

Seasonal variation in fungal biomass

- The effects of soil temperature and moisture in a boreal forest

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Abstract

Climate change involves a series of events promoted by elevated levels of atmospheric CO₂, such as increased temperature and changes in seasonal precipitation patterns, soil temperature, moisture and nutrient availability. Soils in boreal forests are often well-drained and not underlain by permafrost, which makes boreal forests susceptible to droughts. Models predict a 7.4% decrease in soil moisture per degree Celsius of warming in Europe, however, the effect of changes in seasonal patterns on fungi is still unclear. Whether and how changes in soil temperature and moisture will affect fungal biomass is still incoherent. In this study, I tested monthly biomass variation over a whole year in order to determine if seasonal changes in soil temperature and moisture, such as drought, had an effect on biomass, and whether fungal communities in different soil fertility have different responses to disturbances in seasonal patterns. I used DNA extracts from samples collected each month for a whole year. I amplified the ITS region using qPCR and quantified ITS copy number, which were then recalculated to get an estimation of fungal biomass in the sampled soil. Soil temperature and moisture had been measured throughout the year and daily measures were recalculated to monthly averages. This study demonstrates the temporal variation of fungal biomass and how biomass depends on the interaction between temperature and moisture in the soil. Further it shows how fungi in soils of different fertility levels are affected by seasonal changes and extreme weathers such as drought.

Keywords: fungi, biomass, qPCR, temperature, moisture, climate

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1 Introduction

A change in the environment almost always leads to a chain of changes, and thus we no longer speak of “global warming” but global climate change. Climate change involves a series of events promoted by elevated levels of atmospheric CO₂, such as increased temperature and changes in seasonal precipitation patterns, soil temperature, moisture and nutrient availability. For instance, models predict a 7.4% decrease in soil moisture per degree Celsius of warming in Europe, and that a decrease of winter precipitation affects the soil moisture balance (Moore et al., 2016). Low amounts of snowfall towards the end of winter means lower water input from melted snow and so there will be higher risks of drought during summer. Boreal forests are adapted to the seasonal cycles of cool, temperate zones. Soils in these forests are often well-drained and not underlain by permafrost (German & Allison, 2015), which makes boreal forests susceptible to droughts. Cooke & Rayner (1984) developed a framework for fungal strategies which are adaptations for competition, stress, and disturbance. The boreal forests have high variation in fertility, which is determined by acidity, nutrient availability and hydrology (Sterkenburg et al., 2015). According to Cooke and Rayners framework, S-strategists are stress-tolerant fungi which are adapted to endure conditions of continuous environmental stress in low fertility soil. However, in soils with higher fertility and undisturbed conditions, fungi with no stress tolerance but combative strength (C-strategists) will compete for resources and displace S-strategists. There is a third group of strategists, which have a short individual life span, but are fast in growth and reproduction (R-strategists). These are opportunists that jump in wherever the degree of both competition and stress is low.

Many studies have devoted time and effort to predict the response of mycorrhizal fungi to climate change (Mohan et al., 2014). However, these studies have received quite different results. Experimental warming in the arctic tundra has found an increase of ectomycorrhizal biomass (Clemmensen et al., 2006), but warming on an arctic grass species showed reduced abundance of arbuscular mycorrhizal fungi (Olsrud et al., 2010). The effect of drought is still unclear. Mohan et al. (2014)

reviewed studies that tested the impacts of drought, and found that 57% of the studies found a decrease in mycorrhizal fungi, while 43% saw an increase.

Several studies have aimed to understand how climate change may affect saprotrophic fungi and litter decomposition. A model developed by Cox et al. (2000) predicts an increase of respiration in response to warming, but these predictions are thought to be true only if soil moisture is sufficiently high (Aerts, 2006; Davidson & Janssens, 2006). Allison & Treseder (2008) found a clear suppression of fungal activity and reduced abundance under drought conditions. Other studies have shown that there will be a shift in the fungal community, and drought-resistant species will maintain the rate of decomposition (Yuste et al., 2011). It has also been discussed whether ericoid and ectomycorrhizal fungi possess some enzymatic abilities which can decompose soil organic matter and contribute to carbon losses under climate change (Talbot et al., 2008; Bödeker et al., 2014). Whether and how elevated temperature coupled with drought will affect fungal biomass and activity is still incoherent.

The results from different studies may appear contradictory, but it is just a reflection of the complexity of fungal communities. Depending on the intensity and duration of temperature and CO₂ elevation, the fertility of the soil and the composition of the community, responses to experimental climate change will differ. For instance, plants are thought to both allocate more carbon to mycorrhizal fungal biomass during water stress in order for the fungi to access scarce water resources (Augé, 2001), and to respond with reduced CO₂ assimilation by stomata closure (Courté et al., 2010) which would on the contrary mean less carbon allocated to mycorrhizal fungi. According to Simard & Austin (2010, p.285), results from most field studies suggest that plant carbon allocation during soil warming and drying should favor fungal species with high biomass and long distance exploration strategies.

The questions that remain though, is whether fungal biomass and activity is suppressed during drought, and whether fungal communities in different soil fertility have different responses to disturbances in seasonal patterns. In order to answer these questions, I took DNA extracts from soil samples in a boreal forest with known shifting fertility gradient. I used qPCR to quantify total fungal biomass, which is a useful tool to measure biomass variation over time. I tested monthly biomass variation over a whole year in order to determine if seasonal changes in temperature and moisture had an effect on biomass. I hypothesized that (1) fungal biomass is significantly affected by both soil temperature and moisture, and biomass will decrease significantly during drought conditions as a response to water scarcity; (2) fungal biomass in the more stressed, low-fertility plots, will have a stronger response to seasonal fluctuations in soil temperature and moisture, as communities here supposedly are more sensitive to disturbances.

