does not significantly impact NO_2^- concentration in soils.

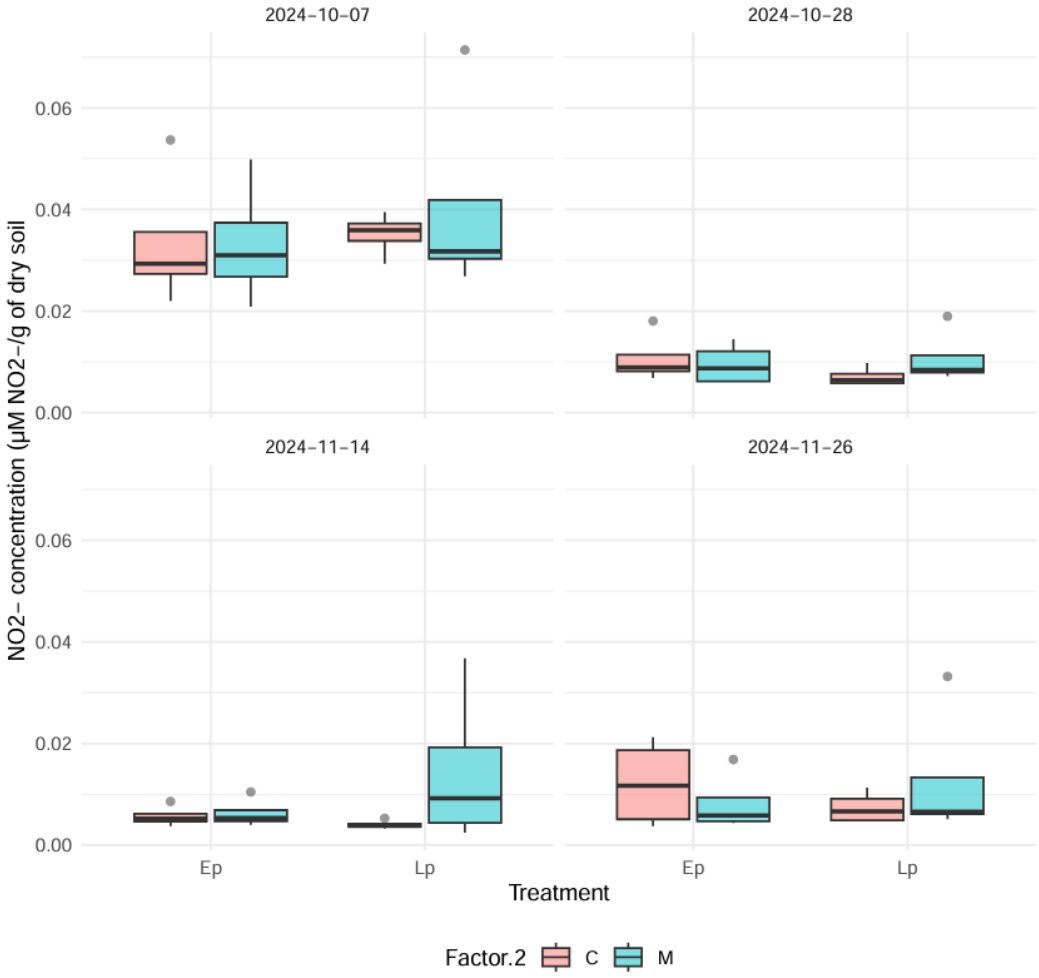


Figure 12 : NO_2^- concentration (µM NO_2^-/g of dry soil) in the different treatments and type of cover crop at four sampling points. Ep=early ploughing, Lp=late ploughing, M, crop mixture, C= control.

According to the boxplot, both treatments (Ep and Lp) exhibit similar overall trends across the experimental period. However, the Lp.M group consistently shows higher variability, particularly on the 14th November, where outliers are observed. However, it is not possible to attest that those relationship are accurate as ANOVA revealed any significant factor.

Ultimately, as NO_2^- is an intermediate in the nitrification process, its lifespan in the soil is relatively short. As a result, studying its concentrations and dynamics is particularly challenging. This explains why no significant results were observed regarding NO_2^- concentrations. In the literature, most studies focus on nitrate concentration in soils, leaving fewer data available for nitrite dynamics. For example, these studies report a decrease in nitrate concentrations in similar contexts (Wyland et al., 1996; Fraser et al., 2013). By rapid absorption of nitrate in soil by roots (Justes et al., 2012).

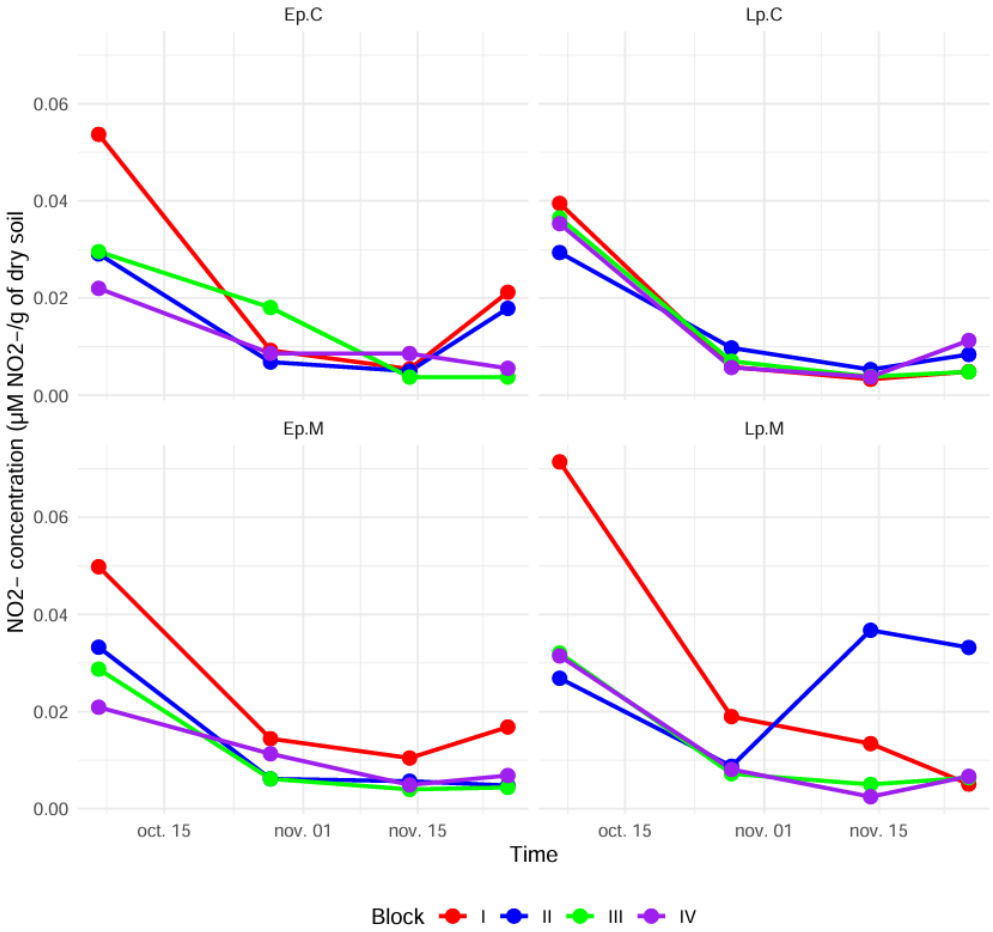


Figure 13: evolution of NO_2^- concentration over time

Over time, NO2- concentrations remained relatively stable and low over the last three sampling dates. Any block effect is revealed. However, a slight increase in concentrations is observed toward the end of the experiment for a majority of samples, as illustrated on the Figure 13. This increase between two last sampling dates is explained by a very higher NH4+ concentration which is the substrate for nitrification and NO2- production.

1.3. NO_3^- concentration

ANOVA 3 revealed Date as a significant factor (p=1.07e-07). There were significant differences in NO_3^- content in soils from the different treatments and type of cover crop as shown in table 8. Ther was no significant interaction between factors does not exhibit any significant effect at any time point.

*Table 9: results of variance analysis of NO_3^- concentrations for two factors. *<0.05, **<0,01, ***<0,001*

NO3- concentrations	07-oct	28-oct	14-nov	26-oct
Treatment	n.s	*	n.s	*
Cover type	n.s	*	*	n.s
Treatment*Cover	n.s	n.s	n.s	n.s

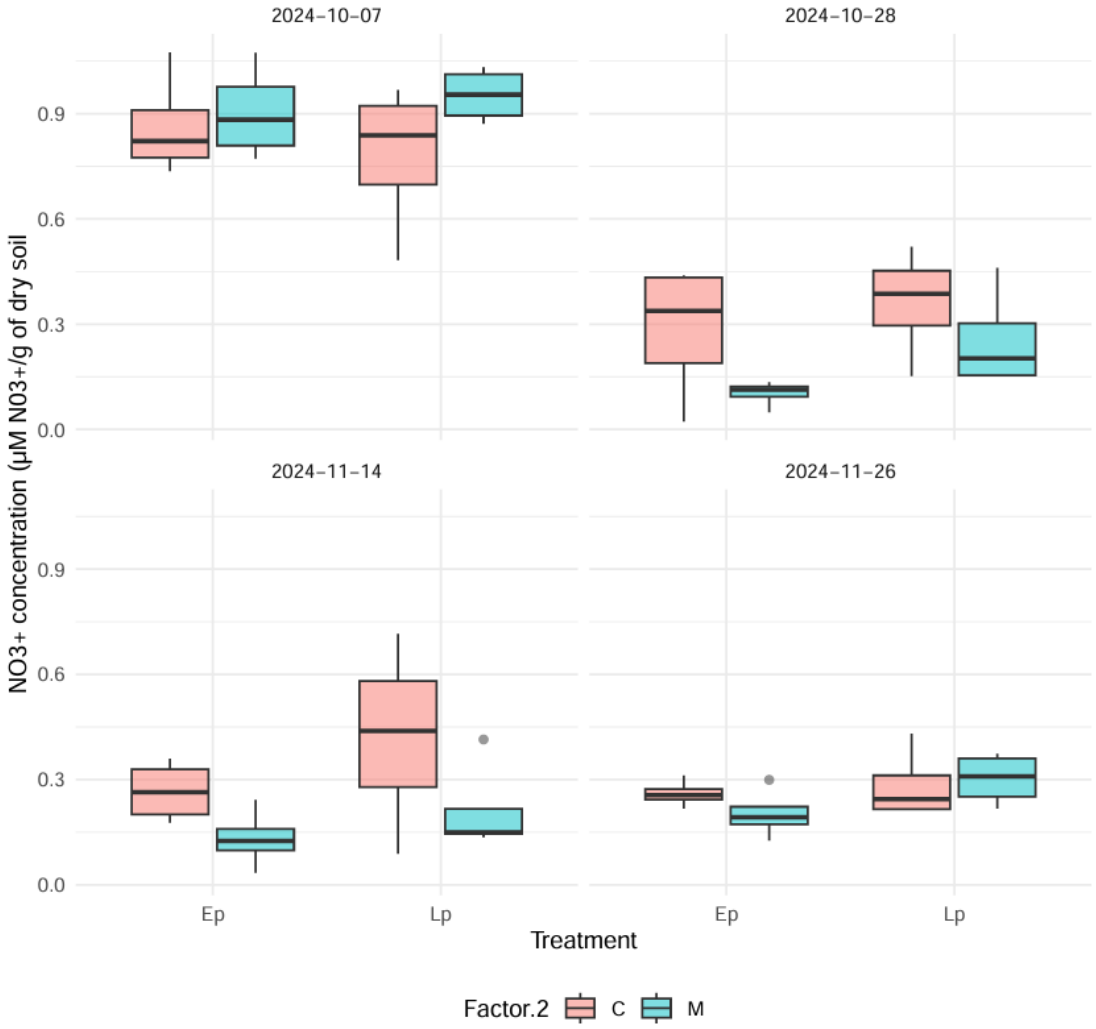


Figure 14: NO_3^- concentration (µM NO_3^-/g of dry soil) in the different treatments and type of cover crop at four sampling points. Ep=early ploughing, Lp=late ploughing, M, crop mixture, C= control.

There is a clear temporal trend of NO_3^- concentration confirmed by an ANOVA analyse incorporating the Date as a factor. Global NO_3^- average shows an increase on NO_3^- concentration despite evolution differs between modalities. Generally, NO_3^- content in soil was lower with the cover crop than the bare soil control except for initial concentration (Figure 14). Those results correspond to the literature as explained before. Higher lower initial concentration for bare soil control would be explained by removal of small cover crop at the beginning of the experiment disturbing mineralisation in those treatment. Lower NO_3^- concentration at the following sample dates underline the great effect of cover crop. Overall, variability in NO_3^- concentrations does not appear to be particularly large, though there are some notable exceptions. Lp variability seems to be a little bit higher than Ep.

Globally, NO_3^- concentrations have great variability during the experiment, particularly in the C group (Figure 14). This suggests potential differences in how Lp processes or accumulates NO_3^- compared to the Ep treatment following trends of N_2O emissions. Furthermore, Ep treatment shows significant differences between factors, particularly on October 28 and November 14, where cover crop values are notably lower. Lp treatment displays higher variability, especially for bare soil on November 14, possibly indicating inconsistent nitrogen transformations or uptake across plots. NO_3^- concentrations of every plots also tends to reach similar and stabilised values after a high variability between plots at the second sampling date. Differences of N_2O emissions between modality on November 26 are less marked than on November 14, following trends of NO_3^- concentrations. This observation indicates that the availability of NO_3^- influenced N_2O emissions in this study.

