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Comparison of the impacts of acid and nitrogen additions on carbon fluxes in European conifer and broadleaf forests

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Abstract

Increased reactive nitrogen (N) loadings to terrestrial ecosystems are believed to have positive effects on ecosystem carbon (C) sequestration. Global "hot spots" of N deposition are often associated with currently or formerly high deposition of sulphur (S); C fluxes in these regions might therefore not be responding solely to N loading, and could be undergoing transient change as S inputs change. In a four-year, two-forest stand (mature Norway spruce and European beech) replicated field experiment involving acidity manipulation (sulphuric acid addition), N addition (NH_4NO_3) and combined treatments, we tested the extent to which altered soil solution acidity or/and soil N availability affected

the concentration of soil dissolved organic carbon (DOC), soil respiration (Rs), microbial community characteristics (respiration, biomass, fungi and bacteria abundances) and enzyme activity. We demonstrated a large and consistent suppression of soil water DOC concentration driven by chemical changes associated with increased hydrogen ion concentrations under acid treatments, independent of forest type. Soil respiration was suppressed by sulphuric acid addition in the spruce forest, accompanied by reduced microbial biomass, increased fungal:bacterial ratios and increased C to N enzyme ratios. We did not observe equivalent effects of sulphuric acid treatments on Rs in the beech forest, where microbial activity appeared to be more tightly linked to N acquisition. The only changes in C cycling following N addition were increased C to N enzyme ratios, with no impact on C fluxes (either Rs or DOC). We conclude that C accumulation previously attributed solely to N deposition could be partly attributable to their simultaneous acidification.

Capsule: Acidification alters soil carbon fluxes in spruce and beech forests due to the altered soil pH. Limited effect of N addition on soil C cycling in both forest types

Keywords: nitrogen, fertilization, acidity, sulphur, organic carbon, respiration, DOC, hydrolytic enzymes, fungi, bacteria

1. Introduction

Human activities have been altering element cycling for several thousand years (Ruddiman, 2003; Shotyk, 1998). Since the Industrial Revolution, emissions of greenhouse gases, nitrogen (N) and sulphur (S) have been increasing (Kopáček and Posch, 2011; Lamarque et al., 2013) and their negative impacts on ecosystems led to the implementation of various international agreements to abate these emissions (United Nations Framework Convention on Climate Change, Convention on Long-range Transboundary Air Pollution, Kyoto Protocol, Paris Agreement). Besides exerting significant negative pressures on global biodiversity (Butchart et al., 2010), atmospheric pollutant deposition has impacted natural biogeochemical cycles of terrestrial ecosystems, including the ecosystem C balance (Hyvönen et al., 2007; Lamersdorf and Borken, 2004; Thomas et al., 2013).

Nitrogen limitation is an inherent feature of many temperate terrestrial ecosystems, thus fertilization by atmospheric N deposition may have increased C pool in biomass and soils of terrestrial ecosystems and thus stimulate the terrestrial CO_2 sink (de Vries et al., 2009). Increased N availability may affect terrestrial C balances via increased net primary productivity (NPP) and/or decreased litter decomposition rates (Janssens et al., 2010; Liu and Greaver, 2010). The potential of a negative effect of increased N availability on decomposition rates was suggested by many experimental N addition studies (e.g. Berg and Matzner 1997; Olsson et al. 2005; Hobbie 2008). Stimulation of litter decomposition is expected at sites with low ambient N deposition and high-quality litter (low lignin), whereas suppression of litter decomposition is suggested at sites with higher ambient N deposition or at sites with low quality (high lignin) litter (Knorr et al., 2005). Despite current studies have focused on the impact of N deposition on C balance, there is clear evidence that acidification (either from S or N deposition) may also affects a range of key processes, including slowdown of litter decomposition (Chen et al., 2015; Liang et al., 2013; Oulehle et al., 2011), as well as mobility and leaching of dissolved organic carbon (Evans et al., 2012). Suppression of litter decomposition at low pH may occur through reduction in the availability of C for microbial community (Persson et al., 1989) and/or by direct effect on the microorganisms themselves (Błońska et al., 2017, 2016; Pennanen et al., 1998; Wei et al., 2013). Input of N to the soil, either from deposition or fertilization, can have an acidifying effect, depending on the fate (immobilization vs. leaching, nitrification) of mineral N in the soil. Thus, it is likely that experimentally induced responses to N fertilisation might in fact be due to changes in soil acid-base status (Evans et al., 2008; Chen et al., 2016). A better, integrated understanding of soil C and N responses to changing inputs of both S and N is needed to predict whether soils will in future act as C sinks or C sources as atmospheric pollutant loadings change. Accounting for, that much of Europe and North America have experienced high historic depositions of S, and receive high N inputs, whilst other regions including large parts of Asia continue to receive high depositions of both S and N (Duan et al., 2016; Larssen et al., 2006; Posch et al., 2015), it is likely that recent measurements of C fluxes from these regions might not be in long-term balance with climatic conditions, but represent ecosystems

77 subjected to multiple anthropogenic pressures, and in many cases undergoing transient change as
78 deposition levels decline or rise.

79 Here, we present a set of replicated acidity/N availability manipulation experiments in adjacent mature
80 broadleaved (beech forest) and coniferous (spruce forest) sites. Spruce and beech represent the
81 dominant tree species in the Central European forests. Both stands are influenced by similar abiotic
82 conditions (climate, deposition, bedrock, original soil type) but differ in vegetation cover as a result of
83 long-term differences in land-use. Furthermore, long-term ambient monitoring from spruce site
84 provided evidence of C soil loss as a consequence of acidification recovery (Oulehle et al., 2011) and
85 both sites were intensively studied to assess the rates of acidification recovery in formerly highly
86 polluted areas (Oulehle et al., 2006). We examined whether there has been a shift in the ecosystem C
87 fluxes in either (dissolved) organic or (gaseous) inorganic forms, as a function of altered acidity and N
88 availability. *In situ* decomposition experiments and measurements of microbial characteristics and
89 enzyme activity provided further insights into C metabolism under changing acidity and N availability.