2 Materials and Methods

2.1 Soil sampling, processing and extraction

DNA extractions used in this study were obtained from a long-term study carried out from September 2012 to the present. Soil samples were collected at a monthly basis from the old-growth forest of Fiby ($59^{\circ}53'30''\text{N}$ $17^{\circ}21'0''\text{E}$), which is a mixed forest with mosaic vegetation types and variation in soil fertility. A sample ‘harvest’ consists of eight plots (2x6 m) distributed evenly (~10 m apart) on a 50 m diameter circle around the eddy flux tower (system which monitor the exchange of carbon between the biosphere and atmosphere, Aubinet et al., 2000), and from every plot three samples were randomly taken each month and directly put on dry ice, stored at -70°C and later freeze-dried. The three samples from one plot were pooled and the soil homogenized by grinding in liquid nitrogen with a mortar and pestle. Using a Nucleospin kit (Macherey-Nagel, Düren, Germany) DNA was extracted from ~50 mg of dry weight soil, following instructions in the manufacturers protocol. The concentration (ng DNA/ μl) was measured using Nanodrop. Extracts were stored at -20°C until use. The field sampling and DNA extractions had already been carried out for other projects. Twelve harvests from 2014, representing each month of the year, were selected for this study.

2.2 Real-time, quantitative PCR

Real-time PCR was performed with BioRad iCycler, and analyzed with iQ5 Multicolor (version 2.1.97.1001), using the Power Sybr Green Master Mix (Applied Biosystems, Warrington, UK). DNA extracts from each plot were diluted to a concentration of 2 ng DNA/ μl . The qPCR reactions with a total volume of 20 μl contained 10 ng DNA template, 0.5 μM forward primer, 0.3 μM reverse primer, 10 nM fluorescein. Fluorescein was added to the PCR mix in order to correct for plate background differences using the ‘dynamic well factors’ setting in the qPCR run. In order to avoid excluding important fungi (as can occur with fITS7) I used gITS7 (GTGARTCATCGARTCTTG) paired with ITS4 (TCCTCCGCTTATTGATATGC) which include all fungi but risk including some plants (Ihrmark et al., 2012). For the standard curve I used amplified plasmids with the ITS region of *Pilidium concavum* and generated a ten-fold dilution series to the concentrations of 30000000 down to 300 copies per reaction. Conditions for qPCRs were as follows: 10 minutes of incubation at 95°C, following 40 cycles of amplification (15 seconds denaturation at 95°C, 20 seconds annealing at 57°C and 45 seconds primer extension at 60°C). I ran a test plate, checking the efficiency of standard curves and testing the samples for inhibition by spiking sample template

with known copy numbers of standard (30000000 copies per reaction). Each plate contained three technical replicates of every sample and standard to avoid gaps in data and reduce risk of error. Negative controls were used to detect contamination. ITS copy numbers were quantified by comparing unknown sample C_T values to the standard curve run in each qPCR plate. When C_T standard deviation was higher than 0.9, the most deviating C_T count was removed and remaining replicates were averaged. Quantity of ITS fragments in template (ITS copies/5 μ l diluted DNA extract) was recalculated to get an estimation of fungal biomass in the sampled soil (ITS copies/mg DW soil).

2.3 Measurements in soil and air

Net CO₂ fluxes were measured from 26 m above ground from an Eddy Flux Tower (Aubinet et al., 2000), at a frequency of 10 Hz. A positive value represents a loss of CO₂ from the ecosystem to the atmosphere and a negative value is an uptake of CO₂ from the atmosphere. The calculated daily net CO₂ flux from 2014 was obtained from David Hadden and Achim Grelle, Department of Ecology at SLU. Daily CO₂ measurements were averaged over the 3 days before and after each sampling date, in order to investigate the relationship between net C fluxes and fungal biomass.

The collected soil data from 2014, included moisture, temperature and fertility (NH₄⁺ and pH). Soil temperature and moisture in each plot was measured every 30 minutes at 5 cm depth (Data logger Em50, Soil Moisture Sensor 5TM, Decagon Devices, Pullman, WA, USA). Soil fertility was analyzed from samples taken three times during the year (May, September and October). Soil pH was determined with deionized water in a 1:3, soil : water ratio, and NH₄⁺ by shaking 5 g of soil in 25 ml 0.5 M K₂SO₄ for 1 h (both as described by Sterkenburg et al., 2015). Daily mean temperature and moisture was calculated by averaging over the 24 hours. I then calculated the monthly average for each plot over the 14 days preceding each sampling date. Mean soil pH and NH₄⁺ measurements for each plot was estimated from average of the three sample dates.

2.4 Data analysis

Statistical analysis was performed with RStudio (version 3.2.5, ©2016 The R Foundation for Statistical Computing) using General Additive Mixed Models (GAMM) with “mgcv” package (Wood, 2016), and Linear Mixed-Effects Models (LME) with the “nlme” package (Pinheiro et al., 2016).

2.4.1 Month variation

Linear Mixed-Effects Model (LME) was used to test the temporal effect on fungal biomass as determined by qPCR. Normality distribution of the data and

homocedasticity were checked using ‘gam.check’ function and data was square root transformed to meet the assumptions. Two outliers with extreme values in fungal biomass were found using ‘boxplot’ function and were removed using ‘droplevels’ function. Data was square root transformed to meet the normality assumptions. Two outliers with extreme values in fungal biomass were found using ‘boxplot’ function and were removed using ‘droplevels’ function. In these analyses ‘Month’ was defined as fixed factor and ‘Plot’ as random to account for independent sampling from each plot. Different models, with and without autocorrelation structure were tested with ANOVA and the best model was chosen based on Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC). Finally, a posthoc test of the best model using Tukey contrasts for pairwise comparisons was used to reveal which months were significantly different in fungal biomass. A line plot was designed to illustrate any temporal and climatic differences in biomass between low fertility plots 1-4 and high fertility plots 5-8, and another plot to visualize any differences in soil fertility between plots.