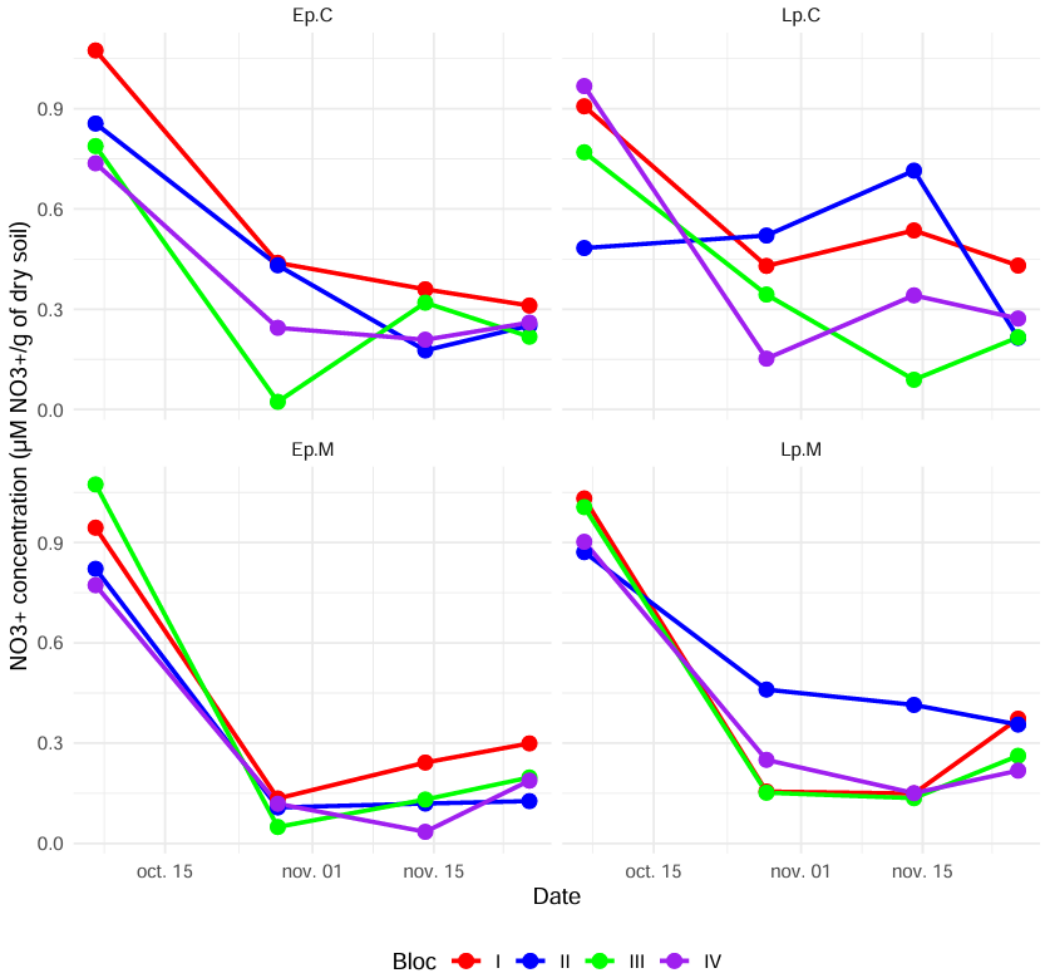


Figure 15: evolution of NO_3^- concentrations over time

Table 10: emmeans of NO_3^- concentration per each significant factor. Degrees-of-freedom method: kenward-roger. Confidence level used: 0,95.

	Treatment	emmean	SE	df	C.I.	Cover type	emmean	SE	df	C.I.
24-10-07	n.s.					n.s.				
24-10-28	EP	0.194	0.0604	4.94	[0.038;0.349]	C	0.323	0.0604	4.94	[0.164;0.472]
	LP	0.308	0.0604	4.94	[0.152;0.464]	M	0.178	0.0604	4.94	[0.023;0.334]
14-11-24	n.s.					C	0.343	0.0608	7.06	[0.199;0.487]
						M	0.172	0.0608	7.06	[0.027;0.316]
24-11-26	Lp	0.231	0.0331	4.05	[0.140;0.323]	n.s.				
	Ep	0.293	0.0331	4.05	[0.201;0.384]					

On October 7 and November 26, treatment has a significant impact on NO_3^- concentrations (p=0.049 and 0.014 respectively). The estimated marginal means show that the Lp level of treatment is associated with a higher average response compared to the Ep level (Table 9). The 95% confidence intervals indicate a potential difference between the two levels, although the overlap at the ends of the confidence intervals suggests caution in interpretation. A higher value of NO_3^- concentration in Lp treatment fits with NO_2^- and N_2O results by a higher nitrification activity or a low denitrification rate. Any conclusion can be done concerning denitrification rates. However lower NH_4^+ concentrations (as shown below) for Lp treatment could indicate higher nitrification rates.

On October 28 and November 14 there is a significant difference between bare soil and cover crop (p=0.013 and 0.024 respectively). The estimated marginal means show that the bare soil of cover crop is associated with a higher average response compared to the cover crop (Tab.10). It is also important to notice that confidence intervals show an overlap of data. This relationship of NO- concentration according to the cover have already been explained and is linked to the presence of plant that take up NO_3^- adding a new fate on NO_3^- compared to bare soil. Indeed, NO_3^- in soil decrease either by plant uptake, leaching, or denitrification (Jackson, 2000).

Globally, it is possible to highlight the greater effect of cover than treatment on NO_3^- concentration.

1.4. NH_4^+ concentration

Variance study

After significant effect of Date on NH_4^+ concentration (p=<2e-16), ANOVA test per sampling date revealed neither significant impact of factors nor factors interaction on NH_4^+ concentration at any time point of the experiment.

*Table 11: results of variance analysis of NH_4^+ concentrations for two factors. *<0.05, **<0,01, ***<0,001*

NH4- concentrations	07-oct	28-oct	14-nov	26-oct
Treatment	NA	n.s	n.s	n.s
Cover type	NA	n.s	n.s	n.s
Treatment*Cover	NA	n.s	n.s	n.s

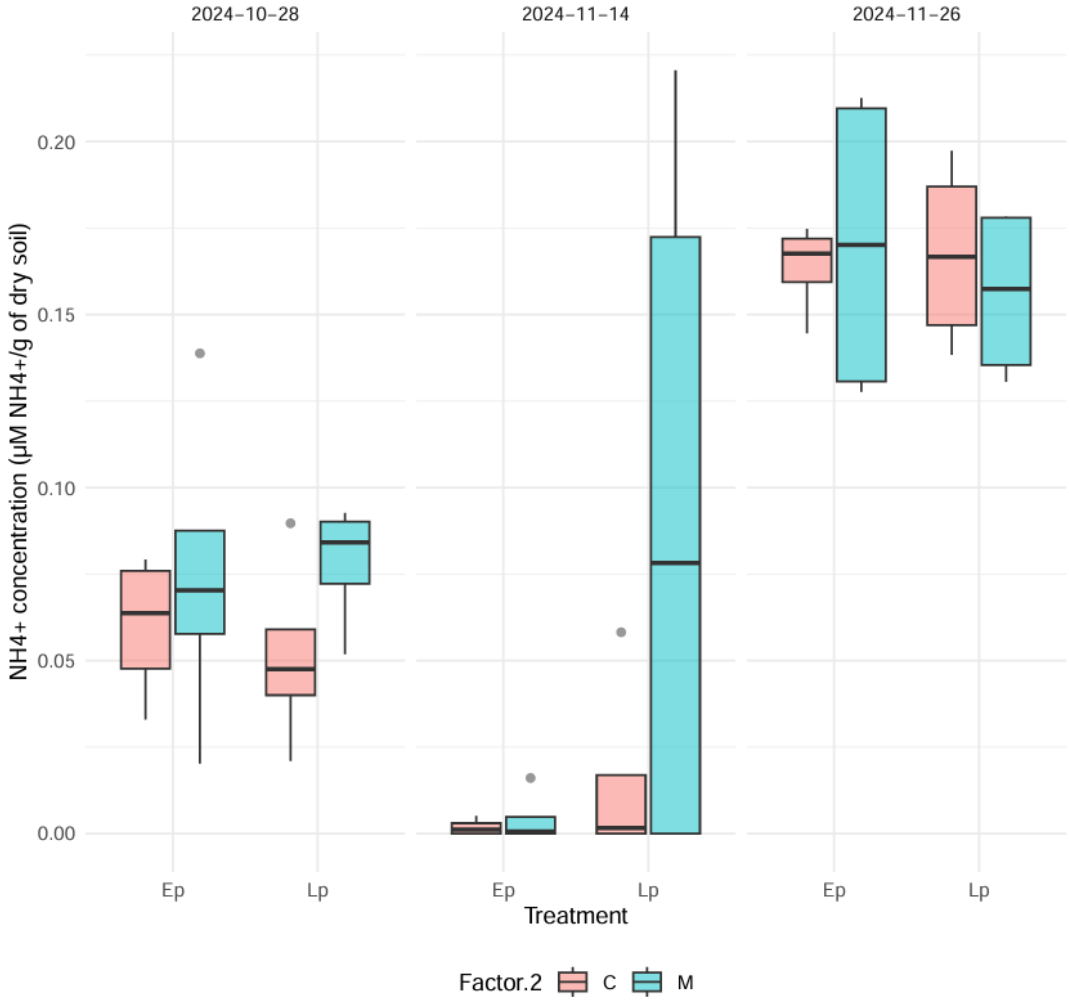


Figure 16: NO_3^- concentration (µM NO_3^-/g of dry soil) in the different treatments and type of cover crop at four sampling points. Ep=early ploughing, Lp=late ploughing, M, crop mixture, C= control.

On November 14, NH_4^+ concentrations in the Ep treatment were consistently low for both types of cover crop. In contrast, the Lp treatment showed more variability. It is caused by numerous negative NH_4^+ concentrations at this moment. Those values were adjusted to zero for the analysis, which likely contributed to the low overall NH_4^+ concentrations and high variability observed in the Lp treatment on November 14. Hence the low NH_4^+ values on this date could be due to measurement errors. The generally low NH_4^+ values in the field could also be problematic since very low NH_4^+ concentrations leads to very low of limit of detection (De Neve et al., 2002). Additionally, the pronounced variability in may be linked to specific field positions, such as NH_4^+ or PC4 (Figure 16), which could have been

influenced local soil characteristics or microclimatic conditions. Heterogeneity between plots is likely more pronounced when concentrations are low as for NH_4^+ concentrations. It exists a great decrease between October 28 and November 14 which is principally due to those measurement issues. It can also be explained by a greater bacterial and archaea activity enhancing nitrification process between the two sampling dates while there is no clear accumulation of organic matter.

Throughout the sampling period, NH_4^+ concentrations were generally higher in the presence of cover crop, except on the final sampling date. It could be due to the presence of biomass which feed SOM in soils. Over time, NH_4^+ concentrations varied considerably also explained by heterogeneity of plots, with an increase observed at the end of the experiment for both Ep and Lp treatments. Great variations are explained by variable weather conditions in temperature and rainfall. The results highlight the importance of temporal variation as a primary driver of NH_4^+ dynamics in this study influenced by climate conditions.

To explain increasing NH_4^+ concentration between November 14 and 26, frost events further contributed to increasing ammonium availability by destroying part of the microbial biomass and releasing nutrients (Mørkved et al., 2006). Warmer temperatures after frost event (10°C on November 25) enhanced microbial activity, leading to greater decomposition of this new SOM and subsequent NH_4^+ accumulation (Hofman et al., 2004). Elevated soil moisture caused by rain likely stimulate organic matter mineralization by microbial activity, contributing to greater NH_4^+ production as highlighted in a study of Dai et al. (2020). Furthermore, reduced nitrification rates due to lower temperatures for a few days could have limit the transformation of NH_4^+ into NO_2^- and NO_3^-, thereby preserving NH_4^+ in the soil and allow its accumulation in soils. A decrease in nitrification can be inferred from a significant increase in NH_4^+ concentration in the soil, while the concentrations of NO_2^- and NO_3^- only slightly increase between the two sampling dates. By the way, this decrease can also explain diminution of N_2O emissions.

NH_4^+ concentrations observed in the Lp treatment showed patterns inversely related to previously observed N_2O emissions between November 14 and 26. Decreasing nitrification rates and breakdown of cover crop tissue after frost adding SOM to the soil explain why there is an accumulation of NH_4^+ in soils. The Lp treatment consistently exhibited greater variability and higher NH_4^+ concentrations compared to the Ep treatment, particularly in the M group. This trend is also explained by greater microbial activities in this treatment. This raises the possibility of a correlation between N2O emissions

and NH_4^+ concentrations, except on the November 26 when NH_4^+ concentrations are greater with cover crop.

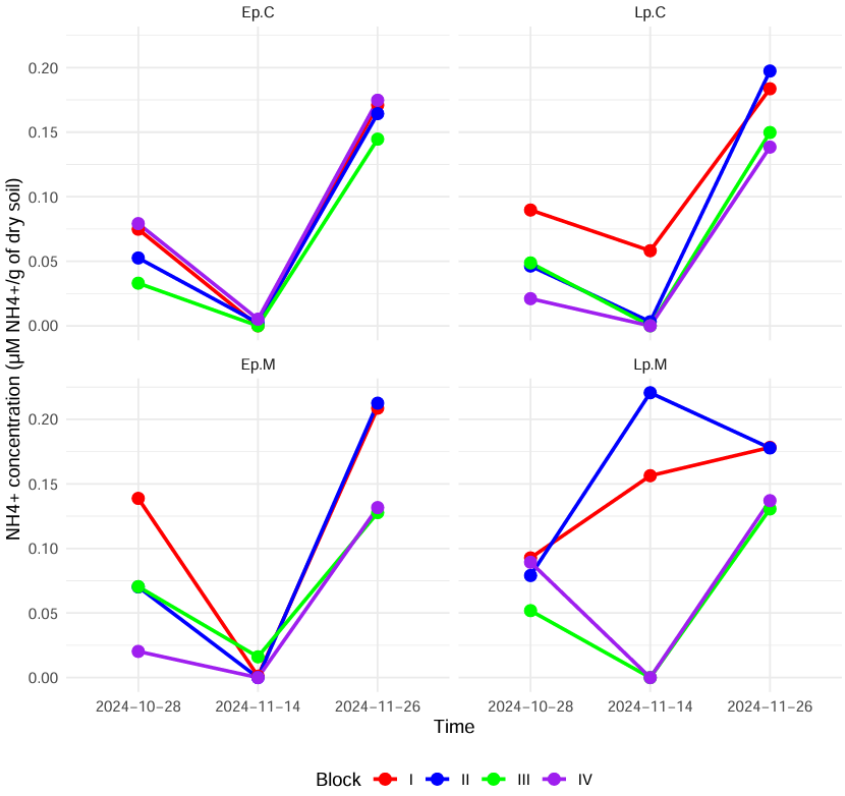


Figure 17: evolution of NH4+ concentration over time

Overall, the trends within each treatment type appear to remain approximately consistent, indicating no significant effect of the Block factor on NH_4^+ concentrations. However, the concentrations for the Lp.M treatment evolve differently, particularly for samples P4M and P2M. These two samples likely contribute to the high variability observed in the previous graph for the Lp.M treatment.

