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ARNAUTS, Natascha; Portillo-Estrada, Miguel; WEVERS, Jolien; Carnol, Monique; Bosman, Bernard & RINEAU, Francois (2023) Investigating the response of soil nitrogen cycling to grass invasion. In: PEDOBIOLOGIA, 97 (Art N° 150874).

DOI: 10.1016/j.pedobi.2023.150874
Handle: http://hdl.handle.net/1942/41643

Investigating the response of soil nitrogen cycling to grass invasion

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Abstract

In heathlands, high mineral N input causes replacement of *Calluna vulgaris*, the dominant plant, by fast-growing grasses such as *Molinia caerulea*. The vegetation shift signifies altered litter quality from low- to high-quality litter due to differences in lignin content. Litter quality usually affects decomposition processes, which can, in turn, alter nutrient cycling. Therefore, the change in plant dominance in this ecosystem possibly alters soil carbon and nutrient cycles, and consequently, ecosystem services (e.g. biodiversity conservation, groundwater recharge, …). We hypothesise that, because of its higher litter quality, nutrient turnover becomes faster with grass encroachment. We tested this hypothesis in a field set-up consisting of 14 plots presenting a gradient of increasing grass dominance (from 0 to 100%). We measured nine soil parameters and assessed possible associations between grass dominance and the soil parameters using multivariate analysis and linear mixed models. We found that grass dominance significantly impacted net N mineralisation and the root biomass. Our results showed very low net N mineralisation rates (0.09±0.04 mg N (kg soil)$^{-1}$ day^{-1}) and relative nitrification rates (1.99±0.62 %). At high grass levels, acid phosphatase activity was significantly lower than at lower grass percentages. These results show that grass encroachment has a minimal impact on heathland soil biochemistry at this point. Still we consider that it may take many years to translate a change in litter quality and dynamics into a change in soil functioning.

Keywords

Heathland, grass encroachment, soil nutrient cycling, soil enzymes, net nitrogen mineralisation, relative nitrification

1. Introduction

Since the beginning of the nineteenth century, a combination of climate change and anthropogenic activity (combustions, agriculture, land-use change) has caused an increased N deposition in soils under multiple forms, which has led to a shift in plant dominance in the heathland (Aerts and Berendse, 1988; Galloway et al., 2004; Heil and Bruggink, 1987; Lavelle et al., n.d.). The heathland area cover has enormously decreased in Western Europe. The heathland surface in Belgium and the Netherlands, for example, has reduced by more than 95 % compared to the beginning of the 19th century (Odé et al., 2001). *Calluna vulgaris* (L.) Hull or common heather thrives on this nutrient-poor podzol soil but is not adapted to high mineral N input conditions, hence, loses competitive advantage to grass (Aerts and Berendse, 1988; Aerts and Heil, 1993; Bobbink et al., 1992). As *C. vulgaris* ages, the shrubs begin to have a more open canopy allowing grasses, mostly *Molinia caerulea* (L.) Moench, to germinate underneath and subsequently replace heather. The change in plant dominance can lead to several alterations in both ecosystem services (such as tourism, biodiversity conservation, groundwater recharge, C sequestration (de Bello et al., 2010; Dise, 2009; Jackson et al., 2002; Saintilan and Rogers, 2015; Sauer et al., 2007; Wessel et al., 2004)) and ecosystem functioning, especially for the carbon (C), N and phosphorus (P) cycles (Bardgett et al., 2013; Hooper and Vitousek, 1998).