2.4.2 Climate effects

The General Additive Models (GAM) was used to test the effects of climatic factors (soil moisture and soil temperature) on fungal biomass as determined by qPCR. The interaction between temperature and moisture was visualized from the obtained GAM model. In this model, these climate factors were considered as fixed together with ‘Month’ effect (since the months were not randomly picked, but chosen to estimate the effects of time and growth phase), whereas ‘Plot’ was again defined as random. Fungal biomass was plotted in relation to the interaction of moisture and temperature, to create a linear prediction of biomass response to climatic changes.

3 Results

Averaged fungal biomass ranged between 6.74E+06 and 4.15E+07 ITS copies/mg DW soil. Figure 1 shows the biomass averaged over all plots and the standard deviation between the plots. There is an increasing variation between plots with increasing biomass. The Linear Mixed-Effect Model (LME) showed a significant temporal effect on fungal biomass ($p<0.0001$). The post hoc test revealed that early months (January-March) have significantly lower biomass than late months (September-December), and mid-late summer months (July-August) have significantly lower biomass than November (table 1).

Data show that temperature in 2014 increases from February to July, where it reaches a peak and thereafter decrease again (figure 2, for variation between plots see supplemental figures). From January to February there is a small decrease. Moisture had the opposite pattern, with a decrease from January to July, and then increase again from July to December, with an exception of a decrease in September (same figure).

Averaged across all plots, using max/min values, the coldest month was February (0.15°C) and the warmest month was July (30.5°C). Soils were on average dryer during summer months (June-August) where the driest month was July (0.7% VWC). Highest moisture was measured in December (32.8% VWC). Averaged monthly temperatures show no visible differences between low and high fertility plots. Soil moisture, however, is on average higher in plots 1-4 compared to plots 5-8. The plotted soil fertility shows that plots 2-4 has lower nitrogen and pH than plots 6-8 (figure 3, for variation between sampling points see supplemental figures).

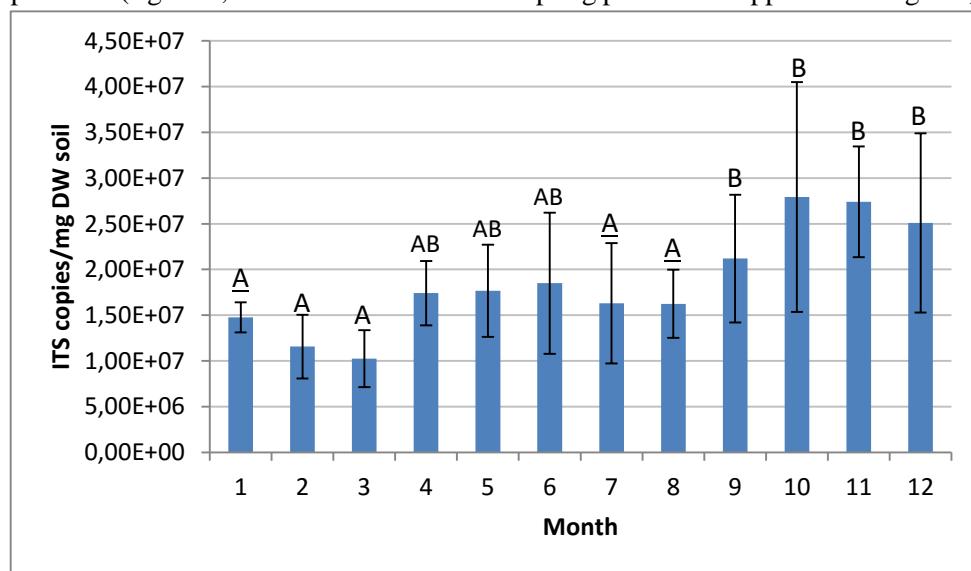


Figure 1. Monthly biomass fluctuations averaged across all eight plots (columns) and the variation (SD) between plots (error bars).

Table 1. Tukey's Posthoc test for pairwise comparison of biomass across all 12 months (N=8 samples per month).

Linear Hypotheses:	z value	Pr(> z)	
11 - 1 == 0	4.092	<0.01	**
9 - 2 == 0	3.510	0.0226	*
10 - 2 == 0	4.548	<0.001	***
11 - 2 == 0	5.490	<0.001	***
12 - 2 == 0	3.878	<0.01	**
9 - 3 == 0	4.101	<0.01	**
10 - 3 == 0	5.118	<0.001	***
11 - 3 == 0	6.081	<0.001	***
12 - 3 == 0	4.447	<0.001	***
11 - 7 == 0	3.700	0.0115	*
11 - 8 == 0	3.582	0.0174	*

Plot 1 and 5 were not visibly different in nitrogen, however, plot 1 has lower pH and are therefore considered different. The General Additive Model (GAM) indicate a significant effect of soil factors on fungal biomass ($p=0.0307$), although the temporal effect was stronger ($p<0.001$) (table 2).

The interaction plot (figure 4) shows that fungal biomass is dependent on the interaction between soil temperature and moisture, and illustrates how fungal biomass responds to seasonal changes in soil moisture and temperature. The linear prediction plot estimated that biomass decrease during drought (high temperature