1.5. Potential nitrification rates

There was a significant effect of the treatment (Ep vs. Lp) on potential nitrification rates ($p = 0.017$). No significant effects were observed for the type of cover or the interaction between the two factors. An additional analysis has been realised to confirm that type of plant does not have any impact on nitrification in Appendix 4.

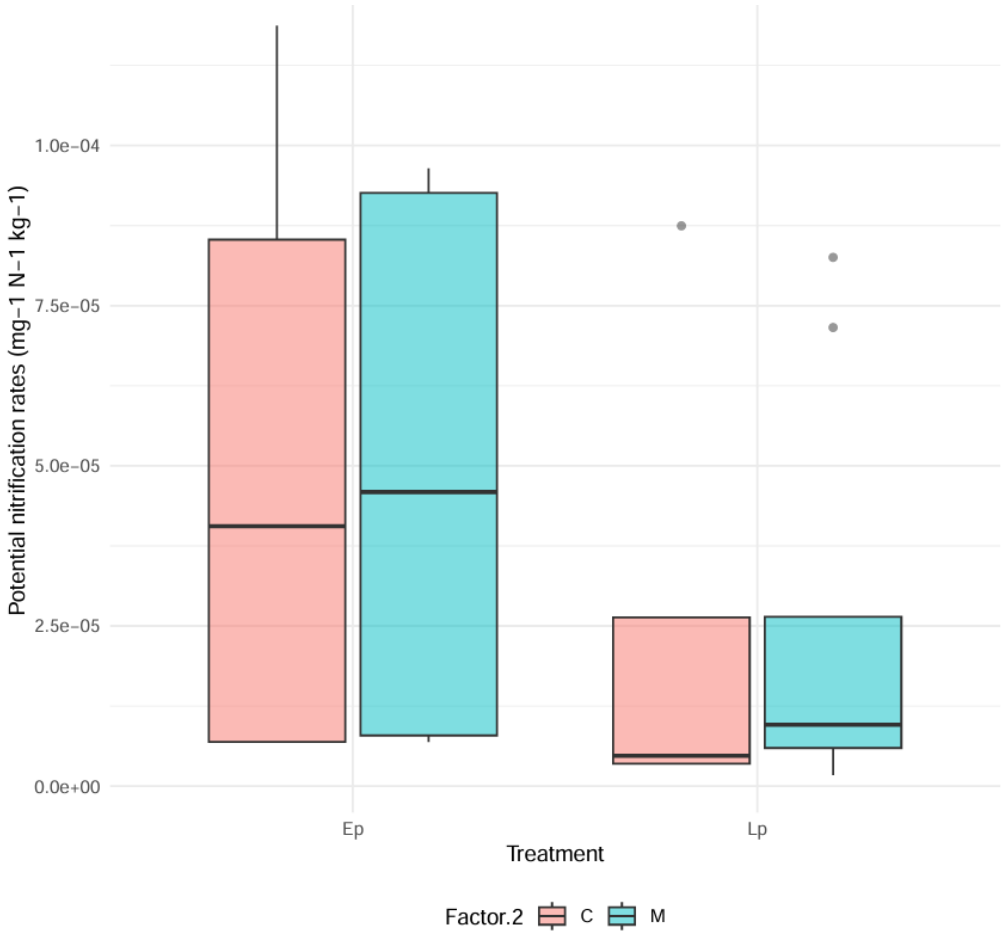


Figure 18: potential nitrification rates ($mg\ N\ kg^{-1}h^{-1}$) in the different treatments and type of cover crop on November 26. Ep=early ploughing, Lp=late ploughing, M, crop mixture, C= control.

The very low differences and no significant results are attributed to the low biomass of the cover and the short time experiment. Same results have been shown in Mendis works whose did not see any impact of the cover on thermal properties of cover crop because of the insufficient biomass of cover crop and the short term experiment (Mendis, 2021).

Table 12: emmeans values of nitrification rates per treatment. Results are averaged over the levels: cover. Degrees of freedom method: kenward-roger. Confidence level used: 0,95

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Ep	5.06e-05	1.93e-05	3.66	-4.98e-06	1.06e-04
Lp	2.48e-05	1.93e-05	3.66	-3.08e-05	8.04e-05

Estimated marginal means revealed distinct trends between the two treatments with higher potential nitrification rates for Ep.

On Figure 18, the potential nitrification rate was more variable in Ep treatment suggesting higher variability in microbial activity. In contrast, Lp has a more consistent distribution, with lower overall

nitrification rates and fewer outliers. These findings highlight that early ploughing promotes higher nitrification activity.

1.6. Water content

Concerning water content, a very significant effect of the date has been observed. However, there is no significant effect of the other factors on the water content at any time point. A study comparing properties of clay soils with ploughing and no-till practices also showed no effect of tillage practices on water content (Laine et al., 2018).

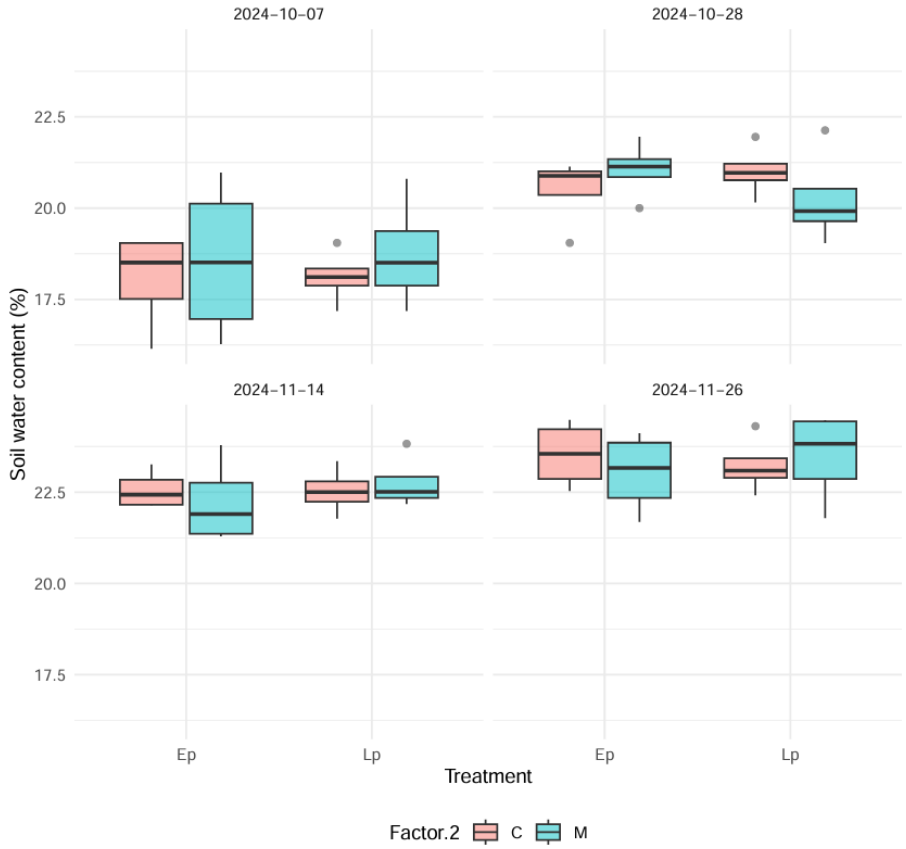


Figure 19: soil water content (%) in the different treatments and type of cover crop at four sampling date. Ep=early ploughing, Lp=late ploughing, M, crop mixture, C= control.

Soil water content increased over time in all treatments which can be explained by the accumulation of rain and low evapotranspiration during the autumn ("L'eau et le sol," December-30-2024). As explained in a paper of University of Picardie, lower temperatures provide less energy to evaporation of soil water and tend to maintain water in soil. Differences of water content between subplots are due to differences in properties of soil and location of on the field.

1.7. Dried biomass

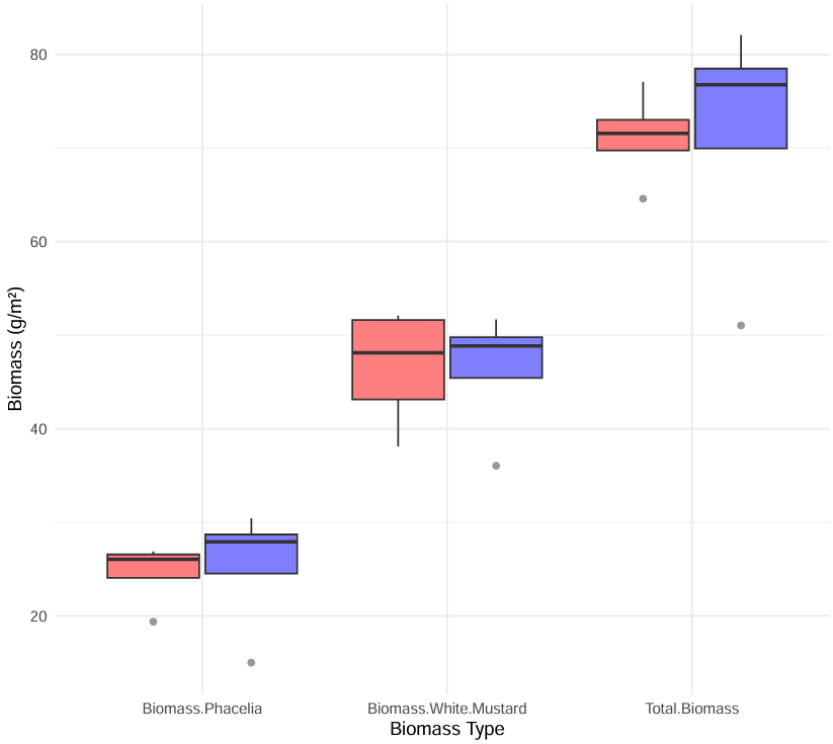


Figure 20: soil Dried biomass (g/m²) in the different treatments and type of cover crop on November 26. Ep=early ploughing, Lp=late ploughing, M, crop mixture, C= control.

For all plot which are covered, a analyse of variance of Treatment factor has been done for Biomass of phacelia, white mustard and total. From this analyse, no significant differences between Ep and Lp has been revealed neither for Phacelia and White mustard. White mustard contributes more to the total biomass than do phacelia.

2. Multivariate analysis

2.1. Global analysis of data base

The aim of this part of the analysis is to explain interaction between variables and the effects of factors on these interactions. By adopting this approach, it becomes possible to obtain a comprehensive overview of the dataset, thereby enabling a deeper understanding of the dynamics between variables.

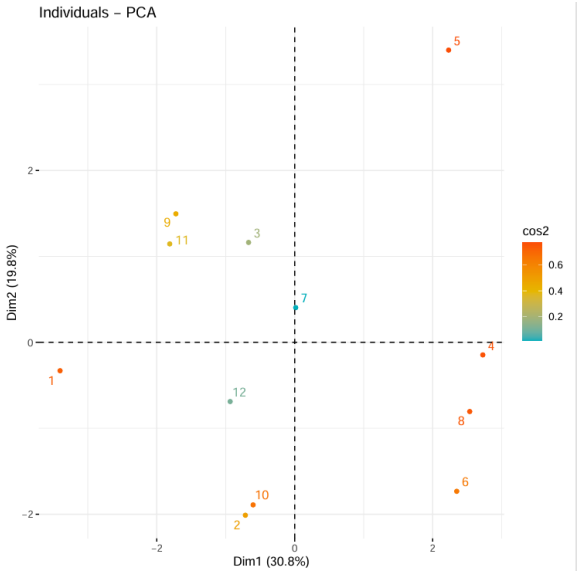


Figure 21: representativity of all observations in the first factorial plan

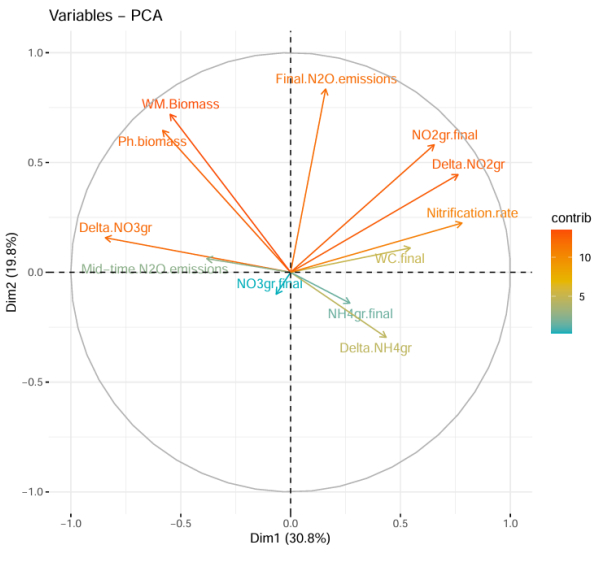


Figure 22: contribution of all variables to the variance of first and second component

On Figure 21, it is possible to see that observations 1 (PM2) and 4 (PC3) are particularly explained by first component, while observations 10 (PC10) and 2 (PC2) are explained by second component. The representation of observation 5 (PM5) differs completely from the other observations partly explaining variability in data.