Heather consists of woody structures that are high in lignin and therefore of low litter quality (Gimingham, 1972; Read et al., 2004; Van Diepen et al., 2015). That is not the case for grass, and thus, it is a preferable source to degrade by soil organisms (Chapin et al., 2002). Both plant species also have a different annual growth cycle. *C. vulgaris* is a perennial plant that blossoms in late August and September (Gimingham, 1972).Conversely, *M. caerulea* is an annual plant, even though roots and tussocks may persist from year to year in the soil. As a result, pure heathland is characterised by a constant low-quality litter input, while grass-dominated areas experience a high but brief input of high-quality litter (Ward et al., 2015). The latter may prime microbe-mediated C decomposition in the grass-dominated parts of the heathland, at least in the short-term (Breland and Hansen, 1998; Kuzyakov et al., 2000; Pascual et al., 1998). The changed litter input could alter the composition of soil microbial communities, which could lead to a potential change in their functioning (i.e. an adapted extracellular enzyme production due to the changed substrate availability). Soil N cycling is often characterised as the net N mineralisation rate, as it ensured N in a plant-available form (Cabrera et al., 2005). Nitrification measurements add to the information on the N cycle by knowing which proportion of ammonium (NH_4^+) is converted into nitrate (NO_3^-), which is moderately leached in heathland soil. Therefore, high nitrification rates may result in high NO_3^- concentrations in drainage and groundwater, causing eutrophication (Wang et al., 2015). The P cycle is closely coupled to that of N. When N is present in excess in the soil, and plant biomass increases, the nutrient requirement, in general, will rise, making P limiting (DiTommaso and Aarssen, 1989; Stevens et al., 2004). P input in terrestrial ecosystems is limited (Read et al., 2004) and, therefore, primarily internal re-cycling from organic to inorganic forms. Consequently, the soil P concentrations will reach an internal steady-state condition (Vitousek and Howarth, 1991). In many studies, N and C are investigated together, as they are both essential elements for living organisms. Under current circumstances, studies have shown an important, secondary aspect of elevated N deposition in soils: C sequestration (Field et al., 2017). Literature describes several effects of N deposition on C. For example,

vegetation shifts as described for heathland are also found for other ecosystems, resulting in reduced C sequestration (Berendse et al., 2001). Mack et al. (2004) showed in her work that N deposition resulted in a net C loss due to increased decomposition. In contrast, a heathland study showed a C sequestration increase (Field et al., 2017). Although there are different outcomes, literature shows the close link between C and N.

For forest ecosystems it has already been elucidated how nutrient cycling, and more specifically N cycling is affected by external influences (Vitousek et al., 1982). Previous studies on heathland helped to understand the heathland ecosystem's response to a specific treatment, gaining more information on the effect of N deposition and climate change (Bobbink et al., 1992; Emmett et al., 2004; Field et al., 2017; Helliwell et al., 2010; Rastetter et al., 1991; Stevens et al., 2004). Our goal was to understand to which extent grass invasion impacts soil nutrient dynamics in a Belgian dry heathland. To do so, we measured soil parameters throughout an experimental gradient with a gradually rising grass cover. An important advantage of this in situ approach is that we examined possible associations between grass dominance and the soil biochemistry (Lekberg et al., 2018; Toju et al., 2018). We hypothesised that grass dominance accelerates soil processes and nutrient turnover rates due to the differing litter quality of heather and grass.
To elucidate which factors are of importance in heathland soil, we chose a range of soil variables to measure on heathland soil samples. The water content and root biomass were measured to present some general information across the grass gradient, since these two intertwined variables have a significant impact on nutrient cycling (Metzger et al., 2017) We also measured total C (TC), total N (TN), organic matter (OM), net N mineralisation and relative nitrification. This paper focusses on understanding the influence of grass invasion on the mineralisation of N and P; therefore we also selected two enzymes as a part of our measurements: chitinase and acid phosphatase, to add to the information we gather from the analysis mentioned above.
The influence of grass invasion on the measured soil variables is examined in this paper using two different statistical descriptors: on the one hand, an exploratory approach (principal component analysis) and, on the other hand, a mechanistic approach using a linear mixed model to study possible associations between grass invasion and soil variables.

2. Materials and Methods

2.1 Site

The study was carried out in the Mechelse Heide (50°59'07.0"N 5°38'01.7"E) in Limburg, Belgium. The site is located at an altitude of 104 m, with a mean annual temperature of 10.3 °C and an average annual precipitation of 839 mm. This area is dominated by the dwarf shrub *C. vulgaris* or common heather, with local encroachment by the subdominant species *M. caerulea*, purple moor grass. All references made to 'grass' throughout this research article refer to *M. caerulea*. *Deschampsia flexuosa,* commonly known as waivy-hair grass, together with *Erica cinerea* or bell heather can be found in certain parts of this nature reserve nearby the sampled plots. However, for this research, the plots were chosen so that only *M. caerulea* and *C. vulgaris* were present. The dry heathland is managed by mowing, burning and sod-cutting (Gimingham, 1972). We selected 14 in situ plots of varying grass cover, similar plant age, similar management history, and flat slope within a total area of 287 500 m2. Each plot covered an area of circa 500-1000 m². In the plots, *C. vulgaris* plants were aged 5-12 years old with a gradient of grass cover ranging from 0 to 100 % grass (Fig. 1). The sampled plots did not undergo measurements to manage grass invasion in at least 3 years prior to the sampling.

