Long-term agricultural management maximizing hay production can significantly reduce belowground C storage

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Highlights:

- Liming and mineral fertilization strongly reduced soil carbon (C) stocks
- Aboveground biomass (hay) production was greatly improved by soil amendments
- Plants in infertile soil allocated more C to belowground structures
- Plants in fully fertilized plots invested more into aboveground biomass
- Plant and mycorrhizal community composition shifted along nutrient gradient

Abstract

Liming and fertilization of grasslands have been used for centuries to sustain hay production. Besides improving hay yields, these practices induce compositional shifts in plant and soil microbial communities, including symbiotic arbuscular mycorrhizal (AM) fungi. However, in spite of increasing interest in soil carbon (C) sequestration to offset anthropogenic CO_2 emissions, little is known about the long-term effects of these agronomic interventions on soil C stocks. We examined how plants, AM fungi, and soil C respond to more than seven decades of annual applications of lime, mineral nitrogen (N), and mineral phosphorus (P) to test the hypotheses that (1) management practices increasing aboveground plant production decrease C allocation to roots, AM fungi and the soil; and (2) the relative availability of N and P predicts belowground C allocation in a consistent manner. Our study was conducted at the Rengen Grassland Experiment, established in 1941. Lime combined with N increased hay yields and promoted development of AM fungal hyphae in soil, while reducing relative C allocation to roots. Simultaneous enrichment of soil with lime, N, and P further boosted hay production, promoted grasses and suppressed other plant functional groups. This treatment also decreased soil organic C and strongly suppressed AM fungi in the soil, although the response to P varied among different AM fungal taxa. Our results indicate that agricultural management practices aimed at maximization of hay production may, in the long run, significantly (-20.4%) reduce belowground C storage. This is a great concern with respect to the intended use of grasslands as anthropogenic CO_2 sinks because the fertilization-induced decrease in soil C stocks can partly or fully negate the C sequestration potential of the grassland ecosystems as a whole.

Keywords: arbuscular mycorrhiza, nitrogen, phosphorus, lime, soil carbon, long term ecological research

1. Introduction

Grasslands cover about 40% of the terrestrial surface of the planet and about half of this area is currently used in agricultural production (Lal, 2007). Because soils contain more organic carbon (C) than both the atmosphere and biota combined and because grassland soils generally have much larger C storage capacity than annually cropped soils, grasslands are extremely important for global soil C sequestration (Conant *et al.*, 2001; Lal, 2013). Long-term productivity of grasslands is influenced by many factors including climate, geographic location, and management practices, and particularly by the level of fertilization (Sala *et al.,* 1988; Conant *et al.*, 2001). For sustainable hay production, soil organic matter is critical because it supplies plants with nutrients, helps to reduce soil erosion, and increases cation exchange and water holding capacity (Schulten & Schnitzer, 1997; Daynes *et al.,* 2013). Consequently, management practices that restore and/or maintain high levels of soil organic matter are desirable. This in combination with recent interest in soil C sequestration, as a means to offset rising CO_2 levels in the atmosphere, has led to a keen interest in optimizing grassland management for C storage (Conant & Paustian, 2002; Lal, 2008). An earlier review of 115 studies from around the world demonstrated that grasslands fertilization, especially with nitrogen (N), tends to be associated with increased soil C (Conant *et al.,* 2001; van Groeningen *et al.*, 2006), suggesting that fertilization is potentially an important driver of soil C sequestration. However, the relationship is difficult to predict because it may critically depend on the type of fertilizer, and is likely to be nonlinear (Wander *et al.,* 1994). One reason for this is that belowground C allocation by the plants and subsequent C transformations within soils likely depend on plant and microbial responses to relative, not absolute nutrient limitations (Cleveland & Liptzin*,* 2007; Kaiser *et al.,* 2014).

The soils and roots of plants in natural and managed grasslands are abundantly colonized by arbuscular mycorrhizal (AM) fungi. These fungi, belonging to the phylum Glomeromycota, form symbiotic relationships with plants that are often mutually beneficial for both partners. Plant hosts supply carbohydrates in exchange for fungal provisioning of mineral nutrients, especially phosphorus (P) and zinc, some of which would otherwise be unavailable for plants (Allen, 1991; Jansa *et al.,* 2003). Besides facilitating nutrient uptake, AM fungi can provide

benefits to colonized plants, such as resistance against pathogens and drought tolerance (Newsham *et al.,* 1995; Smith *et al.,* 2010). High densities of AM fungal hyphae in soil form underground networks that contribute to creation and stability of soil aggregates and reduce soil losses through erosion (Chaudhary *et al.,* 2009). Because many of these features are considered beneficial for the sustainability of agricultural production, there is a great interest in increasing mycorrhizal symbioses through agricultural management (Gianinazzi *et al.,* 2010; Fitter *et al.,* 2011).