According to eigen values, it is interesting to study the first four components which represent the majority of variance with first and second components explaining about 50% of total variance (Figure 22). Delta NO_3^- explains a large partion of variance through its high contribution to first dimension (Dim1). On the other hand, final N_2O emissions play a major role to explain the variance captured by second component (Dim2). By combination of two Figures, it is possible to conclude that observations in plot PM2 (obs1) is principally driven by NO_3^- variation while PC3 (obs4) is driven by nitrification rates.

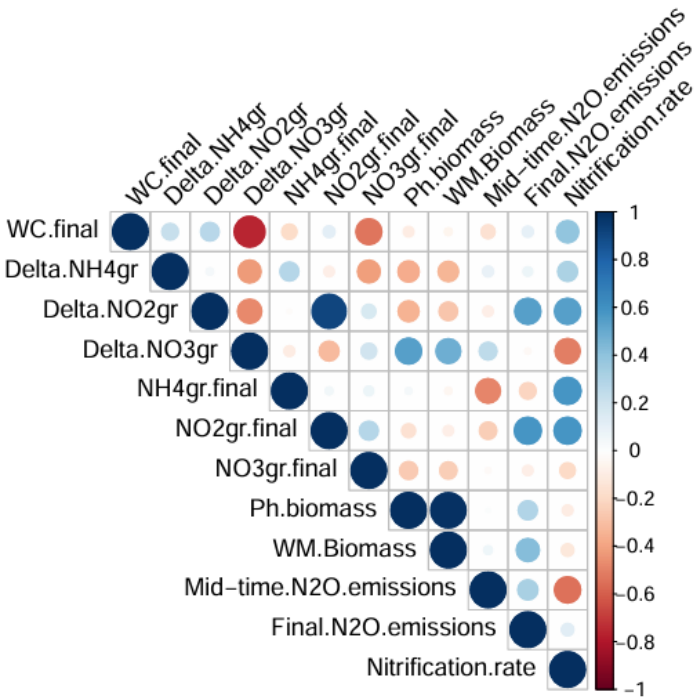


Figure 23: correlations between every variable of dataset

Only significant correlation has been conserved in complements. This suggests that phacelia biomass and white mustard are positively correlated (0.92, p=2.21e-5). It could principally be due to differences is soil properties or issues during seeding. Globally, there were high differences in cover on the field. Furthermore, final NO2 concentration is positively correlated with its variation (0.77 p=5.25e-3). This relationship appears intuitive and does not require further analysis. Furthermore, the variation in NO_3^- concentration and the final water content are negatively correlated (-0.78 p=4.12e-3).

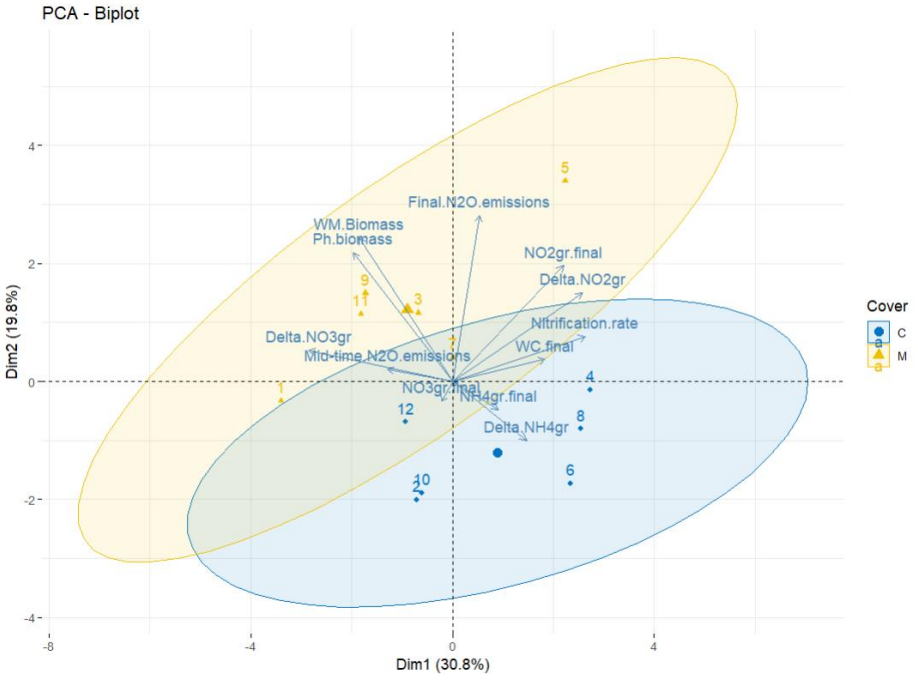


Figure 24: repartition of variables according to the cover crop type

The ellipses represent the dispersion and concentration of data points for each group (Figure 24). They illustrate that the C and M groups are well-separated along the Dim1 and Dim2 axes, suggesting that these two groups exhibit distinct characteristics principally driven by both biomasses. A larger ellipse indicates greater variability within a group. In this case, the two groups are distinct, with relatively similar variability. This observation led to a decision to modify our approach, as it became apparent that the formation of these two groups (which is observed exclusively for this factor and not others) is primarily driven by biomass. This result is logical, as biomass is inherently zero for bare soil. Consequently, we will first analyse interactions between variables without considering biomass. Following this, we will aim to explain how the different variables influence biomass.

2.2. Without biomass

Without considering biomass, it is possible to notice that contribution of variables changed with bigger part of variance explained by two first components (54,5%) (Figure 25). Final NO_2^- concentration and its variation during the experiment are particularly well represented by both first and second component. Nitrification rates (correlation = 0.8633721) contribute a lot to the variation of first component as well as variation of NO_3^- and NO2 concentrations (correlation = 0.7911508 and -0.7576670 respectively).

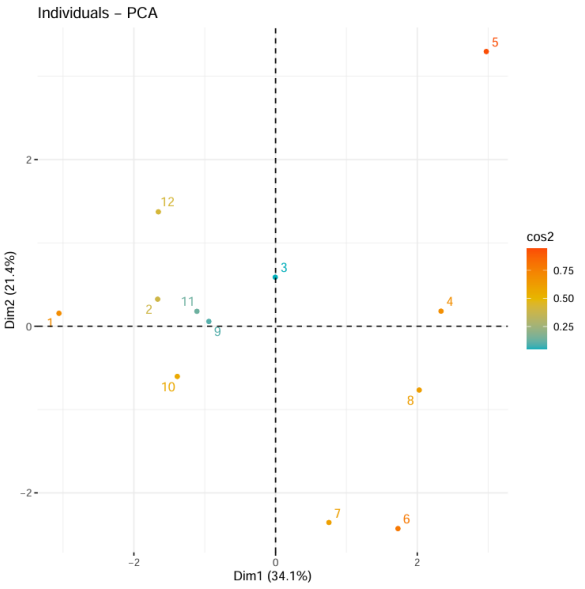


Figure 26: dispersion of observations in first factorial plan of second data set

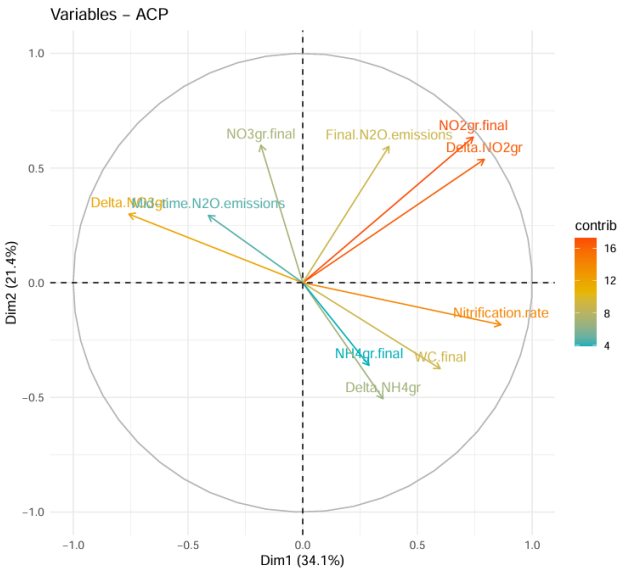


Figure 25: contribution of variables to formation of first and second component of the second data set

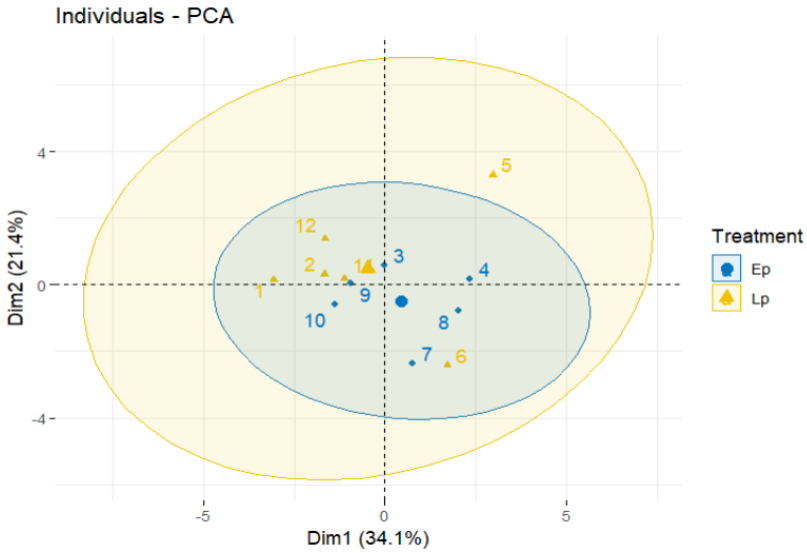


Figure 27: dispersion of observations in first factorial plan for second data set according to the treatment

There is no clear distinction in the representation of observations based on the treatment. However, variability is visually higher for Lp group. This variability is mainly explained by presence of observation 5 (PM4), which is mainly driven by $NO2^-$ concentrations, and observation 6 (PC4), which is driven in the opposite direction by $NO2^-$ concentrations, both represented in the first factorial plan. Those two observations are located in the same plot (Lp block II) which has already shown particularities in results of univariate analysis. As a reminder, PM4 showed a completely different evolution of $NO2^-$ concentration compared to other subplot. It is also the plot with the higher final N_2O emissions. Furthermore, Lp treatment showed higher variability except for nitrification rates in univariate analysis.

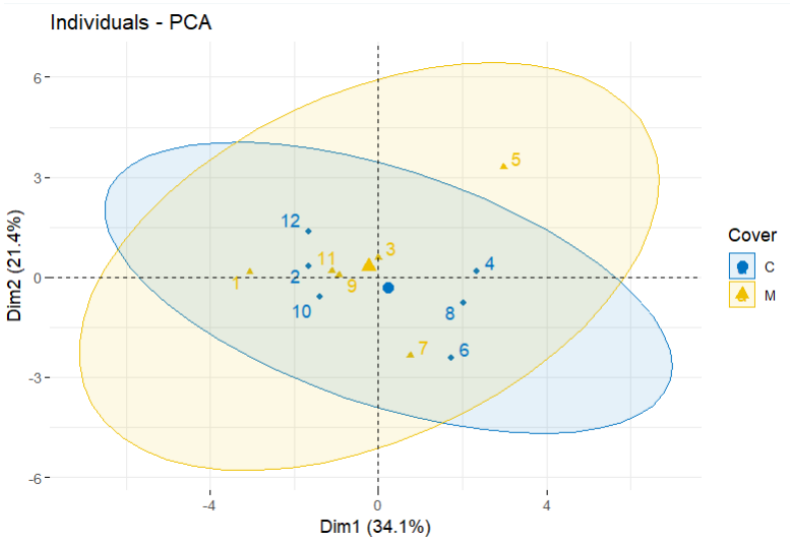


Figure 28: representation of observations in first factorial plan for second data set according to the cover type

Distinct representations of Dim1 and Dim2 are displayed on the graph (Figure 28) for each group, C and M. The slightly higher variability of the M group is also explained by observation 5. Two distinct groups in plots with no cover crop are due to their proximity on the graph: 12,2 and 10 (=Left subgroup) against 6,8 and 4 (=Right subgroup) indicating similarity within these subgroups. Those two groups are more influenced by the first principal component. Data shows no direct impact of variation of N_2O concentration. However, variations of NO_3^- concentration which are negative for observations ij the right subgroup explain this separation. Unfortunately, no clear distinction based on the block or treatment can be made as the low number of observations not allowing a clear interpretation. On the other hand, observations 7 and 6 are relatively close despite having different covers, suggesting some overlap or similarity. The similarity could stem from their location in the field within the same block.

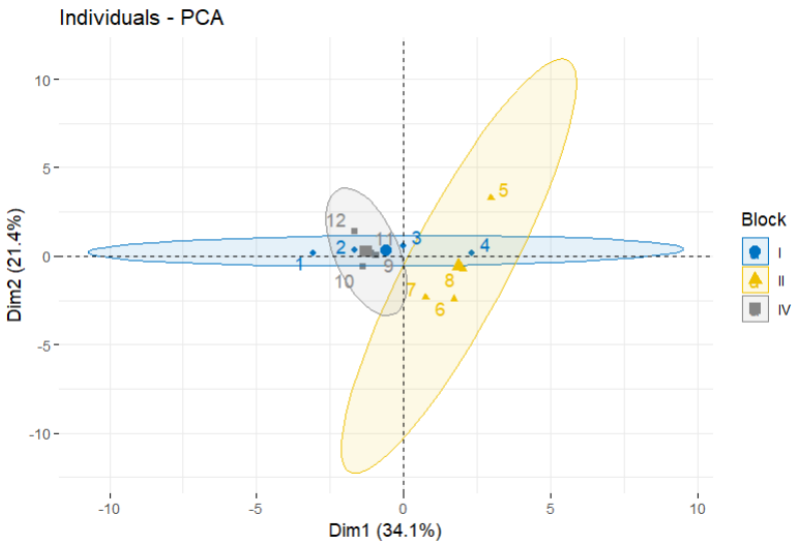


Figure 29: representation and variability of observations in the first factorial plan for second data set according to the Block factor

The PCA revealed clear distinctions between blocks (I, II, and IV), highlighting the significant influence of the block effect on the measured variables. Block I is strongly influenced by the first principal component, which is linked to variations in NO_3^- and NO_2^- concentration as well as potential nitrification rates. In contrast, Block II is primarily driven by the final N_2O emissions, while Block IV is closely associated with mid-time NO_2 emissions. This indicates that nitrogen dynamics in soil are driven differently depending to the block. The variability within Block IV is notably lower than that of Blocks I and II indicating greater homogeneity in the observations of Block IV. Block IV is located in the higher part of the experiment site where slopes are much gentler compared to block I and II (Figure 4).