2.2 Sampling

Soil samples were taken in April 2019: 12 randomly placed quadrats (1 m²) per plot, in each quadrat one soil core was taken in the centre (10 cm deep, 7 cm diameter) using an auger. We took a picture zenithally of each quadrat from 1.5 m distance to estimate plant cover (see below for more details). These 12 soil cores were randomly pooled by groups of three into four composite samples (representing four replicates per plot), which were stored on ice during transport. Once in the lab, the litter layer was removed, the cores were sieved to pass a 3-mm mesh and roots were kept at 6°C for further analysis. 30 Aliquots of 2 g of homogenized, sieved soil were frozen at -20°C for enzymatic analysis, and determination of TC, TN and OM. Bags of 150 g of sieved soil were stored at 6°C for N cycling measurements.

2.3 Determination of plant cover

All 12 quadrat pictures were separately analysed by dividing them into 36 compartments using a 6x6 grid. In each compartment we estimated the relative proportion of grass, heather and bare soil (adding up to 100%). The vegetation cover in the quadrat (thus for each picture) was then computed as the average value from the 36 compartments (Fig. 1). To determine the vegetation specifically for the four composite samples in each plot, the average cover of three pictures was calculated (each picture taken of the exact sampling location). We expect microbial functioning to be significantly influenced by its environment due to local effects of the vegetation. We have therefore chosen to determine the plant cover at the quadrat scale than at the plot scale. The method used to determine the vegetation cover was based on the paper of Roush (Roush et al., 2007).

2.4 Soil water content

During sampling, soil water content was measured at 10cm depth with WET-sensor type wet-2 (Delta-T Devices, Cambridge, United Kingdom) at the exact location were soil samples were taken. Four replicates per plot were taken, the average of these was used in statistical analysis.

2.5 Root biomass

The roots collected during the sieving were washed with demineralised water, dried in an oven at 60 °C for 72 hours, and weighed.

2.6 Net N mineralisation and relative nitrification

The water holding capacity (WHC, Haines-funnel system (Jenkinson and Powlson, 1976)) and gravimetric water content (overnight drying at 105 °C) were estimated, and all samples were adjusted to 60% WHC before incubations. Net N mineralisation and relative nitrification were measured using an aerobic 28-day incubation method (Hart et al., 1994). $N\text{-}NO_3^-$ and $N\text{-}NH_4$ were determined before and after the incubation of soil (20°C, in the dark) by extraction with a 1M KCl solution (1:5, w:v). Samples were analysed colorimetrically using an AutoAnalyzer 3 (Bran+Luebbe, Germany). The net N mineralisation rate was calculated by subtracting the initial from final inorganic N concentrations. The relative nitrification was calculated by dividing the net $N\text{-}NO_3^-$ by the net N mineralisation. Results were expressed per mass fresh soil.

2.7 Enzymatic activity measurements

Enzymatic activity of chitinase and acid phosphatase was measured using a fluorimetric assay (Table 2). In this assay, 4-Methylumbelliferyl N-acetyl-ß-D-glucosaminide and 4-methylumbelliferyl phosphate were used as fluorescent substrates for respectively chitinase and acid phosphatase. For this analysis, a quench control, a standard blank and a substrate blank were measured in parallel to correct for interference and absorption of the product by molecules naturally present in the soil.

The procedure was described by (Saiya-Cork et al., 2002) with the following modifications: 1 g of soil sample was suspended in 25 ml sodium acetate buffer (50 mM, pH 5) and ground during 3 min with mortar and pestle to extract enzymes from the soil. The microplates were incubated in the dark at 25 °C for 1 hour. To stop the reaction and elevate the signal, 10 µl of NaOH (1 M) was added to each well and was shaken at 500 rpm during 5 seconds. Fluorescence was measured using a Fluostar Omega Microplate Reader at 365 nm excitation and 450 nm emission.