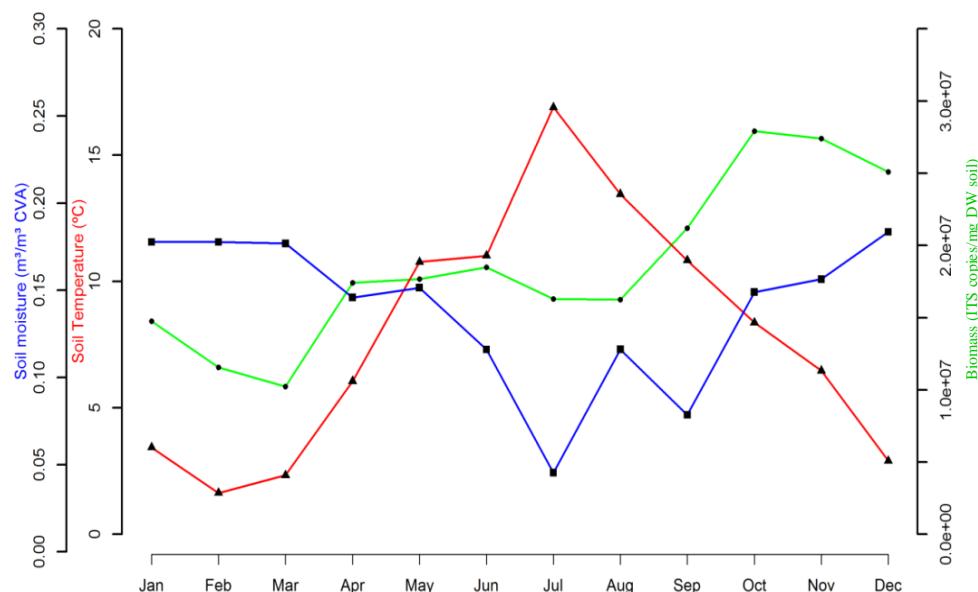


Figure 2. Annual soil temperature and moisture and biomass averaged across all plots. Blue line show soil moisture (m^3/m^3 CVA), red show temperature ($^{\circ}C$). Green line is biomass (ITS copies/mg DW soil).

and low moisture), but also during winter (low temperature and high moisture) (same figure).

As seen in figure 5 (see supplemental figures for variation between days), there was a net loss of CO₂ in January and February. Between March and June there is on balance a net uptake of CO₂, which is the beginning of the plants' growing season. During summer months (June-August), there was a high net release of CO₂ from forest soil to the atmosphere, which coincides with the drought period. In September was on balance a net uptake of CO₂ from the atmosphere, however, throughout October-December the system had a net release of CO₂ from the biosphere to the atmosphere.

The plotted standard curves had an efficiency around 75%, and R² ranged between 0.93 and 0.98.

Table 2. General Additive Model showing the effect of soil temperature and moisture compare with temporal effect (month).

Approximate significance of smooth terms:			
	F	p-value	
Moist,Temp	2.92	0.0307	*
Month	30.90	2.41e-07	***

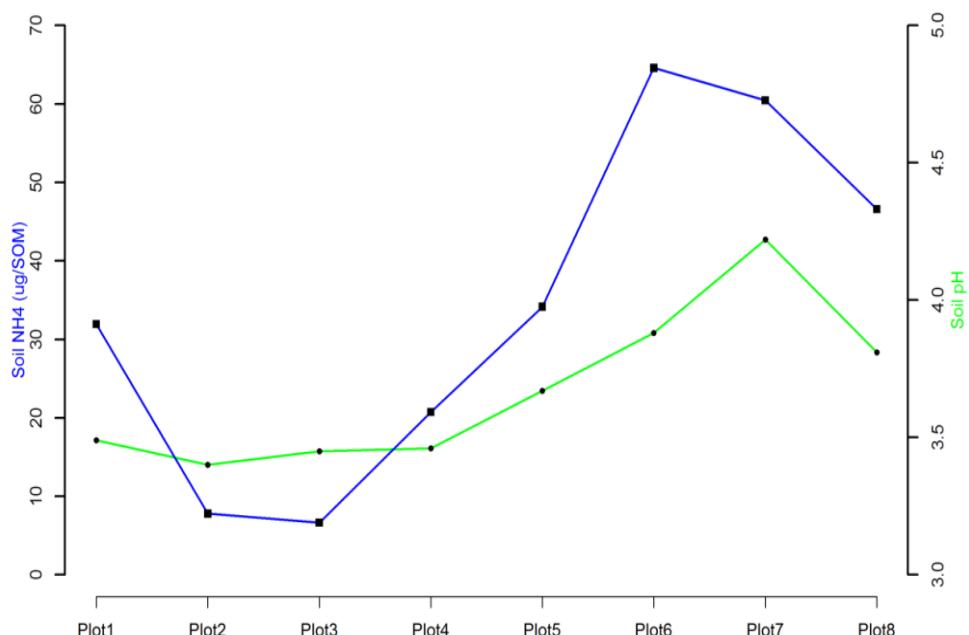


Figure 3. Soil fertility determined by pH and nitrogen levels in each plot. Both factors are visually higher in plots 5-8 compared to 1-4.

4 Discussion

The plotted standard curves had a relatively low efficiency, which determines the sensitivity of a reaction (Applied Biosystems 2011). However, the results were accepted since the efficiency was about the same for all plates and the coefficient of determination (R^2) was within acceptable ranges. The efficiency, judged by the slopes of the curves, was also the same for standards and samples. There was no inhibition of the samples, so the low efficiency was probably due to how the kit worked in general or the length of the amplicons which were considerable long (300-400 bp). Further, the standard contained ITS2 from one fungi whereas my amplicon was a mix of fungi with different ITS2 lengths.

The exponential growth of the amount of product makes qPCR is a rather rough estimate of biomass, and ITS2 copy number is not necessarily an accurate measure of biomass. Other markers for estimating fungal biomass was considered, such as ergosterol, but due to limited time for this study the presented method was chosen. For the purpose of this study which is comparing total fungal biomass changes over