The PCA results confirm the initial decision to account for the block factor in all statistical analysis, as the block effect significantly influences the relationships between variables. These findings underline the importance of incorporating the block factor into the analysis to accurately understand the nitrogen-related variable dynamics in the experimental setup.

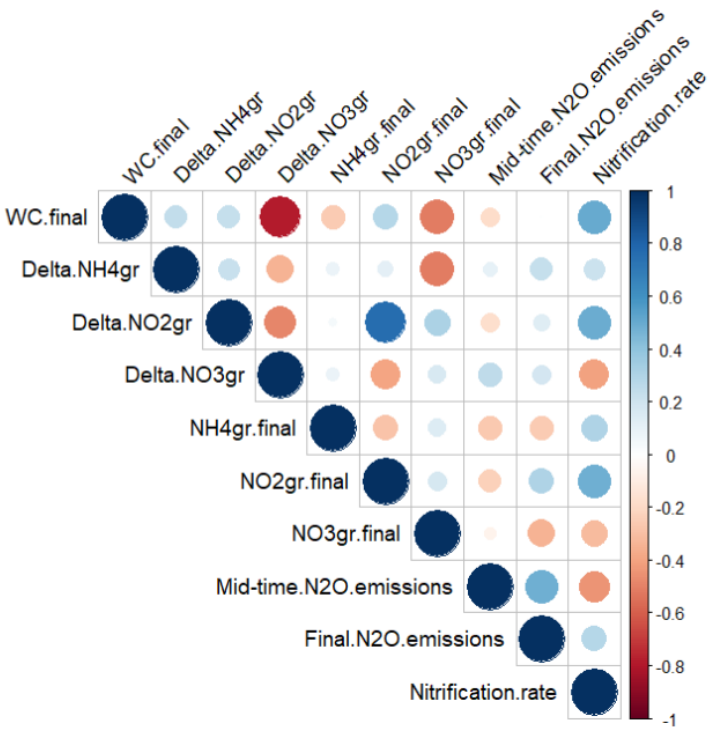


Figure 30: correlations between every variable of second data set

After an analysis of significant correlation, only the interaction delta NO_3^- and final water content ($p=0.00412$) and final NO_2^- concentration and his variation during the experiment (0.00525) were conserved. It is also interesting to notice that nitrification rate variable is correlated with all other variable, even if weakly. It is possible to conclude that nitrification rates have an influence or a role to play to explain trends of those variables.

Study of correlation between final water content and variation in NO_3^- *concentration*

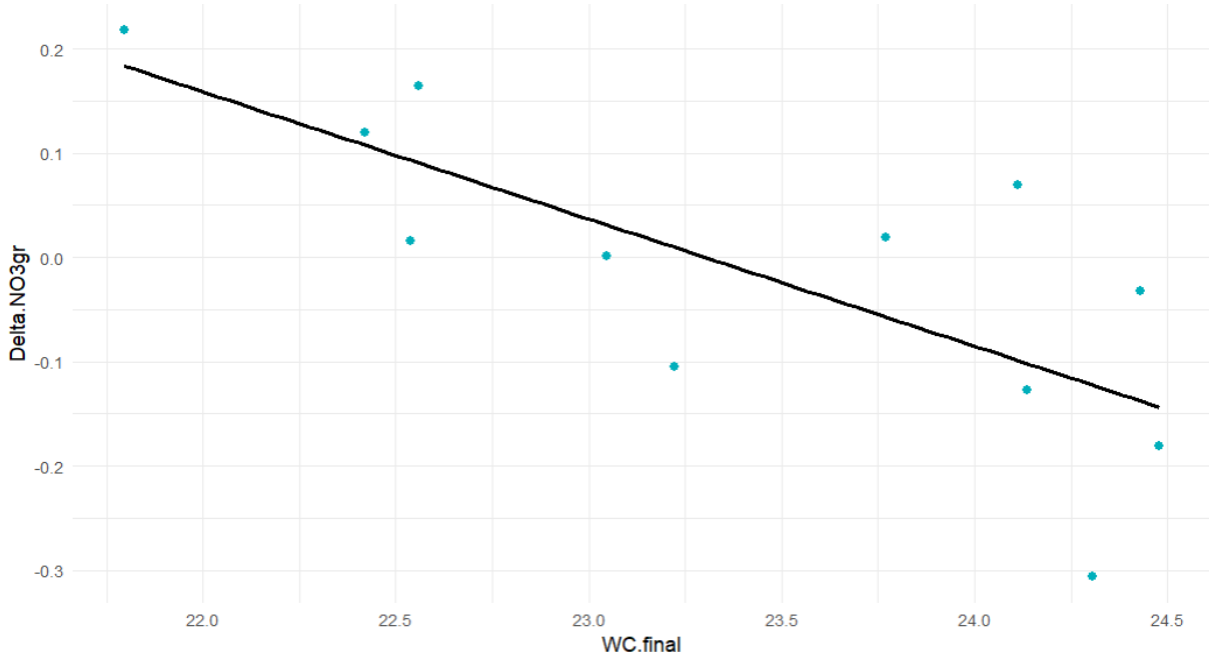


Figure 31: correlation between final water content and variation of NO_3^- concentration for the second dataset

Water content and variation in NO_3^- concentration are negatively correlated (cor= -0.78). NO_3^- variation becomes more negative when water content is higher. It could be explained by a combination of mechanisms. When water content is higher, nitrate added is distributed in a greater volume of water reducing its variation. Furthermore, the high water content in soil is also indicative of this rainy year as previously mentioned. Nitrate could also have been leached, explaining negative variation of nitrate concentration (Dou et al., 2024).

Lower initial soil moisture content result in a deeper nitrate migration, suggesting that under drier conditions, nitrates are more likely to leach into deeper soil layers rather than being lost through surface runoff (Chang-bao et al., 2008). However, analysis of the results reveals a discrepancy with the literature, as PM11, which has a strongly negative delta, exhibits the highest nitrate concentration.

2.3. Biomass

Globally variables are really well represented by components showen by the position of arrows at the limit of the circle on figure 33). Contribution of variables is quite high too. Indeed, more than 70% of the variance is explained by first and second dimensions. Furthermore, final NO_2^- concentration and its variation across the experiment which contribute to the first component compared to final water content and mid-time N_2O emissions which partially contribute to the second component.

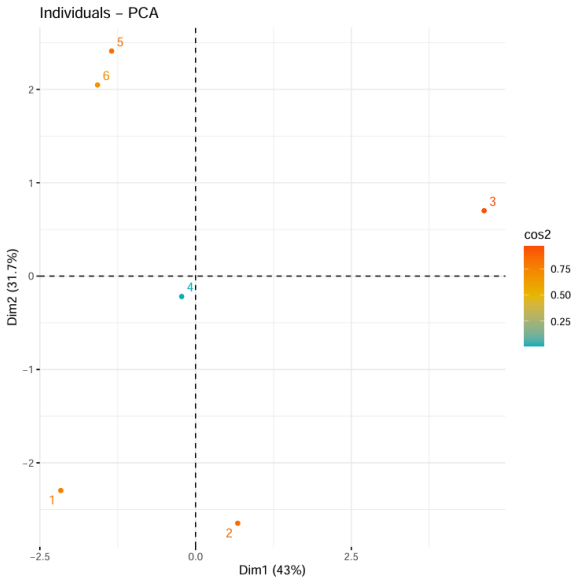


Figure 33: representation of observations in first factorial plan for cover crops subplots

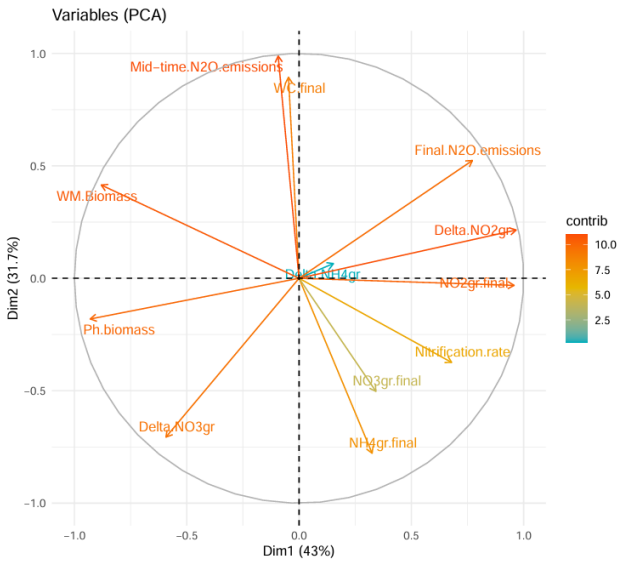


Figure 32: contribution of variables in variance of first and second component for cover crops subplots

On Figure 32, individual 4 (PC3) is the observation with the lowest quality of representation by the two first principal components while the others are relatively represented well. Thus, it is less influenced by those components. Thanks to an analysis of cos^2 of each individual for the first three components, it is possible to attest that this observation is well represented by third component which is principally driven by delta NH_4^+. Concerning other objects, observation 3 is principally explained by NO_2^- concentrations while the others are influenced by both first and second dimension.

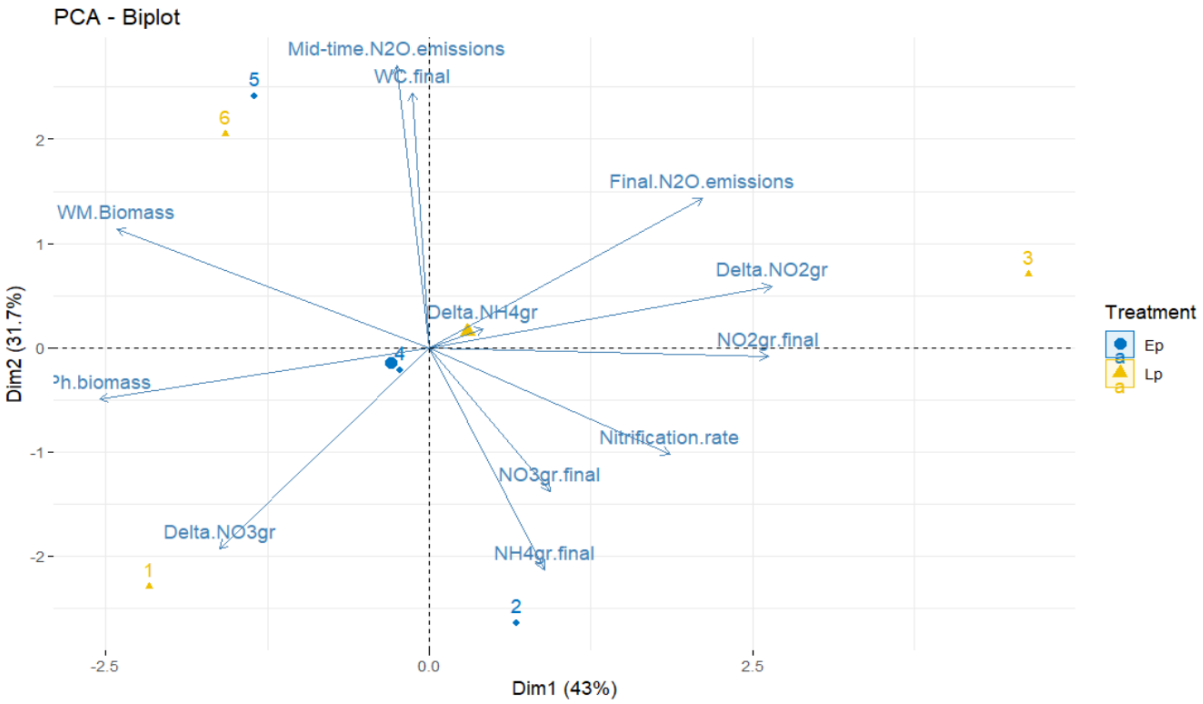
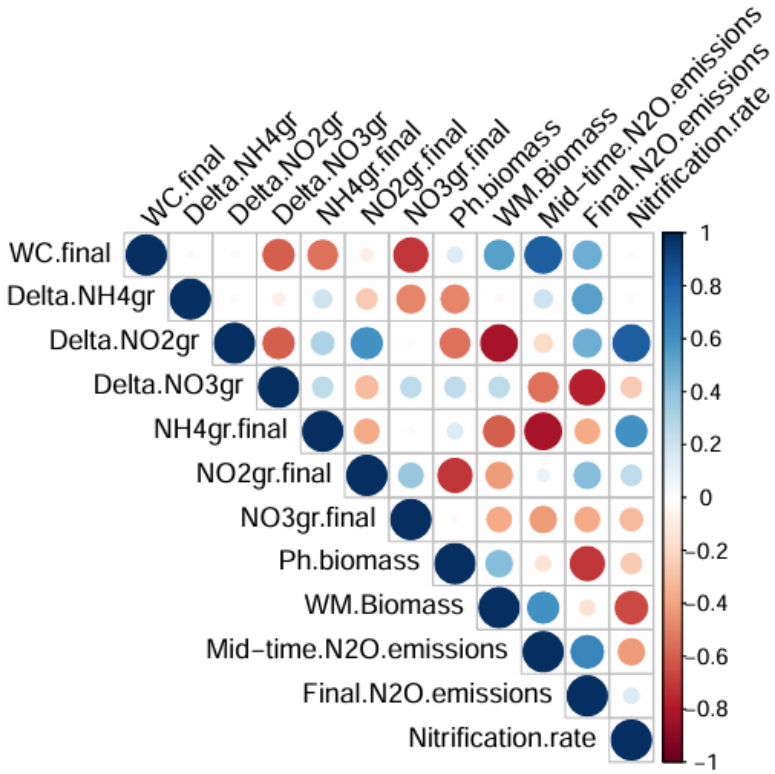


Figure 34: biplot of representations of individuals and variables in first factorial plan of cover crops subplots

Figure 34 indicates that observation 1 (PM2) is highly influenced by delta NO_3^- and observation 3 (PM3) is influenced by the variation of NO_2^- concentration. Observations 5 (PM5) and 6 (PC5) are more driven by the second component.