90 ## 2. Material and Methods

91 ### *2.1. Sites and experimental design*

92 In 2013 two parallel acidity and N availability manipulation experiments were established, at two
93 adjacent forests (50.59 N, 13.26 E) selected to represent major European forest types – mature Norway
94 spruce forest (*Picea abies* (L.) Karst.) and mature European beech forest (*Fagus sylvatica* L.). The spruce
95 stand consists of Norway spruce monoculture planted in the early 1930s, and the beech stand consists
96 of European beech monoculture (Oulehle et al., 2016b). The distance between stands is approximately
97 1 km and both stands are underlined by gneiss bedrock. The dominant soil type is dystric cambisol
98 (Dambrine et al., 1993). Soils at both sites were acidified by acid deposition during the 20th century,
99 which resulted in low soil pH and low base saturation. Despite similar bulk S deposition, total
100 deposition is higher in the spruce stand compared to the beech stand. Throughfall fluxes of inorganic
101 nitrogen are similar in both stands (

; Oulehle et al. 2011, 2016b). Ground cover vegetation in the spruce stand (60%) is formed mainly by *Avenella flexuosa* L. Drejer with admixture of *Vaccinium myrtillus* L., *Dryopteris dilatata* (Hoffm.) A. Gray, *Calamagrostis villosa* (Chaix) J. F. Gmel. and *Galium saxatile* L. The ground cover vegetation in the beech stand (1.5%) comprises mainly of *Calamagrostis villosa* (Chaix) J. F. Gmel. and *Avenella flexuosa* L. Drejer.

At each forest stand, soil acidity and N availability were manipulated for 3 years by systematic addition of S and N treatment solutions. At each experimental site, sixteen 3 x 3 m plots were assigned to control (Ctrl), nitrogen (N), acid (S) and acid + nitrogen (S+N) treatments in a randomized blocked design, thus four replicates per treatment were available. In October 2012 plots were established (mineral soil lysimeter installation), and in April 2014 treatments began following a period of pre-treatment measurements (April 2013 – March 2014). Treatments consists of monthly additions (April – November) of sulphuric acid (H_2SO_4) and ammonium nitrate (NH_4NO_3), mixed with rainwater collected at sites and applied using 15 l watering cans evenly across the plots, followed by addition of extra 15 l rainwater to properly seep in soil all added chemicals. The treatment dose was equivalent to 50 kg S ha^{-1} $year^{-1}$, while the additional water addition was equivalent to 2.5 % of average annual rainfall. Nitrogen treatments gave an input of 50 kg N ha^{-1} $year^{-1}$. NH_4NO_3 addition, albeit ammonium is a weak acid, did not significantly alter pH of added solution (applied solution pH was ≈ 5.4).

Table 1. Stand characteristics, soil properties (BS – base saturation), biomass properties (net primary productivity – NPP) and measured sulphur (S), dissolved inorganic nitrogen (DIN) deposition and dissolved organic carbon (DOC) and nitrogen (DON) leaching. Data from Oulehle et al. (2016b).

	Site description				Soil (Forest floor)					
	Latitude	Longitude	Temperature	Precipitation	Forest age	pH	BS	C/N	C	N
			°C	mm	years		%	g g^{-1}	kg m^{-2}	
Spruce	50.591	13.253	6.5	1092	85	3.80	19	24.9	3.74	0.15
Beech	50.589	13.267			155	4.34	70	20.8	6.54	0.31
	Biomass				Ambient deposition 2013-2015				Soil leaching (Forest floor)	
	NPP	Litterfall	Litter C/N	Biomass C/N	Bulk S	Throughfall S	Bulk DIN	Throughfall DIN	DOC	DON
	kg C ha^{-1} $year^{-1}$		g g^{-1}		kg ha^{-1} $year^{-1}$				g m^{-2}	
Spruce	9373	1650	42	133	4.0	14	9.6	14	17.9	0.56
Beech	7980	2882	52	178		7.5		16	18.2	0.52

123

124 ### 2.2. Soil solution sampling and analysis

125 Every 3-5 weeks during the snow/ice free period (usually April – November) samples were taken,
126 ideally after rainfall when soil moisture conditions permitted collection of soil water. In every case soil
127 water was collected before treatment application. Soil water samples were collected using Rhizon®
128 suction samplers (Rhizosphere Research Products, Wageningen, NL), comprising 10 cm long, 2.5 mm
129 diameter porous membranes attached to 50 ml syringes. Four to six Rhizon samplers were inserted
130 randomly into the forest floor of each plot, to give one composite sample per plot. In the mineral soil,
131 Prenart Super quartz standard suction samplers (Prenart Equipment, Frederiksberg, DK) were inserted
132 into 30 cm soil depth. Soil solution was collected by applying suction into a 2 l collecting bottle.

133 Samples were stored at 4 °C, and analysed for pH using Radiometer TTT-85 with a combination
134 electrode, nitrate (NO_3^-) by high-performance liquid chromatography (Knauer 1000); ammonium
135 (NH_4^+) was determined by indophenol blue colorimetry. DOC (dissolved organic carbon) was
136 determined by a Tekmar-Dohrman Apollo 9000 analyzer (Tekmar Dohrmann, OH, USA). Samples for
137 DOC determination were filtered (0.4 µm glass fiber Macherey-Nagel GF 5) before analysis.

138 ### 2.3. Soil CO_2 flux measurements

139 Soil CO_2 efflux (Rs) was measured monthly between 2013 and 2016 during the snow-free period (April–
140 November/December) using a LiCor infrared gas analyser LI-8100A (LiCor Biosciences, NE, USA)
141 attached to a LiCor Survey Chamber (8100-103 20 cm survey chamber; LiCor Biosciences, NE, USA). In
142 each plot, four soil collars (287cm^2) were placed into the forest floor to a 5 cm depth (64 soil collars in
143 each forest stand) in March 2013. The CO_2 efflux was calculated based on a linear increase in chamber
144 CO_2 concentration over time.

145 We used the van't Hoff equation (Davidson et al., 2006) to model the relationship between soil
146 temperature and soil respiration:

$Rs = \alpha e^{\beta T}$ (where $Q_{10} = e^{\beta 10}$) Eq. 1

where Rs is the *in situ* measured respiration rate, α and β are fitted parameters, T is *in situ* measured soil temperature and Q_{10} is the calculated temperature sensitivity of respiration. Rs was calculated on an hourly basis using on site measured soil temperatures and summed for the whole year to estimate an annual CO_2 efflux.