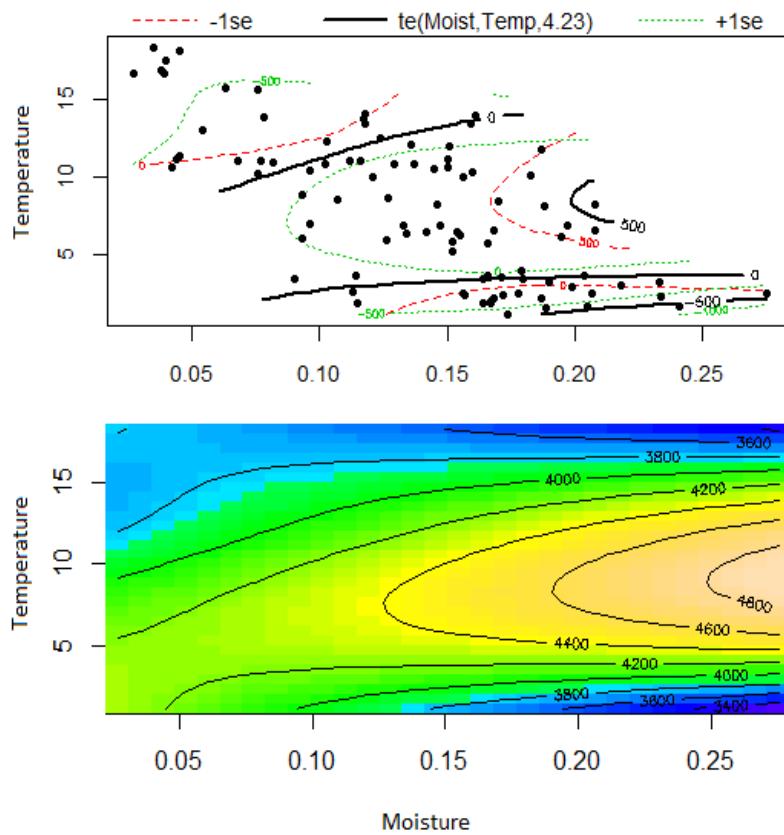


Figure 4. Interaction plot (above) and Linear prediction plot (below) illustrates the interaction of soil temperature and moisture on fungal biomass. Yellow is the highest biomass and blue is the lowest.

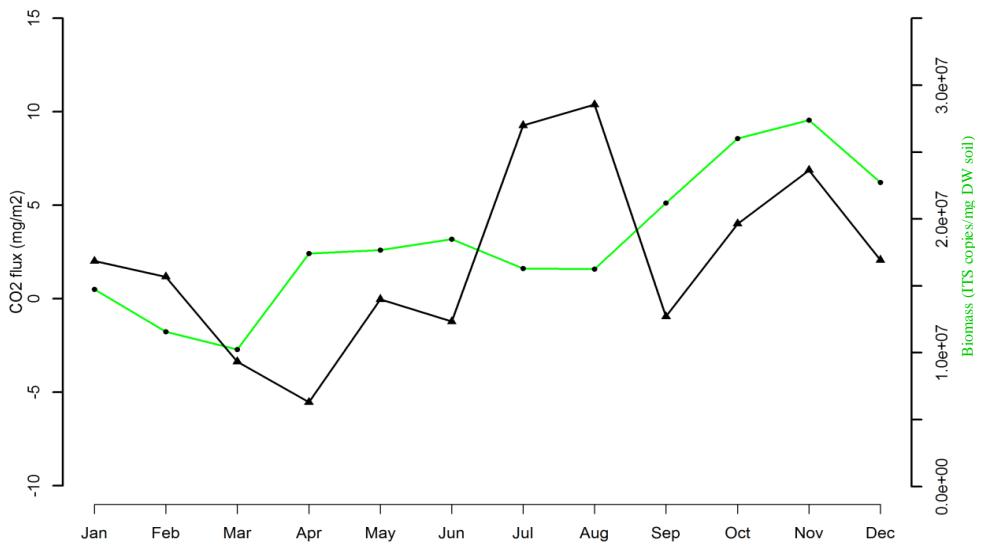


Figure 5. Daily mean net CO_2 flux averaged over three days before and three days after the soil sampling for fungal biomass (black line). A positive value (above 0) represents a loss of CO_2 from the ecosystem to the atmosphere and a negative value (below 0) is an uptake of CO_2 from the atmosphere.

time, I do not expect the efficiency to have significant effect on my final results. Effort was put into avoiding any biases for samples representing different time point and to spread out all uncertainties, which lowers the chance of finding differences and makes the test conservative.

This study demonstrates the temporal variation of fungal biomass and how biomass depends on the interaction between temperature and moisture in the soil. Further it shows how fungi in soils of different fertility levels are affected by seasonal changes and extreme weathers such as drought. There was a high increase of total fungal biomass when comparing the beginning and end of the year. With the exception of winter ending, summer drought and late autumn, which had a decrease, a general annual increase is found. The drought period was evidently a direct effect of extreme heat and low precipitation during July, however, in January and February average soil temperature was above 0°C , which indicates insufficient soil moisture recharge as described by Moore et al. (2016). Instead of an increase of moisture when the snow melted and the soil frost thawed, there was a fast decrease in moisture immediately following the end of winter.

Although there is no clear statistically significant decrease of total fungal biomass during drought, there are indications that it does affect fungal biomass depending of soil fertility, and it is possible that the extent of drought was not enough to affect the more resistant fungi (Yuste et al., 2011). Fungal biomass in low fertility soil had a stronger response to drought than biomass in high fertility (figure 6), even though soil in low fertility was in general moister. In accordance with Cooke & Rayner (1984), stress-tolerant fungi (S-strategists) in low fertility soil are “persistent as long