The following relations were significantly correlated:

- Delta NO_2^- & nitrification rates
- Final NH_4^+ concentration & Mid-Time N_2O emissions
- Mid-Time N_2O emissions & Final water content
- Delta NO_2^- & White mustard biomass

Figure 35: correlations between every variable of cover crop subplots

Study of the interaction between variation of NO_2^- concentration and nitrification rates

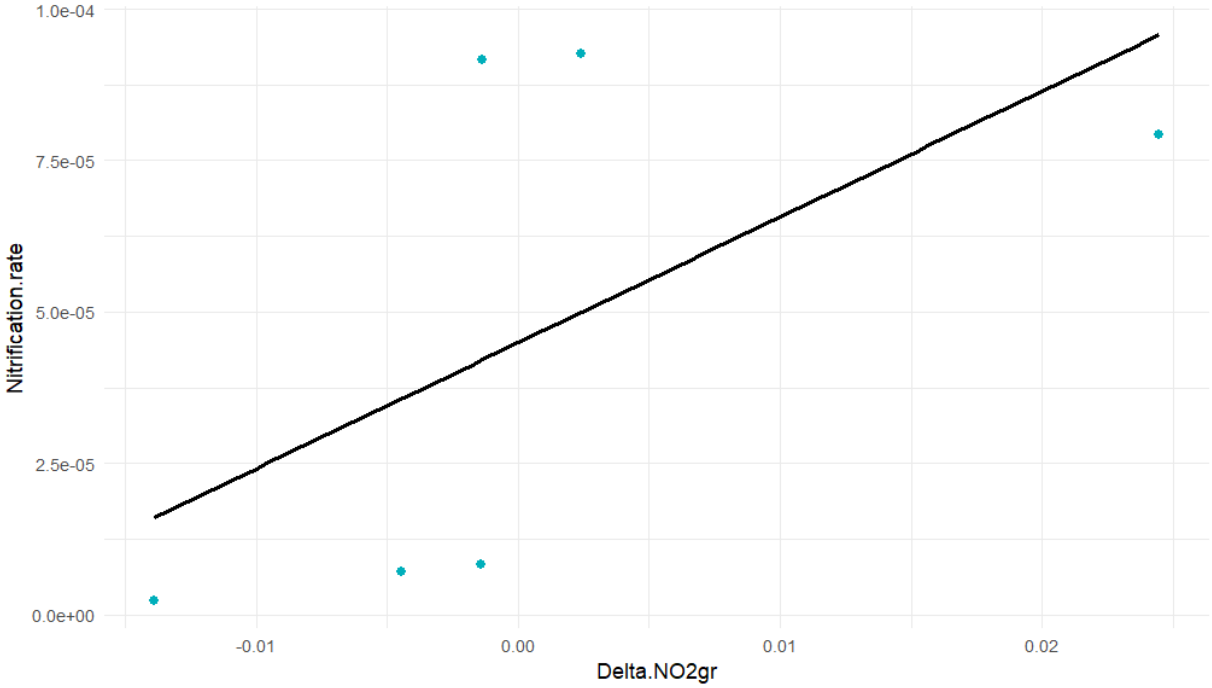


Figure 36: correlation between variation of NO_2^- concentration and nitrification rates of cover crop subplots

Nitrites are an intermediate substrate of nitrification process, as explained in N cycling. If nitrification rates are increased, NO_2^- variation is positive with higher increase of NO_2^- during the experiment. NO_2^- concentrations are closely influenced by AOB activity involved in NH_4^+ oxidation (Kinh et al., 2017). Furthermore, adding nitrite to simultaneous nitrification systems enhanced ammonia removal efficiency by up to 4.5 times and significantly increased the populations of AOA and AOB (Xiang et al., 2023). However, it is important to note that nitrification rates have only been calculated at the end of the experiment and were not constant on every time point of the experimentation as showed by NO_3^- and NH_4^+ dynamics. Those results should be treated with caution.

Study of the interaction between final NH_4^+ concentration and mid-time N_2O emissions

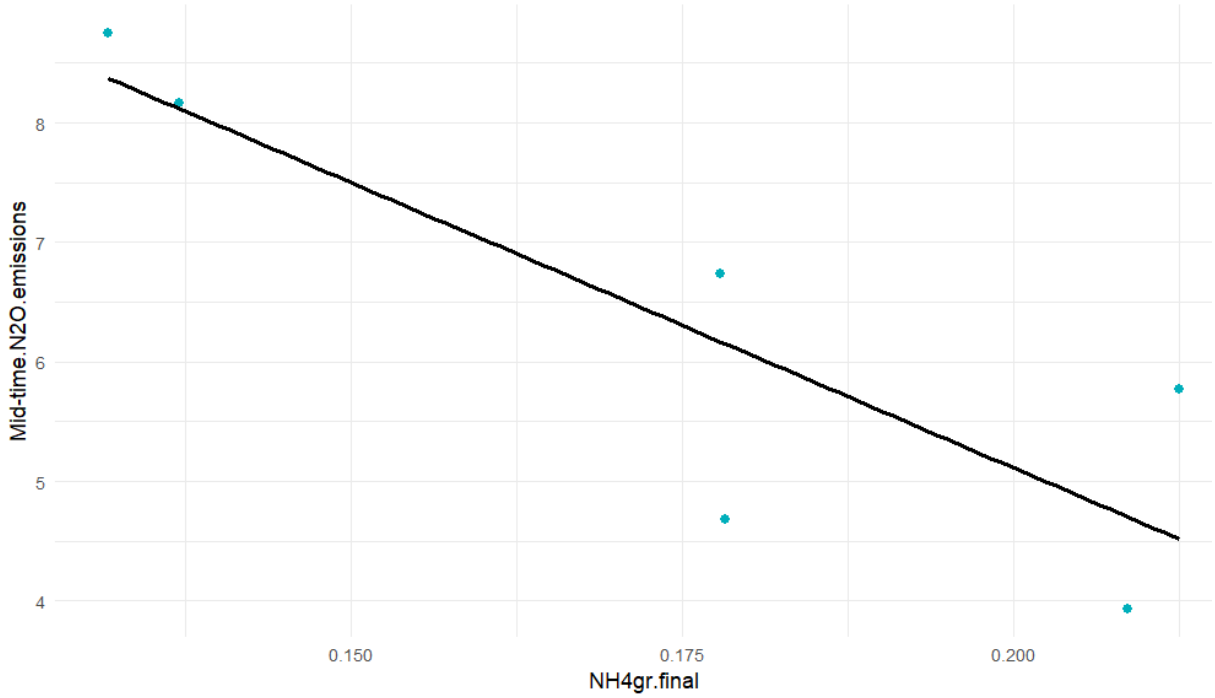


Figure 37: correlation between mid-time N_2O emissions and final NH_4^+ concentration of cover crop sublots

The low N_2O emissions observed on November 14, correlated with the high NH_4^+ concentrations in the soil on November 26, could be explained by the effect of cold temperatures on microbial activity. On November 14, microorganisms were actively consuming NH_4^+, leading to the production of N_2O as a by-product of nitrification. On November 26, the drop in microbial activity, likely due to colder conditions, resulted in an accumulation of NH_4^+ in the soil and a reduction in N_2O emissions. This highlights a transitional phase, potentially influenced by freezing temperatures.

Study of the interaction between mid-time N_2O emissions and final water content

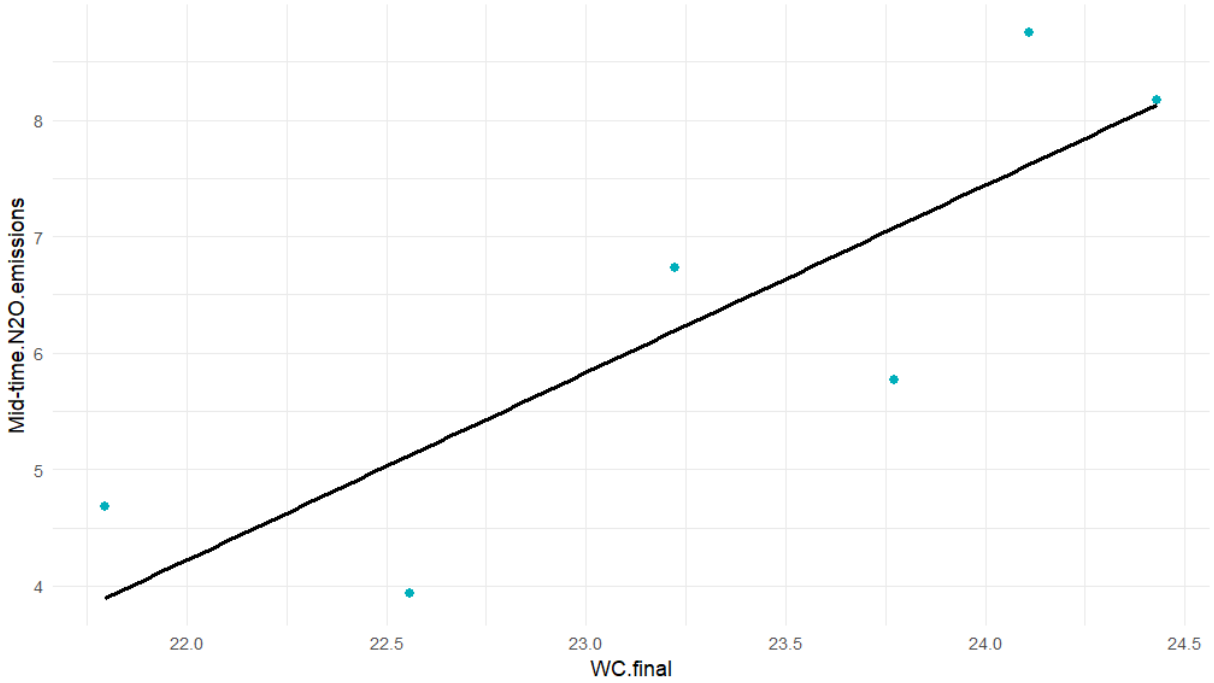


Figure 38: correlation between mid-time N_2O emissions and final water content of cover crop subplots

The relationship between rainfall and N_2O emissions is well-documented in the literature. Studies confirm that N_2O emissions increase following precipitation events (Le Gall et al., 2014). Specifically, "N_2O emissions from denitrification are closely linked to soil moisture levels, with higher emissions observed in wetter soils" (Poincot, n.d.). Another study demonstrated that N_2O emissions begin to rise within an hour after rainfall ends and can persist for up to six hours afterward (Poincot, n.d.). High soil moisture limits oxygen diffusion, boosting microbial activity responsible for denitrification (Viard et al., 2013). While nitrification occurs in drier, oxygen-rich soils, wetter soils promote denitrification, leading to higher N_2O emissions due to anaerobic conditions and nitrate accumulation (Viard et al., 2013).

Study of the interaction between variation of NO_2- concentration and white mustard biomass

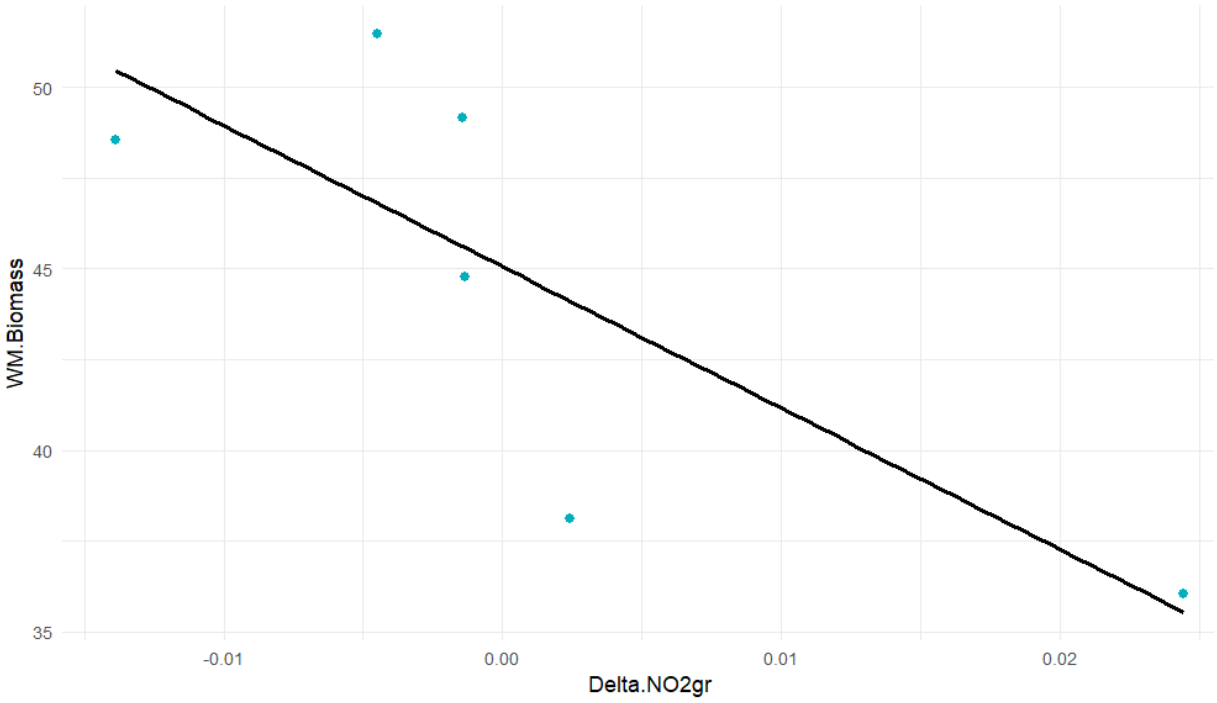


Figure 39: relationship between white mustard biomass and variation of NO_2- concentration in soils for cover crop

As noted in the literature review, the most assimilated form of mineral nitrogen by plants is NO_3^-. Other studies, like Koba et al (2014), also highlight a relationship between biomass and plant uptake. Indeed according to this study, higher biomass is often associated with increased nitrate uptake due to greater root surface area and enhanced metabolic activity (Koba et al., 2014). Given that nitrate serves as a pool for denitrification, its reduction could lead to a decrease in NO_2- production. However, on Figure 39, the relationship between biomass and NO_3^- is not very high. It means that another mechanism explains this relationship. By competition with microorganisms, higher uptake leading to less substrate (NH_4^+) for microorganisms, thereby reducing the substrate for NO2-. Similar results in this study (Kuppe et al., 2024).