2.4. Microbial characteristics and DNA extraction

Soils for microbial characteristics were sampled every year in May. Microbial C (C_{mic}) and N (N_{mic}) were calculated as the difference between the chloroform fumigated and non-fumigated soil samples (sulphate extractable C and N), and amended using extraction efficiency factors of 0.38 for microbial C (Vance et al., 1987), and 0.54 for microbial N (Brookes et al., 1985). Microbial respiration was assessed as CO_2 production after one week incubation of soil in closed vials placed in the dark (10 g of fresh soil, 60 % of water holding capacity, 15 °C), using gas chromatography (Agilent GC HP 6850, USA).

Into a FastPrep™ Lysis Matrix E tube (MP Biomedicals, Solon, OH, USA) approx. 0.5 g of soil was added. Then Hexadecyltrimethylammonium bromide (CTAB) extraction buffer, containing 5% CTAB (in 0.7 M NaCl, 120 mM potassium phosphate, pH 8.0) and 0.5 ml phenol-chloroform-isoamylalcohol (25:24:1), was added and agitated at speed 5-6 for 45s in a FastPrep Instrument (MP Biomedicals, Solon, OH, USA). Following bead beating, chloroform extracted samples were precipitated in a PEG 6000/1.6 M NaCl solution. 70% ethanol was used to wash pellets and re-suspended in molecular biology grade water. Quantus™ (Promega, USA) was used for fluorometrical quantification of total DNA.

Quantative PCR (qPCR) of bacterial and fungal genes of ribosomal small subunit rRNA (SSU rRNA) was performed using the FastStart SybrGREEN Roche® Supermix and Step One system (Life Technologies, USA) as described previously (Bárta et al., 2017; Gittel et al., 2014). R^2 values for the standard curves were > 0.99. Slope values were -> 3.37 giving an estimated amplification efficiency of > 93%.

Detection limits (i.e. lowest standard concentration that is significantly different from the non-template controls) were less than 100 gene copies for each of the genes per assay. All samples, standards and non-template controls were analysed in duplicates.

2.5. Extracellular enzyme activities

Extracellular enzyme activities were assessed in soils sampled in May and October each year. Microplate fluorometric assays under standard conditions were used to determine extracellular enzyme activities. All hydrolytic enzyme activities were determine in 1 g soil which was suspended in 100 ml of distilled water and for 4 min sonicated to allow disruption of the soil particles. Then 200 µL of the soil suspension was added into 50 µL methylumbelliferyl (MUF) of substrate solution for β-glucosidase (BG), exocellulase (cellobiohydrolase - CEL), phosphatase (PME) and N-acetyl-glucosaminidase (NAG) determination or to 50 µL of 7-aminomethyl-4-coumarin (AMC) substrate solution for leucine-aminopeptidase (LEU) determination (Bárta et al., 2014). 50, 100 and 300 µM concentrations of each fluorogenic substrate were tested and the one with the highest enzymatic activity was picked for further analysis. Plates were incubated for 120 min at 20°C. Excitation wavelength of 365 nm and emission wavelength of 450 nm were used for fluorescence quantification using Infinite F200 microplate reader (TECAN, Germany). To assess enzyme activities stoichiometry we calculated C_{enz}/N_{enz}, C_{enz}/P_{enz} and N_{enz}/P_{enz} ratios, where C_{enz} = BG+CEL, N_{enz} = NAG+LEU and P_{enz} = PME (Sinsabaugh et al., 2009).

2.6. Data analysis

After testing the data for normality (Kolmogorov-Smirnov), skewness and homogeneity of variances using Bartlett's test, pH, DOC and soil respiration were analysed for the pre-treatment period (May 2013 – April 2014) and for each full year of manipulation data (2014, 2015, 2016) using repeated measures ANOVA (treatment as a fixed factor) for each of the stands and for each time period. Fisher's LSD multiple comparison test was used at $p < 0.05$ to detect which treatments were different. Effects of forest stand, season (spring and autumn sampling - for enzyme activities only) and treatments on

195 microbial biomass, fungi and bacteria abundances, fungi to bacteria ratios, enzymatic activity and
196 enzyme stoichiometry were determined using a general linear model (GLM) for each time period. We
197 refer only to the treatment effects in depicted figures. All data were analysed using statistical software
198 NCSS v11.

199 ## 3. Results

200 ### *3.1. Soil solution chemistry*

201 Pre-treatment soil water chemistry, in both forest floor and mineral soil, was similar among plots and
202 no significant differences were detected in respect of soil water pH and DOC at either the spruce or
203 beech stands (Figure 1, Suppl. Fig. 1).

204 From the onset of treatment in April 2014, immediate and significant changes in forest floor soil water
205 pH occurred at both stands. In acid treatments (S and S+N), soil water pH was reduced by 0.41-0.48 pH
206 units at the spruce stand and 0.41-0.51 pH units at the beech stand (based on 2015-2016 means).
207 Nitrogen addition alone did not induce any significant changes in soil water pH. Consistent reductions
208 of mean annual DOC concentrations in response to pH manipulation were detected at both forest
209 stands, although these were not significant at the beech stand. DOC concentrations were reduced in
210 acid treatments by 30% - 39% at the spruce stand, and by 45% - 53% at the beech stand (based on
211 2015-2016 means). Nitrogen addition treatments did not lead to significant changes in forest floor soil
212 water DOC concentrations (Figure 1). Despite the alteration of soil water chemistry in the forest floor
213 due to treatments, soil water chemistry in mineral soil (30 cm depth) revealed only limited changes of
214 pH under acid treatments in the beech stand, and no significant changes in DOC concentrations in
215 mineral soil water in either forest stand (Suppl. Fig. 1).