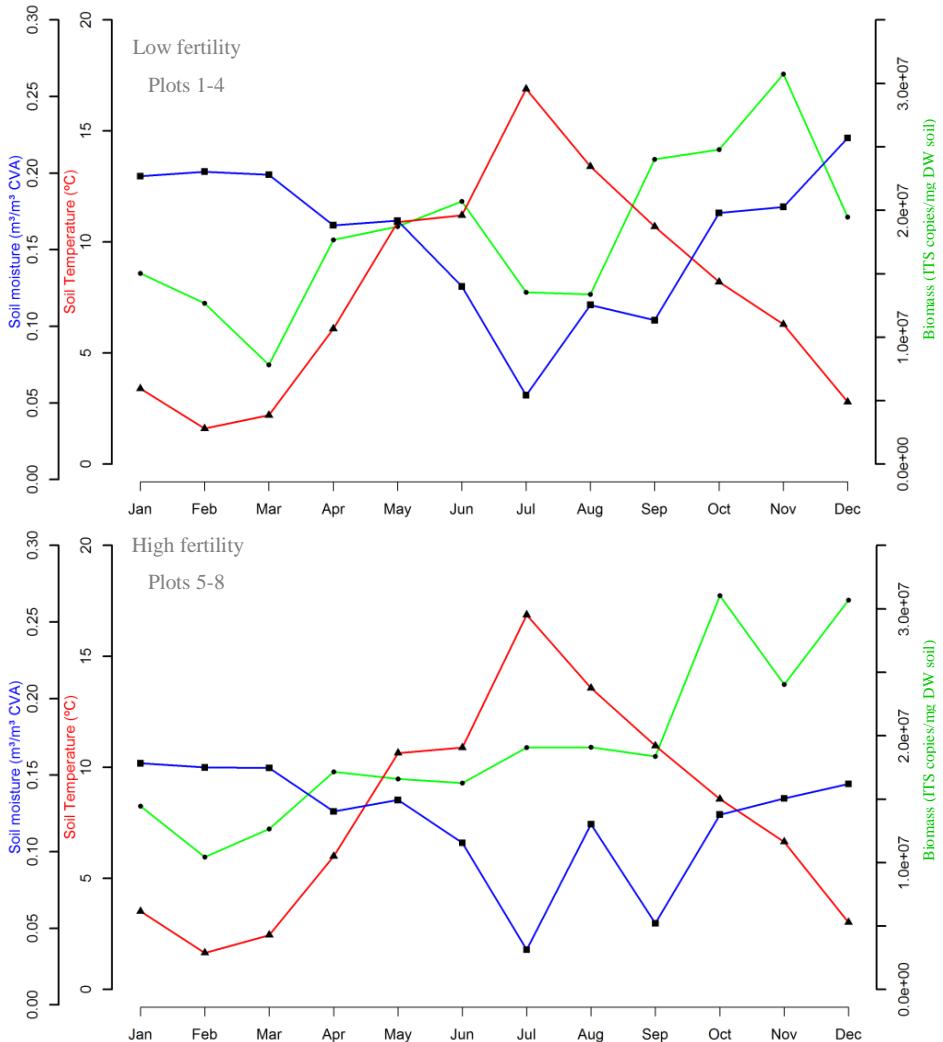


Figure 6. Monthly biomass, soil temperature and moisture. Low fertility (above) shows averaged biomass and soil condition for plots 1-4, and high fertility shows averaged biomass and soil conditions for plots 5-8.

as stress conditions [are] maintained" (p.107) and extreme weathers such as heavy drought probably caused a disturbance in those conditions. Such extreme weathers are expected to increase with global climate change (Moore et al., 2015) and it is possible that summer drought periods will become longer and perhaps more intense. Biomass in richer soils is likely composed of C-strategists (Cooke & Rayner, 1984), which are persistent and efficient at exploiting available resources. This could be an explanation as to why biomass in the more fertile soil does not decrease during drought. The third strategist, which thrives in high fertility soils and can manage fluctuating environments, is the R-strategists (Cooke & Rayner, 1984). They are

individually short-lived and put all efforts and resources into asexual reproduction. There is a possibility that these could be responsible for the very fast increase in biomass after frost in the winter and drought in the summer.

Additionally, Ruehr et al. (2009) observed during drought that inputs of carbon to soil pools was reduced, and another study showed increased decomposition of soil carbon with (Bradford et al., 2008). In this study I found that the drought period coincides with a high net release of CO₂. The net uptake of CO₂ increases already in the end of winter (between February and March) and continue to do so in April, which correspond with the fast increase of fungal biomass that begins in March and peaks in April. This suggest an early start of plant CO₂ assimilation and allocated carbon to mycorrhizal fungi, which initiates fungal biomass growth and promotes the return of nutrients and water back to the plants. The annual cumulative flux of CO₂ (figure 7) shows that respiration rates as integrated over the whole year were higher than photosynthesis and there was an overall loss of soil stored carbon. In a recent study, Hadden & Grelle (2016) found a consistent annual loss of CO₂ to the atmosphere since 2010, and that the number of days per year with net CO₂ uptake has declined while days of net CO₂ loss has increased.

It is necessary to continue investigating fungal responses to climate change and how these affect the ecosystem, in order to predict undesirable outcomes and develop

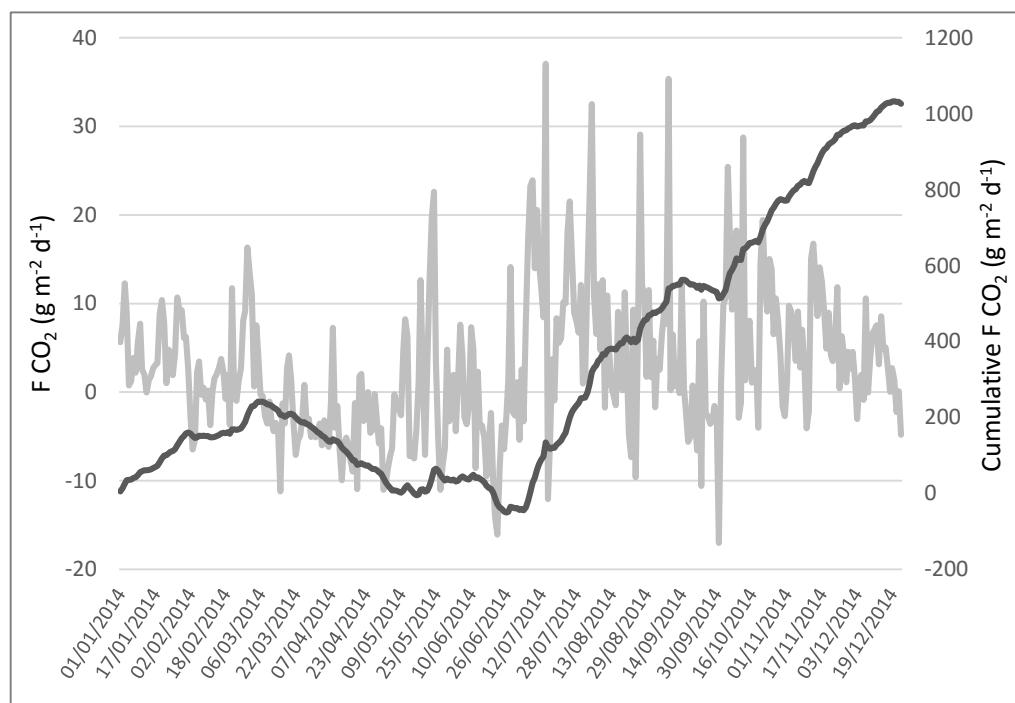


Figure 7. Daily (light grey) and cumulative (dark grey) flux of CO₂ for the year 2014. Increasing values indicate that CO₂ release from ecosystem is higher than uptake. Declining values show a dominating CO₂ uptake.