Relationship Variation of NO_2- *concentration, WM biomass, and potential nitrification rates*

An indirect correlation between plant biomass and nitrification rates is observed, aligning with findings in the literature. This relationship could be explained by competition for ammonium between plants and microorganisms, reducing the availability of NH_4^+, the key substrate for nitrification (Kuppe et al., 2024). Additionally, higher plant biomass may limit oxygen availability for microorganisms due to increased root respiration, further inhibiting nitrification (Hasson, 2012). While the role of biological nitrification inhibition (BNI) cannot be confirmed with the current data (Norton et al., 2019) , the observed trend supports established research linking higher plant biomass to reduced nitrification rates.

2.3.1. White mustard biomass

Tableau 14: influence and importance of explicative variables on WM biomass

Variable	Influence on WM biomass	Overall
Mid-time.N2O.emissions	0.60000000	0.71382194
WC.final	0.54285714	0.50313502
Ph.biomass	0.42857143	0.97311089
Delta.NO3gr	0.25714286	0.38356755
Delta.NH4gr	-0.02857143	0.07177292
Final.N2O.emissions	-0.14285714	0.58715111
NO3gr.final	-0.37142857	0.63496447
NO2gr.final	-0.42857143	1.20280863
NH4gr.final	-0.60000000	0.90649359
Nitrification.rate	-0.65714286	1.20929421
Delta.NO2gr	-0.82857143	1.12477840

Tableau 13: PLS model of WM biomass. Validation: RMSEP. Cross-validated using 6 leave-one-out segments.

	Intercept	1 comps	2 comps	3 comps	4 comps
CV	6.904	3.649	3.564	3.038	2.969
adjCV	6.904	3.432	3.337	2.789	2.731
TRAINING:	% variance explained				
	1 comps	2 comps	3 comps	4 comps	
X	37.31	69.33	80.74	97.61	
Ph biomass	92.69	96.65	99.51	99.66	

It appears that 3 or 4 components are optimal for effectively predicting biomass, with an RMSEP around 2.969. The model achieves a high level of explanation of biomass as early as the 2nd component (96.65%). This indicates that only a few components are sufficient to capture the essential relationship between the explanatory variables and biomass (Delta NO_2-, final NO_2-, Nitrification rates). It is also possible to note that phacelia biomass is very close to 1. Thus, it is possible to conclude that NO2-concentration and nitrification rates influence directly White mustard biomass.

2.3.2. Phacelia biomass

Tableau 15: influence and importance of explicative variables on phacelia biomass

Variable	Influence on Ph biomass	Overall
WM.Biomass	0.42857143	0.54498541
Delta.NO3gr	0.25714286	0.48376690
WC.final	0.14285714	0.09278683
NH4gr.final	0.14285714	0.10624363
NO3gr.final	-0.02857143	0.39925155
Mid-time.N2O.emissions	-0.14285714	0.14176658
Nitrification.rate	-0.25714286	0.36899143
Delta.NH4gr	-0.48571429	0.25247012
Delta.NO2-gr	-0.54285714	0.67857145
NO2-gr.final	-0.71428571	0.70602004
Final.N2O.emissions	-0.71428571	0.72806161

Tableau 16: PLS model of phacelia biomass. Validation: RMSEP. Cross-validated 6 leave-one-out segments.

	Intercept	1 comps	2 comps	3 comps	4 comps
CV	5,436	4,426	4,199	3,935	3,627
adjCV	5,436	4,120	3,861	3,611	3,323
TRAINING:	% variance explained				
	1 comps	2 comps	3 comps	4 comps	
X	38,39	58,43	81,72	98,34	
Ph biomass	90,24	96,90	99,00	99,63	

All variables have an overall value <1, meaning that effect of those variables on phacelia biomass is not intensive and do not require further investigations (Table 16). However, it is possible to notice that drivers oh phacelia and white mustard are different. Phacelia has final N_2O emissions and white mustard has nitrification rates and N_2O dynamics as highlighted by biomass PCA. Furthermore, phacelia has an impact on white mustard but inverse relationship is not real. The higher biomass of white mustard creates greater contrasts, making it easier to observe and identify more pronounced relationships compared to phacelia.

VI. Personal contribution

During the course of this study, I was actively involved in various stages of the project. In the field, I carried out essential tasks such as marking experimental plots, conducting manual weeding, sowing, collecting soil samples, and measuring N_2O emissions. In the laboratory, I performed analyses to determine concentrations of NO_2^-, NO_3^-, and NH_4^+, as well as potential nitrification rates. Additionally, I processed and analysed the data, ensuring statistical rigor and interpreting the results to draw meaningful conclusions. These contributions encompassed both practical and analytical aspects, highlighting my active role in the project's success.

VII. Conclusion and perspectives

This research aimed to investigate the potential of using BNI cover crops, specifically plantain, phacelia, and white mustard, to influence nitrogen dynamics in agricultural systems and provide insights into their impact at the rotational scale. The results obtained from this study partially met the objectives set out initially.

From a field perspective, cover crop mixture showed different nitrogen dynamics by decreasing NO_3^- concentrations compared to bare soil. The ploughing treatment also played a role in dynamics of NO_3^- and N_2O emissions. White mustard displayed higher biomass production, which resulted in more pronounced relationships and easier observation of trends, compared to phacelia. The study highlighted the relationship between soil moisture and N_2O emissions, as well as the influence of microbial activity under varying soil and weather conditions. However, no definitive evidence of BNI capacity was demonstrated for the tested cover crops with the available data, leaving room for further exploration. The analysis confirmed some correlations supported by existing literature, such as the interplay between soil moisture and N_2O emissions, with wetter soils favouring biomass activity and higher emissions. Additionally, the microbial response to environmental conditions, including temperature and moisture, explained part of the variability observed in nitrogen dynamics.

Based on these findings, recommendations to producers include selecting cover crops based on biomass production. For instance, white mustard, due to its higher biomass, may offer better nitrogen retention and contribute to reducing nitrate leaching during the off-season. Producers should also aim to regulate soil moisture through adequate drainage or tillage practices to minimize excessive N_2O emissions. Additionally, adjusting the timing of tillage or incorporation of cover crops can influence microbial activity and nitrogen transformations, potentially reducing losses.

Perspectives

While this study provided valuable insights, several aspects could be improved or further investigated to enhance our understanding of BNI crops and their role in sustainable agriculture. It would be interesting to prioritize long-term experimentation during warmer season. Indeed, as shown by this study and several authors, plant growth would be an important variable explaining the lack of results.

It would be interesting to investigate microbial populations and their functional genes could provide direct evidence of BNI activity and clarify the mechanisms involved. Furthermore, collect more data on soils properties or environmental conditions to investigate the impact of more factors influencing nitrogen dynamics. Experiment in controlled environmental conditions could help isolate specific

factors, such as soil moisture or temperature, and their impacts on nitrogen dynamics. Measurement of N2O emissions and nitrification rates more frequently to better understand dynamics at each time of the experiment, as well as biomass to analyse its link with dynamics nitrogen throughout the entire experiment

Including additional BNI crops or other cover crop species could help identify those with the strongest inhibition potential. Finally, it would also be interesting to investigate more deeply the impact of agriculture management systems on BNI cover crops to provide practical recommendations tailored to producers' needs.

By addressing these perspectives, future research can contribute to refining the use of BNI crops, improving nitrogen management strategies, and promoting environmentally friendly agricultural practices.

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IX. Appendix

Appendix 1

Choose of weight extraction: Initially, 15 grams of soil were used for extraction. However, based on the results of initial NH_4^+ analyses, it has been decided to test which soil amount would be more efficient and representative of reality. For this purpose, two different soil amounts, 5 and 10 grams, were compared using the same volume of solvent.

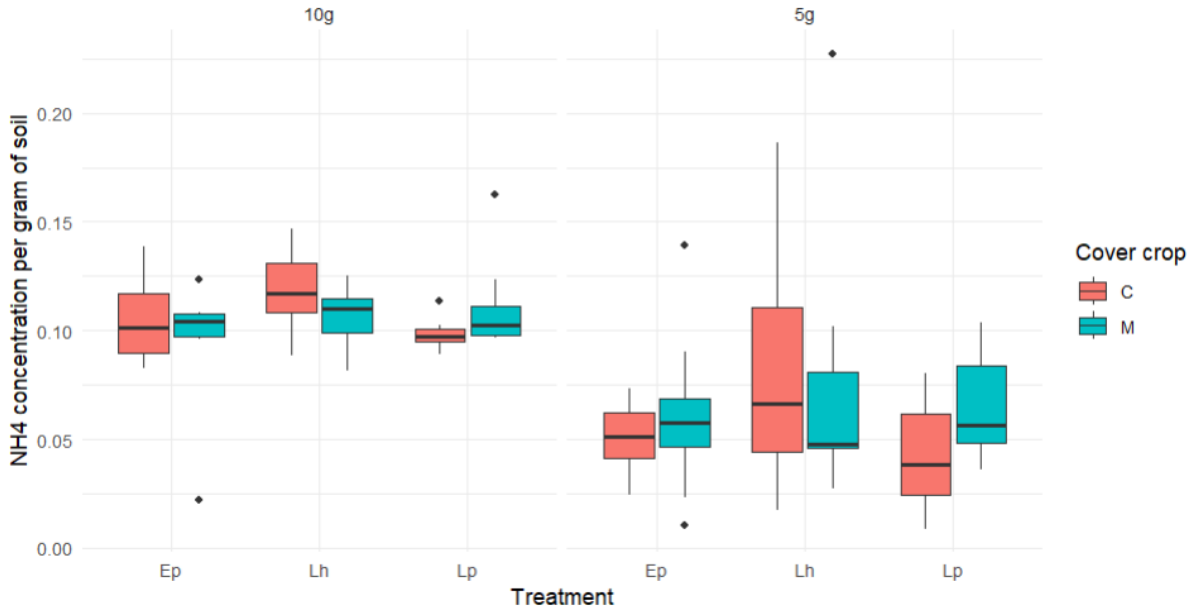


Figure 40: comparison of amount of soil extracted (5g and 10g) to determine NH_4^+ concentration

The analysis shows that an extraction with 10 grams of soil manage to extract more suggested by a greater concentration in sample solution. Indeed, it exists a statistically significant impact of the extraction weight on the results concerning ammonia concentration per gram of soil ($p = 1{,}81e^{-8}$). For this study, the interest is to take the extraction weight with the most variability to be sure to represent the variability of mineral nitrogen in soils. That is the reason why it has been decided to continue the experiment with 5 grams of soil extracted. Finally, the graph shows most contrasted concentrations for 5 grams extraction compared to 10 grams.

Appendix 2

Slurry test – Determination of the potential nitrification rate (PNR)

Modified from Hart et al 1994.

Materials

a. Orbital shaker that holds several 250 mL Erlenmeyer flasks securely.
b. 250-mL Erlenmeyer flasks, 2 per sample + 1 for the blank.
c. 15-mL falcon tubes, 7 per sample + 3 per batch (for the blanks).
d. 2-mL centrifuge tubes, 7 per sample + at least 3 per batch (for the blanks).
e. 10-mL pipette
f. 10-mL pipette tips with ends cut off to create 0.5 orifice (to not clog when pipetting soil slurry), 7 per sample.
g. 1000-µl pipette + tips (7 per sample + 3 per batch)

Stock solutions (10 samples):

a. Potassium monobasic phosphate (KH_2PO_4), 0.2 M. Dissolve 27.22 g of KH_2PO_4 in 1 L of water.

b. Potassium dibasic phosphate (K_2HPO_4), 0.2M. Dissolve 34.84 g of K_2HPO_4 in 1 L of water.

c. Ammonium sulphate ($(NH_4)_2SO_4$), 50 mM. Dissolve 6.607 g $(NH_4)_2SO_4$ in 1 L of water.

d. Potassium sulphate (K_2SO_4), 50 mM. Dissolve 8.713 g K_2SO_4 in 1 L of water.

Store all 4 solutions in the fridge. If storage is planned for more than a few days, it is better to prepare the solutions from sterilised (autoclaved) water.

Preparation of combined solutions:

- *1.5 mM of NH4+ and 1 mM of PO43- (for the PNR)*
- *Ammonium-free control*

In two 1-L volumetric flasks, mix:

- 1.5 mL KH_2PO_4 stock solution AND
- 3.5 mL K_2HPO_4 stock solution AND
- EITHER 15 mL $(NH_4)_2SO_4$ stock solution OR 15 mL K_2SO_4 stock solution
- Bring up to volume, 1 L.