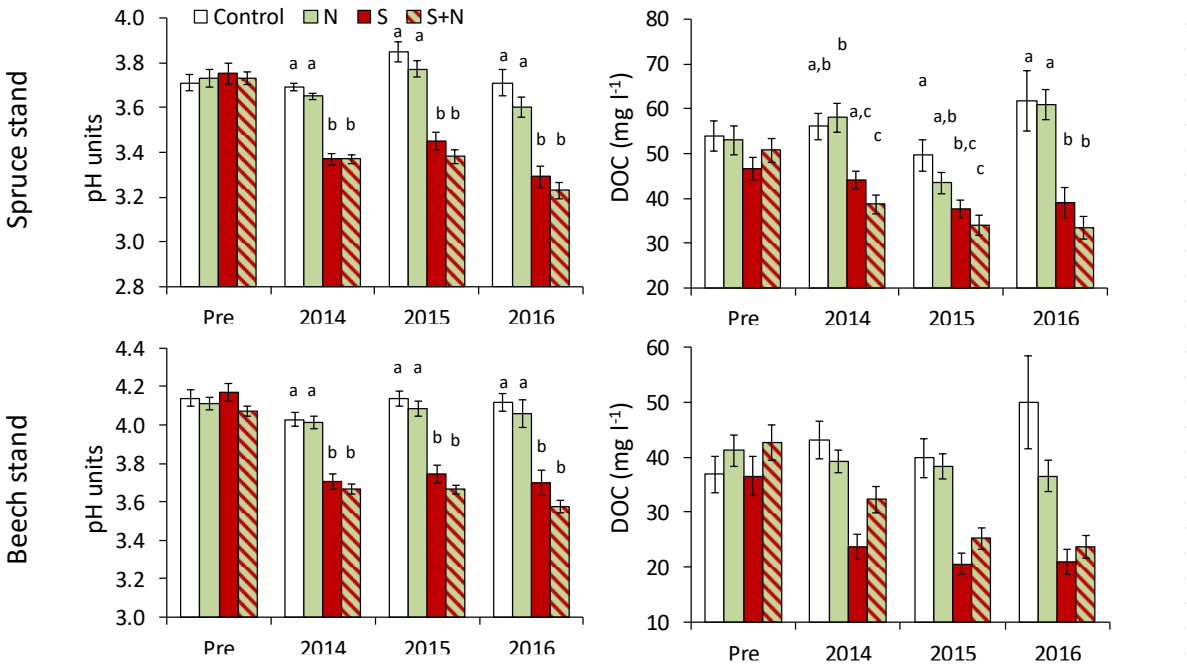

Figure 1. Mean and standard error of forest floor soil water pH and DOC at each forest stand for control, nitrogen (N), sulphur (S) and sulphur + nitrogen (S+N) treatment plots (n = 4 per treatment and forest stand). Data are shown for the pre-treatment period (year 2013) and for the last three full years of manipulation. Significant treatment effects by site and time period denoted by letters (P < 0.05).

3.2. Soil respiration

Significant exponential relationships existed between soil respiration and soil temperature thus soil temperature exerted a strong control over soil CO_2 efflux, with coefficients of determination (R^2) ranging between 0.88 and 0.90 at the spruce stand and 0.79 and 0.83 at the beech stand (soil temperature ranged between 1.3 and 9.4°C during the respiration measurements). Annual mean soil respiration in the spruce stand (3.30 µmol CO_2 m^{-2} s^{-1}) and beech stand (3.39 µmol CO_2 m^{-2} s^{-1}) did not significantly differ. Rather high variability of the soil CO_2 efflux among individual collars, combined with strong (seasonal) temperature dependence made it difficult to detect treatment effects on total soil respiration. Nevertheless, consistent reductions of mean annual soil respiration were observed under the S only treatment in the spruce stand in 2015 and 2016 ($p < 0.05$), by 6 % - 10 %. A non-significant,

though consistent, reduction of Rs under combined treatment was measured in spruce stand. No significant effect of N addition on soil respiration was detected in the spruce stand. In the beech stand, neither acidity nor N manipulations affected soil respiration (Figure 2). Calculated mean annual C efflux was 9.9 t C ha^{-1} $year^{-1}$ in the spruce stand and 9.7 t C ha^{-1} $year^{-1}$ in the beech stand (based on 2013-2016 means for control plots; Eq. 1). Acid treatment in the spruce stand thus reduced C soil efflux by 0.53 – 0.95 t C ha^{-1} $year^{-1}$.

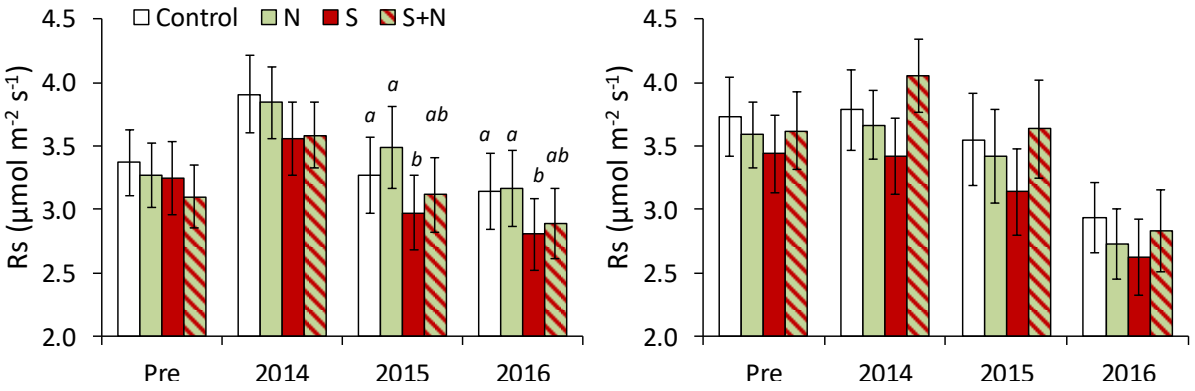

Figure 2. Mean and standard error of soil respiration at each forest stand for control, nitrogen (N), sulphur (S) and sulphur + nitrogen (S+N) treatment plots (n = 4 per treatment and forest stand). Data are shown for the pre-treatment period (year 2013) and for the last three full years of manipulation. Significant treatment effects by site and time period denoted by letters (P < 0.05).