The net fluorescence units (NFU) and enzymatic activity were calculated using the following formulas:

$$NFU = \frac{assay - sam}{\frac{quench\ control - samp}{standard\ blank}} - substrate\ blank \quad (1)$$

$$Enzymatic\ activity = \frac{\frac{NFU \times conc\ standard \times volume\ standard}{blank\ standard}}{volume\ sample \times \frac{mass\ soil}{volume\ buffer} \times time} = \left[\frac{\mu mol}{h \times g}\right] \quad (2)$$

This method was chosen because it corrects for quenching.(Clarke et al., 2001; Freeman et al., 1995)

2.8 Total carbon and total nitrogen

The samples were air-dried at 70 °C for 48 hours and they were ground to pass a 0.5-mm sieve in an ultra-centrifugal mill (Model ZM 200, Retsch GmbH, Haan, Germany). The total soil C and N were determined by dry combustion, based on the Dumas method using an elemental analyser (Model FLASH 2000, Thermo Fisher Scientific, Germany) (Culmo, 1988). We checked on a set of test samples that there were no carbonates present in the soil beforehand. The amount of carbonates in the soil was measured beforehand on a set of test soil samples taken from the same plots. There was no extra acidification step performed due to absence of carbonates in this type of soil.

2.9 Organic matter

The soil organic matter content was measured on soil that was dried overnight at 60 °C. An acidified potassium dichromate ($K_2Cr_2O_7$) oxidation was used for colorimetric determination. A series of glucose dilutions was used to make a standard curve. Absorbance was measured at 590 nm with a spectrophotometer (Model Novaspec Plus, Fisher Scientific, Waltham, MA, USA). The equation of the glucose standard curve was used to calculate the C content in the samples. These results were multiplied by a factor of two to estimate the organic matter content (Carter and Gregorich, 2006; Pribyl, 2010).

2.10 Data analysis

We performed a principal component analysis (PCA) to examine the relationships between measured variables (vegetation cover, root biomass, organic matter (OM), soil water content (water), total carbon (TC), total nitrogen (TN), net N mineralisation (net min), relative nitrification (rel nitr), and soil enzyme activity of chitinase and acid phosphatase).

To test for possible associations between grass cover and soil variables, we performed a linear mixed model. Firstly, we performed an analysis using the grass gradient as a continuous variable (ranging from 0-100%). We fitted the linear mixed model for each soil parameter as response variable. Each model included grass cover (%) as an explanatory factor, and the plot as random factor. Secondly, the vegetation was divided into four groups based on the percentage of grass coverage: group 1 (0-24.99%), group 2 (25-49.99%), group 3 (50-74.99%) and group 4 (75-100%). These four groups together with the plot as a random factor were fitted into the linear mixed model for each soil parameter measured. We performed this second analysis because we were questioning whether

certain trends were found in our first model, since our dataset is relatively small. For both approaches, when normality and homogeneity of variance were not met, we performed a transformation (square root (1+x), logarithmic or exponential) on the data to meet this requirement. When transformed data also did not meet the normality assumption, we used the transformation with the best fit. *P*-values ≤0.05 were considered significant. A Bonferroni correction was performed to correct for multiple analyses by dividing the significance level (=0.05) by the numbers of tests performed, resulting in a significance level of 0.0056

We performed a cluster analysis on the measurements of acid phosphatase activity in function of grass percentages because we observed a break in the curve of the lower phosphatase activities and less variability from 75 % grass cover. We wanted to determine if a specific threshold value of grass invasion is needed to have a significant effect. The cluster analysis was performed via a complete linkage method using the 'hclust' function in R (supplementary Table A.4. The Euclidian distance and dendrogram classified these data into four groups of grass levels, between which soil parameters were compared using an Anova and Tukey's post hoc. All statistical analyses were performed in the R environment version 3.6.1 (R Core Team, 2019).

3. Results

The PCA revealed two principal components having the most influence (Fig. 2). The two components of the PCA (Fig. 2B) together explained 54 % of the total variation. The first one (36 % of the total variability) was mostly associated with OM, TC, TN and net N min. The second component, representing 18 % of the variability, was mostly correlated with the enzymatic activity of acid phosphatase and chitinase and water content, which are clustered together. Root biomass and rel nitr were also correlated with the second component, although less strong. The samples with high grass cover are spread out over the first component and have negative values on the second component (Fig. 2A). High grass cover tends to correlate with high OM, TC and TN and the net N min rate. We also observed that samples with low grass cover, and therefore higher heather cover were mostly characterized by higher enzyme activity (Fig. 2A,B).