So you will have 2 solutions:

- "PNR" solution - KH_2PO_4, K_2HPO_4 and $(NH_4)_2SO_4$
- "control" solution - KH_2PO_4, K_2HPO_4 and K_2SO_4

Adjust both solutions to pH 7.2 by adding NaOH (or HCl) while the combined solution is stirred.

One litre is enough for 9 samples at 100 ml each (the remaining ~100 ml can be used for the blank flask). However, to use the solution dispenser, use a 2-L bottle and always prepare more than needed (air bubbles may appear in the last fractions, thus keep the blanks for last)

Preparation of flasks

Annotate all flasks with sample name and solution (PNR or control).

For each sample, place 15 g of sieved (2- or 4-mm mesh), field moist soil into 2x 250-mL Erlenmeyer flasks (use only 10 g of material that is high in organic matter, such as soil from O horizons) and record the exact weight for each flask.

Determine gravimetric soil water content of a separate subsample: weigh 15 g of soil in an aluminium dish (also record the weight of the empty dish), oven-dry it at 99°C overnight, then weigh it again.

For each sample, in one of the 2 flasks, add 100 mL of the ”control” solution, using the solution dispenser (2x 50 ml). After thoroughly rinsing the dispenser, add 100 mL of the “PNR” solution in the remaining flasks containing soil. Then fill an additional flask with ~ 100 ml of the “PNR” solution to serve as blank. Finally, cap the “PNR” flasks with parafilm pierced with holes (this will allow gas exchange but minimize evaporation).

➔ *Tip*: don’t fill the PNR flasks first, as the nitrification would already start while you are rinsing the dispenser and filling the control.

Collecting at t = 0h

Take the first sample (t = 0h) from each flask as follows. Shake each flask immediately before sampling so that the aliquot removed has the same soil/solution ratio as the rest of the slurry.

Remove about 5 ml of slurry (or blank) from each flask with the 10-ml pipette fitted with a modified pipette tip, cut of at the end to create a 0.5 cm orifice. Place the samples in a 15 mL centrifuge tube. Make sure the parafilm is properly placed back on the “PNR” flask.

Also collect 5 ml of the “control” solution directly from the stock in a 15-ml tube as a blank for the control samples (only for t = 0h).

Immediately centrifuge all 15-ml tubes at 3000g for 2 minutes.

While the centrifuge spins, place only the flasks that received the “PNR” solution (1 per sample + 1 blank) on an orbital shaker and shake at approximately 120 rpm throughout the sampling duration at 27°C. Once samples from t = 0h are collected, the flasks with the “control” solution are no longer needed.

From centrifuged tubes, draw 2x 1 mL of clear supernatant out of the centrifuge tube with a pipette (using 1000-µl tips) and place in 2x 2-ml tubes, one for NO3-, one for NO2-.

Cap and freeze the tubes until the solutions can be analysed for NO_3^- and NO2-.

Collecting at t = 2h, 4h, 6h + 2 points on day 2

Follow the same steps as above to collect slurry from "PNR" flasks 5 more times during the 2-day period at t = 2h, 4h, 6h, 24h and either 22h, 26h or 28h based on what is convenient (be consistent throughout the experiment).

For the blank flask (containing only the "PNR" solution, no soil), 2 sampling times will suffice, taken at the first (t = 0h) and the last sampling times.

➔ *Tip:* For all sampling times, proceed with the centrifugation, pipetting and freezing as quickly as possible after sampling. If needed, keep tubes on ice.
➔ *Tip:* For soils with high organic matter content (forest soils mainly) filtration is needed (see Hart for more info)

Analysis

Thaw the solutions on ice immediately prior to analysis

When thawed, invert the tubes several times to mix.

Arrange the tubes so that the entire time series of samples from a single flask are together (this will minimise confounding rate estimates with analytical drift), including the control (ammonium-free solution) and each of the 3 blanks.

Analyse the solutions for NO_3^- by colorimetric analysis (see *ad hoc* protocol).

Calculate the rate on NO_3^- production

For each soil sample (flask), calculate the rate of NO_3^- production (mg N L^{-1} h^{-1}) by linear regression of solution concentration versus time. Calculate the rate per unit dry soil according to the following equation:

$$\frac{Rate}{(mg\ N\ kg^{-1}h^{-1})} = \frac{Rate}{(mg\ N\ kg^{-1}h^{-1})} \times \frac{0.1\ L\ + volume\ of\ water\ in\ field - moist\ soil\ sample}{kg\ oven - dry\ soil\ in\ flask}$$

Appendix 3

SOIL EXTRACTIONS

Extract soil samples with 0.5 M K_2SO_4 directly after sampling.

1) Weight e.g 10 g FW soil (equivalent to ~ 9 g DW) in 50-ml falcon tubes
   Soil quantity and buffer volume could be adjusted to any value as long as the proportions are kept. Record the exact fresh weight value for the correction of the calculations.

   Fresh to dry soil ratio (FW/DW) would be also needed to correct calculations (see soil moisture determination). Take another subsample for that purpose. The water content within the soil sample will be also taken into account for the final calculations).
2) Add 20 ml of 0.5 M K_2SO_4
3) Shake tubes for 1 h at 165 rpm in an orbital shaker (if possible, place the tubes horizontally)
4) Settle to decant the soil during at least 1 h (*if working with lower volumes, in falcon tubes, centrifuge for 5 min at 8000 rpm*).
5) Filter the solution through Whatman no. 42 filter paper. Use small funnels. Around 12-15 ml extracts will be collected.

6) Proceed with determination or store the extracts at -20 °C.

MINERAL N DETERMINATIONS:

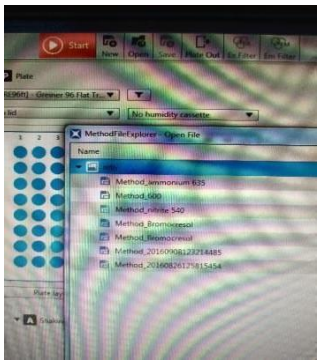
- ✓ For NH_4^+ and NO_3^- samples may need to be diluted (probably 10 or 20 times, but this must be checked for each experiment and incubation time). First, test the needed dilution a couple of samples from the treatments with the highest and lowest expected values (for both NH_4^+ and NO_3^-) before diluting or analysing other treatments.
- ✓ Always plan for more than the theorical number of plates needed, since you may need to

start over a plate because the samples were not diluted enough or else. Generally, a volume of 50 mL of VCl3, 25 mL of sulfanilamide and 25 mL of NEDD may be enough

- ✓ Use the same dilution of K_2SO_4 0.5 M as blank.
- ✓ Use the multichannel pipette (8 channels, 30-300 µl) to add the buffer/reactive solutions on the plate. Use the plastic reservoirs for the solutions.
- ✓ It's important to prepare the buffer/reactive solutions on the day of the measurement as indicated.
- ✓ Measure two technical replicates of each sample

Spectrophotometer:

- First switch on the computer. Start the session.
- Wait 1 min and switch on the spectrophotometer (not before the computer!!!). If the connection is properly established, the light will turn magenta.
- Open Spark Control program.
- Open the appropriate protocol ("Method_nitrite_540" or "Method_ammonium_635").



NO_3^- DETERMINATION

VCl_3-Griess method (Sulfanilamide/naphthylamine) after Miranda et al., (2001). With this method, NO_3^- is reduced to NO_2^- with VCl_3, and the content of NO_2^- is determined by the Griess reagents [NO_2^- previously present in the sample + NO_2^- coming from NO_3^- reduction]. So, in this assay, NO_2^- will be determined. Later, NO_2^- determination will be also needed to calculate the real, NO_3^- concentration of the sample as: $\mathbf{NO_3^- = NO_x^- - NO_2^-}$

Prepare the following solutions (fresh, on the measurement day) using double distilled water. Notice that, for some of them, part of the volume is HCl:

- **0.8 % VCl_3** (Merck 1123930025) **in 1 M HCl** (always open and dispense VCl_3 in a hood)
  [e.g. 400 mg VCl_3 in 50 ml final vol., containing 4.106 ml HCl 37%] Warning! (*See below*) *(or prepare in advance a 1M HCl solution)*

- **1 % sulphanilamide** (Sigma S9251) **in 3 N HCl**
  [e.g. 500 mg sulphanilamide in 50 ml final vol., containing 12.318 ml HCl 37%]
  (or prepare in advance a 3 N HCl solution)

- **0.1% N-naphthylethylenediamine dihydrochloride (NEDD)**
  (Sigma 33461) [e.g. 50 mg NEDD in 50 ml final vol.]

Just before use, mix equal volumes of sulphanilamide and NEDD solutions [Griess reagents]

Prepare the solutions in a volume based on the number of samples that will be measured on a particular day.

Prepare always in excess, around 20% more. e.g. for one 96-well plate: ~12 ml VCl_3 solution and ~12 ml Griess reagents (6 ml sulphanilamide solution + 6 ml NEDD solution). *And other 12 ml Griess for* NO^-

The reaction was carried out in 300 µl in 96-well microplate:

- 100 µl sample (diluted as needed, e. g. 1/10)
- 100 µl VCl_3 solution
- 100 µl of mixed (just before measurement) Griess reagents [50 µl sulfanilamide + 50 µl NEDD]

After 20-30 min, absorbance was red in a spectrophotometer at 540 nm (NO^- containing samples become pink). Be very precise with the reaction time, always the same for all the plates!

Add a NO^- standard curve in each plate!! Prepare it from KNO_3 in a range of 0-20 mg NO^- L^{-1} (concentration based on NO_3 molecules, not on KNO_3!!). *See below.*

Warning! VCl_3 is very volatile!! To prepare it, tare an empty closed falcon tube (with the cap). Take it to the hood, open the product and add an approximate amount to the tube. Close the tube and take it to the scale again. When the weight is similar to the desired amount, just adjust the volume needed

NO^- DETERMINATION

Together with nitrate, NO^- content must be determined in the samples, and subtract the obtained value to the previous one [NO^- previously present in the sample + NO^- coming from NO^- reduction]

Prepare the following solutions as above:

- **1 % sulphanilamide** (Sigma S9251) **in 3 N HCl**
- **0.1% N-naphthylethylenediamine dihydrochloride (NEDD)**

(Sigma 33461) The reaction is carried out in 200 µl in 96-rwell

microplate:

- 100 µl sample (non-diluted or with a lower dilution)
- 100 µl of mixed Griess reagents [50 µl sulfanilamide +

50 µl NEDD] After 15 min, read at 540 nm.

Include a "diluted" NO_2^- standard curve in the plate (as $NaNO_2$), in a range of 0-2 mg $NO_2^- L^{-1}$. *See below*

NO_2^- content is expected to be very low or undetectable in soil samples.

NH_4^+ DETERMINATION

NH_4^+ content was determined by the Berthelot method (Patton et al., 1977). The method is based on the phenol hypochlorite assay (Berthelot reaction).

Prepare the following solutions (fresh, on the measurement day):

- Solution A: 0.33 M **sodium phenolate** (Sodium phenoxide trihydrate, Sigma 318191) in 0.16 M NaOH.

  First, prepare 2 M NaOH dissolving 8 g NaOH in 100 ml of double distilled water.

  Then, mix 2.8 g of sodium phenolate with 4 ml of 2 M NaOH in a final volume of 50 ml with double distilled water. Ensure pH of the solution is around 13 (add 2,8 g in 4 mL of NaOH 2M and then, adjust to 50 mL with water).

- Solution B: 0.02% **sodium nitroprusside** (Sodium nitroferricyanide, Sigma 71778).
- Solution C: 2% **sodium hypochlorite** (Bleach, VWR 90350) (2mL of bleach 14%/100mL total (98mL of water).

The reaction was carried out in 300 µl in 96-well microplate:

- 50 µl sample (diluted as needed)
- 100 µl solution A
- 50 µl solution B
- 100 µl solution C

After 30 min, absorbance was red in a spectrophotometer at 635 nm (samples become

blue). Include a NH_4^+ standard curve in the plate (as $(NH_4)_2SO_4$), in a range of 0-25

mg $NH_4^+ L^{-1}$.

(Concentration based on NH_4^+ molecules!! Notice that there are two in each $(NH_4)_2SO_4$ molecule). *See below.*

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AMPLES for the STANDARD CURVES:

Stock at 100 mg NO3-/L = *163 mg KNO3/ L*

Standard curve **mg NO3- / L**	Stock (100 mg NO3-/L) vol (ul) (Vf 2ml)	ul H2O
0	0	2000
2	40	1960
4	80	1920
6	120	1880
10	200	1800
14	280	1720
20	400	1600
24	480	1520

Stock at 100 mg NO2-/L = *150 mg NaNO2/ L*

Prepare a second **diluted stock at 10 mg NO2-/L**

Standard curve **mg NO2- / L**	Stock (**10 mg NO2-/L**) vol (ul) (Vf 2ml)	ul H2O
0	0	2000
0.1	20	1980
0.2	40	1960
0.3	60	1940
0.4	80	1920
0.8	160	1840
1	200	1800
2	400	1600

Stock at 100 mg NH4+/L = *366 mg (NH4)2SO4/ L*

Standard curve **mg NH4+ / L**	Stock (**100 mg NH4+/L**) vol (ul) (Vf 2ml)	ul H2O
0	0	2000
1	20	1980
2	40	1960
3	60	1940
4	80	1920
6	120	1880
8	160	1840
10	200	1800

Appendix 4

To further investigate whether the type of cover crop might influence nitrification rates in the rhizosphere, a separate analysis was conducted. A new dataset was created to compare the effects of phacelia, white mustard, and bare soil. An additional ANOVA (AV2) was performed on this dataset, which yielded the same results, confirming that the type of plant does not have a significant impact on nitrification rates.

1	C	3.837118e-05
2	PH	3.594784e-05
3	WM	3.809806e-05

➔ The average of bare sol is higher than both WM and mustard.