3.3. Microbial activity characteristics

Microbial biomass C, N and their ratios significantly (P < 0.05) differed among forest stands, with higher biomass C and N in the beech forest (324 µmol g^{-1} and 31 µmol g^{-1}, respectively) compared to the spruce forest (290 µmol g^{-1} and 23 µmol g^{-1}, respectively). Microbial C to N ratio was significantly lower in the beech stand (10.9) compared to the spruce stand (12.7) (Figure 3). Microbial respiration did not significantly differ between stands on average. Microbial biomass C and N were negatively related to soil pH across both forests (Figure 4), but in the spruce site acidic conditions led to higher respiration rates. No significant treatment effects were detected, although there was a tendency for microbial C and N to decrease under acid treatments (S, S+N) in the spruce forest, thus a shift toward higher microbial C/N was detected. Significantly (P < 0.05) higher fungal to bacterial ratios were measured in

the spruce stand (0.045) compared to the beech stand (0.031), consistently with the higher microbial C to N ratios. Despite no significant treatment effects on fungi/bacteria ratio in the spruce stand (Figure 5), significantly lower bacterial gene copies were measured under acid treatments (Suppl. Table 2)**Chyba! Nenalezen zdroj odkazů.**. Therefore, consistent increases of fungi/bacteria ratio under acid treatments (Figure 5) appear to be attributable to decreases in bacterial abundance rather than increases in fungi. In the beech stand, a significant treatment effect on fungi/bacteria ratio was detected in 2016 (Figure 5). This resulted from decreases of bacteria and increases (albeit insignificant) in fungal gene copies (Suppl. Table 2).

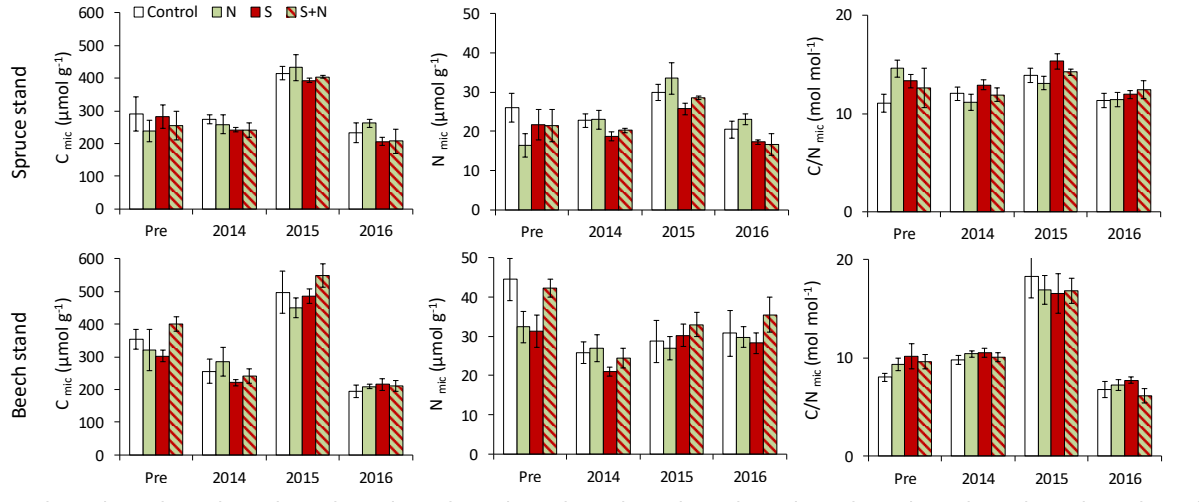

Figure 3. Mean and standard error of microbial C, N and C/N at each forest stand for control, nitrogen (N), sulphur (S)and sulphur + nitrogen (S+N) treatment plots (n = 4 per treatment and forest stand). Data are shown for the pre-treatment period (year 2013) and for the last three full years of manipulation. Significant treatment effects by site and time period denoted by letters (P < 0.05).

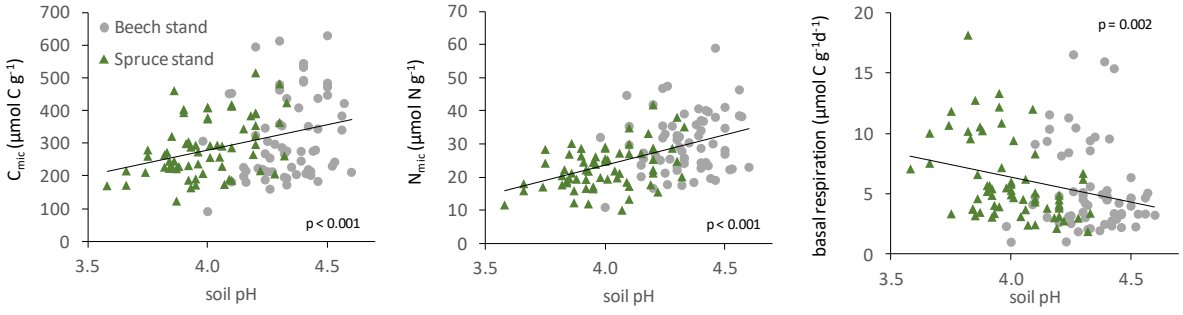

Figure 4. Soil microbial C, N and microbial respiration related to soil pH among forest stands. Linear regression and its significance (p value) highlighted for all data.

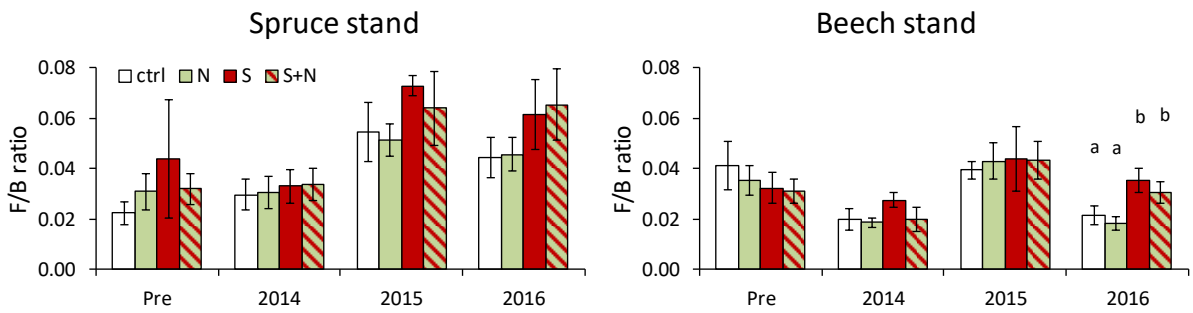

Figure 5. Mean and standard error of fungi to bacteria ratio at each forest stand for control, nitrogen (N), sulphur (S) and sulphur + nitrogen (S+N) treatment plots (n = 4 per treatment and forest stand). Data are shown for the pre-treatment period (year 2013) and for the last three full years of manipulation. Significant treatment effects were assessed by GLM analysis for each stand and time period separately.