The result of the linear mixed model using the grass level grouping was considered significant for a *p*-value < 0.0056 after Bonferroni correction. The highest percentage of grass, i.e. the group of 75-100%, was significantly associated with the phosphatase activity ($\beta = -1.18$, $p<0.0056$), the net N min rate ($\beta = 2.55$, $p<0.0056$), and the root biomass ($\beta = -0.76$, $p<0.0056$). We also found that the group of 50-74.99% grass is significantly linked to the root biomass ($\beta = -1.04$, $p<0.0056$). We also tested the impact of the grass gradient throughout our field sites by fitting a linear mixed model for each measured soil parameter. A *p*-value < 0.0056 after Bonferroni correction was also considered significant for this analysis. These results showed that grass had a significant effect on the net N min rate and root biomass, and not on any of the other measured soil variables (Table 1). The net N min rate in our measurements ranges from -0.148 to 1.6 mg N $(kg\ soil)^{-1}\ day^{-1}$ ($\beta = 0.028$), with two extremely high points in a pure grass plot: samples 8A and 8C with 1.2 and 1.6 mg N $(kg\ soil)^{-1}\ day^{-1}$ respectively (Fig. 3). The grass cover was significantly associated with

the log of the net mineralisation rate (ß = 0.028, p<0.0056). The measured plots have a root biomass ranging from 0.52 g to 11.43 g. We also found the grass cover significantly associated with the log of the root biomass (ß = -0.0091, p<0.0056).

Rel nitr rates were measured between -1 and 4 % with two high and two low data points with differing grass levels (Fig. 3). There was no significant effect found of the grass cover on the chitinase activity of the soil samples (Fig. 3). The variation in the chitinase activity measurements is high. We observed lower phosphatase activities and less variability at levels higher than 75 % grass cover (Fig. 3). We therefore tested whether there is a minimum grass cover level threshold value needed to produce an effect on the acid phosphatase activity. Four levels of grass invasion were distinguished chosen on the dendrogram at height 1.5. This height was selected to keep the number of clusters low enough in order to maintain sufficient replicates per cluster (Table 2).

We compared the soil parameters, using an ANOVA and Tukey test, between the four groups defined by the cluster analysis (Fig. 4, Table 2, supplementary Table A.5). The acid phosphatase activities at group 0 (0.0-30.4 % of grass cover) and at group 2 (45.5-85.4 % of grass cover) were more variable than at group 1 (30.5-45.4 % of grass cover)and at group 3 (85.5-100.0 % of grass cover) (). However, only group 1 and 3 were significantly different from each other (p-value < 0.01). The average acid phosphatase activity of group 1 was 47 % higher compared to the average acid phosphatase activity of group 3 (high grass cover: 85.5-100 %). Therefore, although we did not observe a significant association between the grass cover (as continuous variable) and the acid phosphatase activity, it seems that the groups with higher grass cover were significantly associated with lower acid phosphatase activity

4. Discussion

4.1 Effects of grass invasion

In order to clarify the consequences of the shift of heathland into grassland, knowledge of the measured soil variables is paramount to discover how soil N cycling has been affected by grass invasion. Our hypothesis stated that due to differing litter qualities of *M. caerulea* and *C. vulgaris*, litter decomposition and thereby nutrient cycling rate would increase with grass dominance.

The PCA results showed two main clusters of variables (the first cluster: OM, TC, TN and net N min; the second cluster: soil water content, acid phosphatase and chitinase activity). These parameters possibly explain most of the variability in the measurements. In our sampled plots, the net N min rate was closely linked to the OM, TC and TN; this finding confirms the importance of organic matter input in nutrient-poor soil. N deposition causes a plant biomass increase and thus a need for other nutrients (Yue et al., 2016). Overall we found very low net N min rates, which could be explained by the timing of our sampling. We sampled during early Spring when soil moisture and temperature are more consistent than in Fall, although cycles are much slower compared to Autumn (Bonnett et al., 2006; Franzluebbers et al., 1994). Van Meeteren et al. (2007) demonstrated that the net mineralisation rate was heavily affected by temperature and soil moisture, with a decreasing net mineralisation rate when soil moisture increased at the lowest temperature measured. As we cannot find such a trend in our results, it would be interesting to investigate this in Fall.