ways of regulating the carbon cycle. Fungal biomass and productivity will respond in diverse ways to climate change and extreme weathers, due to micro habitats in the soil controlled by nutrient availability, hydrology and acidity. Further, as shown recently, soil fertility affects fungal community composition (Sterkenburg et al., 2015), and therefore variations in soil conditions must be taken into account when creating predictive models of climate change effects on fungal communities and their activities.

5 Acknowledgements

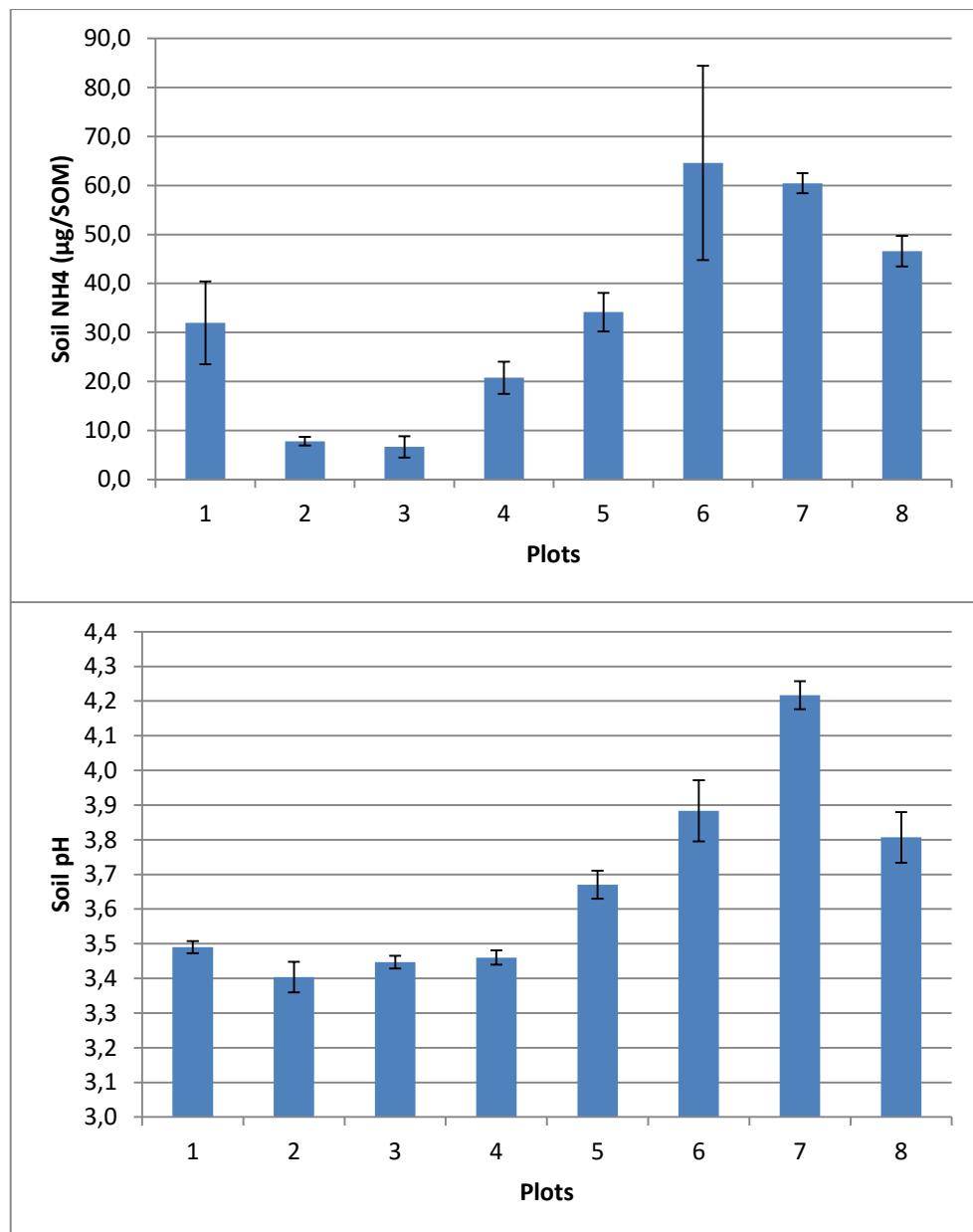
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6 References