3.4. Enzymatic activities

Forest type significantly ($P < 0.05$) affected activity of all enzymes except these involved in C cycling (β-Glucosidase - BG and cellobiohydrolase – CEL). Consistently higher activities of enzymes primarily involved in N acquisition (leucine-aminopeptidase - LEU and N-acetyl-β-D-glucosaminidase - NAG) were measured in the beech stand compared to the spruce stand. Similarly, phosphatase (PME) activity was significantly higher in the beech stand compared to the spruce stand. Stoichiometry of enzymatic activity revealed significantly higher C_{enz}/N_{enz} and C_{enz}/P_{enz} ratios in the spruce stand compared to the beech stand (C_{enz}/N_{enz} = 4.3 in the spruce stand and 1.9 in the beech stand; C_{enz}/P_{enz} = 1.0 in the spruce stand and 0.8 in the beech stand). Conversely, a higher N_{enz}/P_{enz} ratio was measured in the beech stand compared to the spruce stand (0.43 vs 0.23) (based on control means, Figure 6).

Enzymatic activities among treatments did not significantly differ in the pre-treatment period. After treatment started, a tendency towards higher C enzyme activities under all treatments (N, S and S+N) was detected in the spruce stand (Suppl Table 1, Figure 6). These changes resulted in significant ($P <$

0.05) alteration of enzyme stoichiometry by treatments. C_{enz}/N_{enz} ratio was significantly higher under all treatments (mean of 5.3) compared to control (3.9), and C_{enz}/P_{enz} ratio significantly increased under all treatments (mean of 1.3) compared to control (1.0). No treatment effect on N_{enz}/P_{enz} ratio was detected. This suggests that treatments primarily affected C enzyme activities in the spruce stand. In the beech stand, a significant reduction of NAG activity was detected under all treatments. This resulted in a significant increase of C_{enz}/N_{enz} ratio under all treatments (mean of 2.4) compared to the control (1.8). The absence of observed change in the C_{enz}/P_{enz} ratio and a consistent (albeit insignificant, P = 0.097) change in N_{enz}/P_{enz} ratio suggested that treatment effects at the beech stand primarily affected N acquiring enzymes (Suppl Table 1, Figure 6). In the spruce stand, C_{enz} and N_{enz} were strongly negatively related to soil pH (Suppl. Fig. 2) while in beech no such dependence was found.

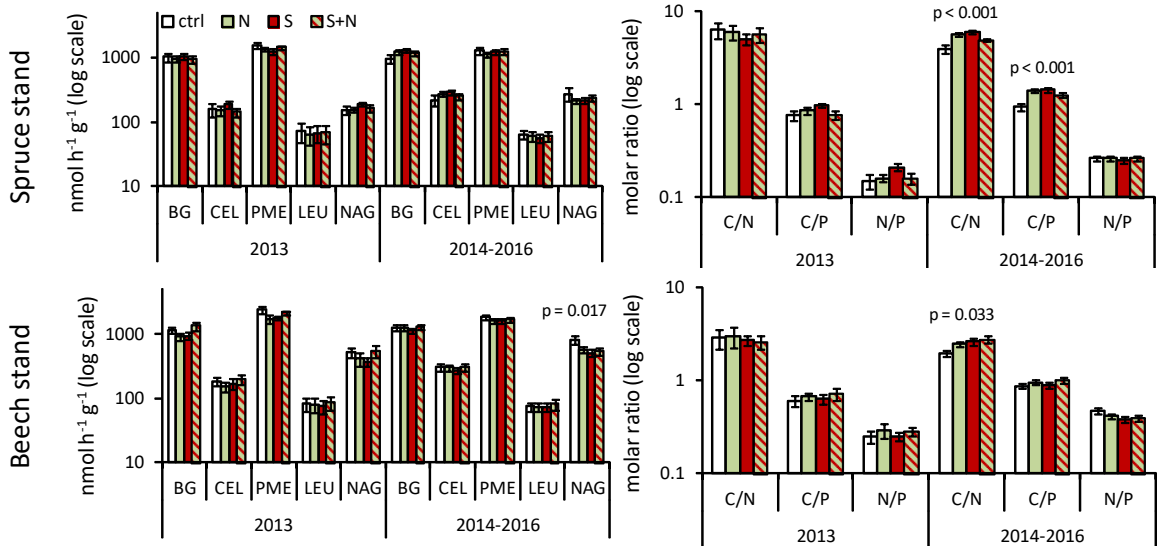

Figure 6. Mean and standard error of enzyme activities and their ratios at each forest stand for control, nitrogen (N), sulphur (S) and sulphur + nitrogen (S+N) treatment plots (n = 4 per treatment and forest stand). Data are shown for the pre-treatment period (year 2013) and for the last three full years of manipulation. Significant treatment effects by site and time period denoted by p value. We calculated enzyme C/N, C/P and N/P ratios, where C = BG+CEL, N = NAG+LEU and P = PME.

306 ## 4. Discussion

307 Our results provide evidence that soil acidification alters C fluxes in both deciduous and conifer forests.
308 Dissolved organic carbon concentrations in soil solution were suppressed by acidification in both forest
309 types, and suppression of soil respiration was detected in the spruce stand. These responses have
310 significant implications for long-term soil C cycling. On the other hand, nitrogen addition alone did not
311 yet affect either DOC concentrations or soil CO_2 efflux in either forest type.