Mycorrhizal fungi are key components of grassland C sequestration (Treseder & Allen, 2000; Zhu & Miller, 2003; Johnson *et al*., 2006; Wilson *et al*., 2009). Hyphae of AM fungi may comprise a large fraction (apparently up to a half) of microbial biomass in grassland soil (Olsson *et al.,* 1999), and therefore their responses to fertilization are highly relevant to soil C dynamics. Although AM fungi are expected to rapidly channel and immobilize a significant portion of plant-derived C through the soil, there is an on-going debate about the importance of this C highway in the buildup of stable soil organic matter and soil C sequestration (Phillips *et al.,* 2013; Averill *et al.,* 2014; Balasooriya *et al.,* 2014). Density of AM fungal hyphae in the soil was previously found to be positively correlated with the responses of soil organic C to long-term N enrichment and burning in a North American tall-grass prairie, while application of fungicides suppressed AM fungal hyphae and reduced soil organic carbon (Wilson *et al.,* 2009). Responses of AM fungi to N fertilization have been shown to vary with availability of soil P. For example, N enrichment of P deficient grasslands often increases AM fungal biomass but N enrichment of P rich grasslands often decreases it (Johnson *et al.,* 2003; Wilson *et al.,* 2009; Liu *et al.,* 2012).

The functional equilibrium model can help explain why N enrichment may either increase or decrease AM fungal biomass. This conceptual model states that plants should preferentially allocate photosynthates to structures that acquire the most limiting resources; plants growing in infertile soil should allocate more to roots and mycorrhizas and plants growing in nutrient rich soil should allocate more to shoots and leaves (Brouwer, 1983; Johnson *et al.,* 2003). Increasing soil N availability in P-limited soils exacerbates P limitation and enhances the importance of symbiotically acquired P; thus plants are expected to increase allocation of photosynthates belowground to AM fungi. In contrast, N enrichment of P-rich soils is likely to remove limitation by any belowground resource and, in accordance with the functional

equilibrium model, plants are expected to shift allocation of photosynthates aboveground to shoots and away from AM fungi (Johnson, 2010).

Changes in nutrient availabilities and consequent shifts in C allocation by plants can have large effects on AM fungal communities (Egerton-Warburton *et al.,* 2001, 2007; Liu *et al.,* 2012). Application of fertilizers systematically increases plant productivity, but also reduces plant diversity (Silvertown *et al.,* 2006; Hejcman *et al.,* 2007; Semelová *et al.,* 2008), potentially driving changes in the fungal communities. The effects of fertilization on individual AM fungal taxa are highly context-dependent and are often confounded with the effects of soil disturbance and crop rotation (Jansa *et al.,* 2006 and references therein). Individual taxa of AM fungi differ in their responses to fertilization; some species increase and others decrease in abundance in response to nutrient enrichment (Douds & Schenck, 1990; Johnson, 1993; Egerton-Warburton *et al.*, 2001).

The aim of this study was to analyze the responses of soil C and AM fungi to 70 years of annual applications of lime and mineral fertilizers at one of the oldest agricultural experiments worldwide – the Rengen Grassland Experiment. Our goal was to test the ability of the functional equilibrium model to predict responses of soil C stocks to grassland inputs. We examined the abundance of AM fungi inside and outside plant roots and used molecular techniques to determine the abundance of several AM fungal taxa. This allowed us to test the hypothesis that lime and fertilizers would reduce plant allocation of photosynthates to belowground structures including the mycorrhizas. We predict that the differences of lime and fertilizer inputs among treatments will have consequences for the long-term buildup of soil C stocks, co-incident with the effects on AM fungi: N enrichment will increase allocation to AM fungi due to increasing relative demand for limiting P, while fertilization with both N and P will decrease allocation to AM fungi, consequently reducing the soil C sequestration.