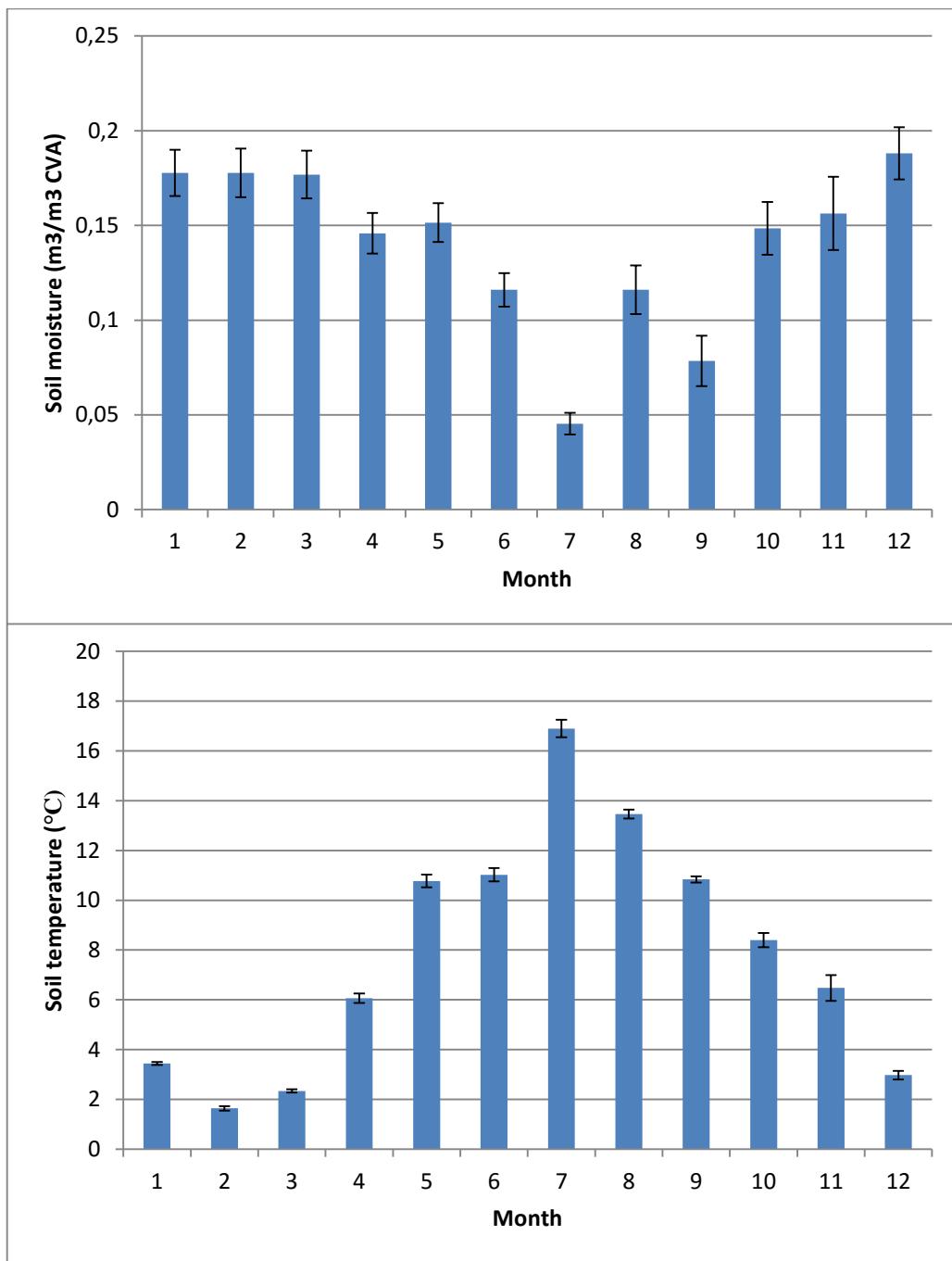
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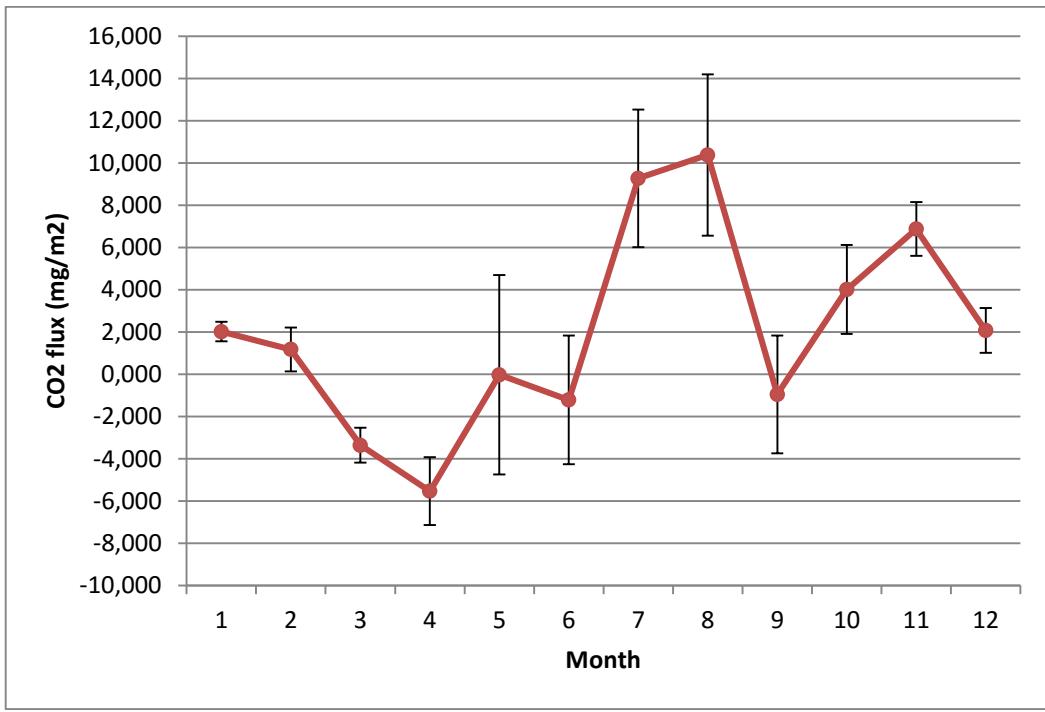
7 Supplemental figures



Soil fertility of each plot. Average of NH4 and pH (columns) from three sampling points in 2014, and the variation between sampling points (standard error bars). N=3 sampling points.



Average soil temperature and moisture (columns) for each month, and variation between plots (standard error bars). N=8 plots.



Annual net CO₂ flux. Average CO₂ flux for each month (line) and the variation between the three days before and after sampling date (standard error bars). N=6 days.