312 ### *4.1. Acidification alters dissolved organic carbon leaching*

313 Monthly addition of sulphuric acid immediately reduced forest floor soil solution pH by 0.4 units in
314 both stands. Due to higher initial acidity in the spruce stand, this equated to a higher post-treatment
315 hydrogen ion concentration of 270 µeq l^{-1} in the spruce stand, compared to 130 µeq l^{-1} in the beech
316 stand. This "acid forcing" induced a consistent decline in soil solution DOC of around 50 %. In line with
317 other lab and field experimental pH manipulations (Clark et al., 2011; Ekstrom et al., 2011; Evans et al.,
318 2012); analyses of long-term monitoring data (Finstad et al., 2016; Monteith et al., 2007); and
319 modelling assessments (Rowe et al., 2014; Sawicka et al., 2017), we therefore found clear and
320 quantitatively significant effects of soil solution acidity on soil solution DOC concentration. Albeit
321 different litter quality the effect of acid treatments on forest floor soil solution DOC concentration was
322 consistent at both sites, suggesting a prevailing chemical (rather than biotic) control over DOC
323 partitioning between soil and soil solution (Oulehle et al., 2013). Whether these effects were mediated
324 by altered dissociation of organic acids in response to changing acidity, or direct effect of changed ionic
325 strength (Hruska et al., 2009) remains unresolved. The neutralization capacity of forest mineral soils
326 at both stands was highlighted by the delayed soil solution pH decrease in deeper depth. This
327 ultimately led to a progressive acidification of mineral soil, due to the leaching of base cations
328 accompanied predominantly by sulphate anion.

329 The absence of soil solution pH and DOC responses to N addition in our experiment is consistent with
330 the interpretation that chemical processes control DOC mobility. The effect of NH_4NO_3 addition on soil

acidity depends on the fate of NH_4 and NO_3; If both NO_3 and NH_4 are taken up by the biota no acidification occurs (regardless of whether nitrification has occurred or not) but if NO_3^- is leached in excess of any leaching of NH_4^+ (due to preferential uptake and/or nitrification of NH_4^+) this will cause acidification, and may suppress DOC leaching (Evans et al., 2008). Contrary to previously suggested positive effects of N deposition on DOC leaching (e.g. Pregitzer *et al.*, 2004) we did not detect any substantial DOC increase following N addition in our systems. However, long-term N inputs may stimulate primary productivity (Liu and Greaver, 2010), and thus ultimately increase the supply of ecosystem C available for leaching as DOC (Rowe et al., 2014), provided that other feedbacks such as changes in plant species and litter quality/quantity do not offset the increase in NPP. In forests subjected to long-term elevated N deposition, as in our case, response of soil solution DOC concentrations may differ from N limiting systems where production of DOC may be more responsive to increased N availability.

4.2. Acidification effects on soil carbon dynamics

Measured total soil respiration (Rs) rates were closely related to the seasonal course of soil temperature, and soil temperature was the overriding control on temporal variation in Rs, with limited effects of soil moisture (Oulehle et al., 2016b). A large part of Rs in forests is derived from autotrophic respiration (Högberg et al., 2001), thus detection of subtle changes in soil CO_2 effluxes, driven by heterotrophic decomposition of fresh litter and SOM, is difficult. Nevertheless, consistent declines in Rs were detected in the spruce stand under acid treatment. The measured respiration decline of 6% - 10% under S treatment is similar to that found in a recent meta-analysis (7%) based on experiments from China (Feng et al., 2017). The reduction of CO_2 effluxes under S treatment corresponds to the 27% – 49% of annual litter production (aboveground litterfall of 1937 kg C ha^{-1} $year^{-1}$ between 2013-2016, Oulehle *et al.*, 2016b). Based on a total S addition of 50 kg S ha^{-1} $year^{-1}$ this corresponds to the suppression of decomposition by 10.6 to 19 kg C kg S^{-1}. The reduction in decomposition per unit S deposition may not be linear, however, as this would require linear relationships between S input and soil water hydrogen ion concentration, and subsequently with Rs. In a previous study (Oulehle et al.,

2011) we demonstrated a continuous loss of forest floor C pool, at a rate of 1160 kg C ha^{-1} $year^{-1}$, since precipitation acidity declined in the early 1990s as a consequence of "acid rain" abatement policy (total loss of 18.6 t C ha^{-1} between 1994 and 2010). We proposed that recent loss of soil C carbon was derived from the soil C accumulated during the acidification period. Based on our calculations with the lower estimate of S deposition effect on C decomposition (10.6 kg C ha^{-1} S ha^{-1}), a C pool of roughly 20 t C ha^{-1} could have been accumulated between the 1960s and 1990s (the time period during which precipitation acidity fell below 4.3; Kopáček *et al.*, 2016). Our experiment thus provides a plausible explanation for observed rates of C accumulation in forest soils exposed to acidification stress and could account for major changes in forest carbon stocks in the forests of Central Europe and other acidification-impacted regions.

Acid treatments in our experiment induced decreases in soil solution pH and DOC concentration in both forests, but only appeared to suppress Rs in the spruce stand. The two forest ecosystems can be characterized by: i) higher soil pH and base saturation in the beech stand; 2) higher substrate concentration and enzyme C to N ratios in the spruce stand; 3) an apparently higher degree of interactions among the microbial community in the spruce stand (Bárta et al., 2017); and 4) contrasting fungal life strategies, with saprotrophic fungi more abundant in the beech soil, and ectomycorrhizal fungi more abundant in the spruce soil (Bárta et al., 2017). Taken together, these features suggest a much stronger connection of soil metabolism to C acquisition in the spruce stand compared to the beech stand. Consequently, a decrease of soil solution pH and following suppression of labile C availability would be expected to have a much stronger impact on SOM decomposition in the spruce stand compared to the beech stand (Klotzbücher et al., 2011). We found a strong positive relation between soil pH and microbial biomass and high microbial respiration and C mining activity under low pH in the spruce stand. It coincided with a consistent (though non-significant, Figure 3) decrease in microbial biomass following acid treatments in the spruce stand (roughly 11 % based on 2016 data) and observed decreases in Rs. These results support our conclusion that the microbial (or at least prokaryotic) community in the spruce stand was most probably more stressed due to more acidic

383 conditions (from the beginning of the experiment, and intensified by S treatments) than the beech
384 community. The only acid treatment effects which were consistent across both forest types were
385 significant changes in enzyme stoichiometry, and partially significant changes in fungi to bacteria
386 ratios. Acid treatments significantly changed the C_{enz} to N_{enz} ratio, with increases following acid
387 treatments in both stands. However, in the spruce stand the increase in C_{enz}/N_{enz} ratio appeared to be
388 driven by increase of C_{enz} activities, which was further supported by the negative relationship between
389 soil pH and C_{enz} activity. In addition, C_{enz}/P_{enz} ratio increased in response to acid treatments in spruce
390 stand, with no change in the beech stand; this supports the assumption of predominantly enhanced C
391 enzyme activities in the spruce forest (Figure 6). More specific studies are needed to unravel the
392 feedback between altered soil pH and microbial biomass and enzyme activities. It is also important to
393 link actual qualitative changes among microbial communities caused by altered soil chemistry with
394 corresponding C fluxes.