We found a significant effect of grass on the net N min rate, which confirms results from other studies and provides additional insight regarding the impact on the dry heathland in Belgium.. The work of Finzi and Canham (1996) has described that mixed species litter causes a significant difference in the net N min rate for forest litter. Our experiment showed significantly higher rates in the grass-invaded plots than single-species *C. vulgaris* plots. The composition of these two species is very different, hence, decomposition rates are also dissimilar. The woody structures of *C. vulgaris* are high in lignin which is not easily decomposable, contrary to *M. caerulea*. Other studies have discovered that rhizosphere decomposition is rapid when soil lignin is low (Bradley et al., 1997; Rahman et al., 2013), which is consistent with our findings.

In addition to an elevated N min rate, we also identified a significantly higher concentration of phosphatase in the grass dominated plots. Many studies show a negative feedback system of this enzyme: low P concentrations induce the productions of acid phosphatase (Olander and Vitousek, 2000). These results taken together imply that P is the liming nutrient in these plots. And that a higher N availability renders a need for more P to be built in (Margalef et al., 2017).

Our results showed a significantly lower root biomass in grass dominated plots. The roots of *C. vulgaris* generally reside in the top 10 cm of the soil, contrary to *M. caerulea* roots which are evenly distributed down to 100 cm depth (Aerts and Heil, 1993). *C. vulgaris* is known to have a more superficial root system. Additionally, the roots of *M. caerulea* are concentrated at a greater depth of the soil profile at locations where they coexist with Ericaceae species such as *C. vulgaris* (Gimingham, 1972). It was found that when roots are at different depths, differences in microbial communities might be found at the level of the rhizosphere while the bulk soil is more homogenous throughout the changing vegetation (Veresoglou et al., 2012). Since we measured at the top 10 cm of the soil profile, and we also identified a significantly higher net N mineralization rate at the grass dominated plots, this implies that microbial communities highly involved in N mineralization present in the rhizosphere are not linked to heather. Therefore, it would be interesting to measure net N min rates at multiple depths of the soil profile in plots where *C. vulgaris* and *M. caerulea* co-exist.

We only discovered an effect of the grass invasion on the net N min rate, phosphatase activity and the root biomass, and not on any of the other variables that were measured as a proxy for soil nutrient cycling. While studies on other ecosystems observed significant effects of an altered vegetation on soil nutrient parameters. Souza-Alonso et al. (2014) found significantly higher TN and TC in invaded mixed forest and shrubland soils, in addition to significantly higher P, magnesium and calcium. Contrary to our findings, the soil nitrification rate was found to be higher in invaded areas of a dry grassland (Pellegrini et al., 2021). These studies showed highly variable results, however, these differed in methodology, so comparisons with our observations are limited.

Our results should, however, be interpreted taking into account the following two arguments. First of all, the humus build-up over the years may have a much stronger influence than the actual litter input. We chose the sampling plots based on their vegetation ranging from 100 % heather (and 0 % grass cover) to 100 % grass cover (and 0 % heather); therefore, we assumed the organic matter layer's composition to follow similar proportions to the plant cover. However, literature has shown that for the decomposition of lignin, the weight halves over the course of 23 years (Huang et al., 1998), yet is not fully decomposed. Consequently, this suggests a *C. vulgaris* litter build-up. Since, the area is historically a heathland with *C. vulgaris* as dominant vegetation (Gimingham, 1972), the organic matter is mainly litter originating from *C. vulgaris* and thus the composition is less contrasting than the plant cover. The grass invasion of the last decade could thus be too recent to have a significant influence. Indeed, literature shows that grass and heather have different organic input dynamics, i.e. a higher biomass turnover rate for grass which shows in the net N min rate (Certini et al., 2015; Van Vuuren et al., 1993).

A study by French (1988) has already demonstrated a lower decomposition rate of the *C. vulgaris* stem compared to *M. caerulea* leaves. Although the aboveground biomass of *C. vulgaris* is on average ten times higher than that of *M. caerulea*, the litter production of both roots and shoots of grass exceeds that of heather for the same area (Aerts and Heil, 1993). Furthermore, *M. caerulea* being an annual plant, its aboveground biomass wilts entirely in winter, which results in a large event of litter input. We sampled in April 2019 when most of the grass litter had been probably decomposed largely over winter, which may explain why we only detected an effect of the grass gradient on the net N min rate and the root biomass. In these data, we see that the change in plant dominance does not affect many soil variables, while they may be still largely influenced by the legacy in plant cover (Brock et al., 2019; Monger et al., 2015). Therefore, we believe it to be of interest to measure grass invasion over an extensive amount of time. Remote sensing data could improve the accuracy of estimates of changing vegetation cover. Another option is to measure litter input into the soil by using litter traps (Talbot et al., 2015). These are difficulties of measuring in a field setup where not all factors can be controlled and should be taken into account when examining the data.

Secondly, it is unknown to which extent the microbial soil community structure, which plays an essential role in the decomposition of organic matter, varies throughout the grass gradient (De Vries et al., 2015). The decomposition rate is dependent on litter quality, and we used the C:N ratio to investigate this throughout the sampled plots. A favourable ratio would vary in the range of 10:1 – 30:1. A high ratio of 100:1 would not be readily utilisable by microorganisms unless additional N sources are available (Larcher, 2003). The soil C:N ratio in our study spans from 20:1 to 31:1 across the gradient.. The litter input seems to be originating from both grass and heather, thus creating a gradual change. However, studies have shown a quick return of the soil microbial communities after treatment (Jensen et al., 2003), indicating that this gradual addition of grass litter creates a brief change, switching back after decomposition (Pellegrini et al., 2021). Only a small fraction of the soil organic matter turns over every year; therefore, the overall changes in soil biochemistry are low, and so are the changes in microbial communities. When looking at soil microorganisms in acidic soils, it is known that fungi mostly drive the nutrient cycles compared to bacteria (Gimingham, 1972; Matthies et al., 1997). Both vegetation species, *C. vulgaris* and *M. caerulea,* associate with different types of mycorrhizal fungi,

303 respectively ericoid (ERM) and arbuscular (AM). While ERM fungi contribute to decomposition, AM do not (Smith and Read,
304 2010). Lindahl et al. (2007) found for a boreal forest that saprotrophic fungi are the primary decomposers of fresh litter. This
305 information in combination with only minimal literature on saprotrophic fungi in heathland, makes it hard to predict how the
306 different types of fungi coexist in our grass invasion gradient.

307

308 ## 4.2 Conclusion

309 We believe to have added to the general understanding of nutrient cycling processes in heathland soil by performing this research.
310 The advantage of this study lies in the fact that we investigated the impact of gradually rising grass levels in a field set-up, which
311 significantly affected the net N min rate and the root biomass. Grass invasion affected the net N min rate and the root biomass.
312 The phosphatase activity is only significantly associated with the highest grass cover (85.5-100%). None of the other soil
313 parameters in our study were significantly affected, indicating that the changing vegetation may have been too recent to have a
314 major impact on soil nutrient pools and cycling. The effect of a shift in plant dominance may have larger consequences at a longer
315 timescale when the soil composition and soil decomposing communities are more subjected to grass invasion. This implies that
316 the heathland ecosystem functioning seems to have a strong inertia. More drastic changes in its functioning may happen long after
317 an eventual disturbance or change in environmental conditions. Which is particularly interesting when considering climate
318 change: we might not see an impact at first but the changes will happen brutally, perhaps many years after the initial
319 environmental changes. While we would be thinking that this ecosystem is very resistant because of its absence of response. For
320 future studies, it would be interesting to focus on the microbial communities in these plots and include long term studies on OM
321 dynamics and litter input.

322

5. Acknowledgements

The authors would like to thank ANB (Agentschap voor Natuur en Bos) and NPHK (Nationaal Park Hoge Kempen) for providing access to the sampling site. The PhD of the first author was financed by the Centre for Environmental Sciences from UHasselt (CMK). The authors would also like to thank PhD students Laure Steenaerts, Wouter Reyns, Laura Coninx, Michiel Huybrechts, Karolien Bijnens and Jana Deckers, and master student Lennert Indestege for their help in the lab and the field; as well as dr. Veerle Stouten for her valuable input.

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