395 It has been proposed that microbes adjust their enzymatic activity to gain the limiting element from
396 the substrate that is rich in this element and that the production of enzymes increases with substrate
397 complexity (Mooshammer et al., 2014). As shown previously, acidity affects the solubility and
398 bioavailability of organic matter (Evans et al., 2012; Scheel et al., 2007). We infer that shifts towards
399 enzymatic C mining after acidification were most probably connected with organic matter stabilization
400 and thus reduced availability to microbes, causing them to produce more C-acquiring enzymes to
401 sustain C supply. This interpretation is also supported by observed negative relationships between BG
402 and CEL and soil pH in the spruce stand and found shifts to fungal prevalence in both stands.

403 Based on our data, qualitative changes in the microbial community under acid treatments can be
404 attributed to decreases of bacteria in both forests, and to an increase of fungi in the beech forest,
405 resulting in higher fungi to bacteria ratios. According to the literature, bacteria are generally more
406 impacted by low pH than fungi (Rousk et al., 2009; Strickland and Rousk, 2010). In addition, fungi on
407 average have higher C to N biomass stoichiometry, slower growth and turnover (Rousk and Bååth,

2011) and probably also higher carbon use efficiency than bacteria (Allison et al., 2005; Six et al., 2006). It means that they can store more C in their biomass per unit of consumed C than bacteria. Thus, the observed shifts in F/B ratio (either due to bacterial decreases and/or fungal increases) might partly contribute to decreases of soil microbial respiration in the field under acid treatments in both stands.

4.3. N addition effects on soil organic matter dynamics

In contrast to the rather limited number of experimental studies of soil acidity effects on SOM cycling in natural or semi natural ecosystems (Chen et al., 2016; Pennanen et al., 1998; Persson et al., 1989; Wu et al., 2016), many studies have linked enhanced soil N availability to the soil C cycle (e.g. Olsson *et al.*, 2005; Hobbie, 2008; Janssens *et al.*, 2010; Liu & Greaver, 2010). In general, meta-analyses of N addition experiments have shown stimulation of biomass productivity or reduction of soil respiration, leading to potentially increased C sequestration. Although ^{15}N tracing studies have suggested only minor N deposition contributions to C sequestration in temperate forests (Nadelhoffer et al., 1999), many field-scale N addition studies have suggested that N deposition could substantially enhance soil carbon sequestration by both forests and heathlands (Field et al., 2017; Prescott, 2010). Our plot experimental design did not allow testing of N addition effects on plant biomass productivity, therefore allocation of C to belowground was assumed constant over the duration of our experiment. Based on our data we did not see any significant effects of N addition on Rs (Figure 2), microbial biomass (Figure 3) and respiration (data not shown). There was no evidence of N addition effects on fungi to bacteria ratios (Figure 5), but we did observed shifts in enzyme C to N stoichiometry (Figure 6), similar to those in the acid treatments, suggesting ongoing qualitative changes in microbial processes yet not detectable on a quantitative basis.

The lack of pronounced effects of N addition on the C cycle at our sites may be attributable to the high pre-existing levels of available N in these systems; both forests have been subjected to elevated N deposition since the 1950s, peaking in the 1980s with bulk deposition estimates of ca. 17 kg ha^{-1} $year^{-1}$ (Oulehle *et al.* 2016b; see

for current N fluxes). Microbial communities may therefore already be adapted to increased N availability at our sites, whereas systems receiving lower levels of ambient N deposition may be more responsive to additional N inputs (Phoenix et al., 2012). Nevertheless many other temperate forested ecosystems, especially those in Europe and North America, have been exposed to high levels of historic N deposition, and our data suggest that heterotrophic processes in these systems may be relatively unresponsive to additional N inputs. Forest ecosystems in parts of the world (apart of P-limited tropical forests) where N deposition is currently increasing, such as parts of Asia, Africa and South America (Dentener et al., 2006; Lamarque et al., 2013), but where fertility (i.e. soil C/N ratio) remains low may be expected to show greater change, potentially including effects on ecosystem C sequestration (Hyvönen et al., 2008).

With regard to dissolved carbon losses (and to a lesser extent also gaseous C fluxes) it is also possible that the absence of responses to N addition also reflects the lack of resulting changes in soil acidity. Previous work has shown that changes in DOC leaching in many N addition studies can be explained by experimentally-induced shifts in acid-base chemistry (Evans et al., 2008), but in our study the addition of NH_4NO_3 over 3 years was insufficient to alter soil solution pH, because little of the added N was leached as NO_3 (ca. 6% of added N in the spruce forest floor and ca. 11% of added N in the beech forest floor, data not shown). Thus the clear, chemically-mediated changes in DOC concentration observed in the acidification treatments were not replicated in the N-only treatment.

4.4. Implications for understanding of ecosystem responses to multiple drivers

A high proportion of biogeochemical research to date has taken place within areas of Europe and North America that have, as a result of industrialisation and agricultural intensification, been exposed to multiple environmental stressors, including the simultaneous deposition of a range of atmospheric pollutants. Nevertheless, experiments carried out within these ecosystems often focus on a single

458 driver, whilst many analyses of monitoring data have considered only a partial range of potential
459 driving variables. Studies of forest carbon sequestration in response to atmospheric deposition have
460 overwhelmingly focused on N deposition, yet our results suggest a potentially similar rate of soil C
461 accumulation in response to S deposition (> 10 kg C kg S^{-1}) as that estimated for N deposition (10 – 15
462 kg C kg N^{-1}; de Vries et al. 2009). Furthermore, S deposition to European and North American forests
463 has changed more rapidly and more dramatically over the last 50 years than N deposition, implying
464 that S may have had a greater influence on the soil C cycle during this period, and also raising the
465 possibility that some observed changes may have been incorrectly attributed to N deposition (Kolář et
466 al., 2015). In order to correctly interpret existing records, and to accurately predict future changes in
467 ecosystem biogeochemistry in response to continuing environmental change, we argue that a more
468 holistic approach to the impacts of multiple environmental drivers is needed.

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