2. Material and methods

2.1. Study site description and experimental design

This study was conducted at the Rengen Grassland Experiment established in 1941 by the University of Bonn in low productivity *Nardetum* grassland in the Eifel Mountains, Germany (50°13' N, 6°51' E). The site is 475 m above sea level, average annual precipitation is 811 mm and the mean annual temperature is 6.9°C. Soil type is a pseudogley. The experimental plots (3 m × 5 m) received annual amendments of lime (mainly as CaCO3) and fertilizers for more than seven decades, reaching levels considered sufficient for sustainable hay production in that area. We sampled four treatment combinations (average inputs in kg ha^{-1} yr^{-1}): Ca (715 Ca), CaN (100 N, 752 Ca), CaNP (100 N, 35 P, 936 Ca), and a control (unfertilized plots). The different amounts of Ca in the different treatments were due to the forms of applied fertilizers (such as Ca_3PO_4 in the CaNP treatment, which were not counterbalanced by reducing lime inputs in the same treatment as compared to the other limed treatments). The experimental plots are arranged in two blocks containing each five randomly arranged replicates of the Ca, CaN and CaNP treatments, totaling 30 experimental units with ten replicate plots per treatment. No differences in the underlying geology or soil properties were detected between the two blocks when comparing concentrations of available P, total N, organic C, mobile Ca in the soil and the soil pH (analyses not shown), justifying pooling the replicates across the two blocks. Since the original field experiment did not contain non-fertilized controls, ten control plots were designated in 1998 on adjacent grassland strip below one of the blocks that has never been fertilized or grazed, and which were cut twice a year as the original experimental plots ever since. By correlating selected soil properties (see above) within the experimental blocks along the gradient covering various distances from the control plots, no significant soil gradient could be uncovered, which would. When including the control plots in the correlation analyses, only soil pH indicated significant shift with the distance from the control plots, driven by the strong difference in soil pH between the controls and the other plots (see Table 1). These analyses thus proved that the control plots could be compared with the other plots without much concern. Further details of the site and experimental design have been published previously (Schellberg *et al.*, 1999; Hejcman *et al.,* 2007).

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174

175 *2.2. Sampling procedure and plant analysis*

176 Aboveground biomass was cut in early June and in mid-October. A sample for laboratory
177 analyses was collected along a strip 1.25 m wide and 5 m long and weighted. Subsamples of 500
178 g from each of the plot were dried at 60°C for 48 h to determine the dry biomass (hay yield). The
179 percentage of surface cover by individual vascular plant species was estimated visually in twenty
180 plots on 20 June 2011 according to a regional plant species list (Rothmahler *et al*., 2000),
181 considering three plant operational groups (grasses, herbs and legumes). The nomenclature of
182 plant species was based on Kubát *et al.* (2002). To eliminate edge effect, relevés were taken in
183 the center of each plot, considering an area of 1.8 m × 3.2 m.

184 Three types of belowground samples were collected: (1) composite soil+root samples, (2)
185 root standing biomass, and (3) new root growth. All samples were transported to the Czech
186 University of Life Sciences in Prague and frozen within 24 hours of collection.

187 (1) On 20 October 2011, eight small diameter cores (2.5 cm diameter ×10 cm depth)
188 were collected across the central region of each plot and mixed into one composite
189 sample. Roots recovered from the composite sample were analyzed for AM fungal
190 colonization and the soil from the composite samples was analyzed for extraradical
191 (soil-borne) AM fungal hyphae and other soil properties as described below.

192 (2) At the same time, one large (5 cm diameter) core was taken from the center of each
193 plot to measure the standing root biomass. The deepest possible core was taken and
194 the resulting core depths varied from 10 to 15 cm. Each core was weighed and its
195 exact length was recorded. Gravimetric soil moisture content was measured on a sub-
196 sample of soil from each root core by weighing the soil before and after drying at
197 70°C for 72h. Each hole created through this sampling was immediately filled with
198 granitic river sand.

199 (3) In mid June 2012, the area was re-sampled using the same corer and sampling depth
200 to estimate new root production during eight months of root growth.

201 Roots were removed from the large cores by soaking them in soapy water for 5 to 10 min, and
202 then rinsed with tap water through a 1mm sieve. Roots were also carefully removed from the
203 new growth (sand) cores by rinsing with tap water through a 1 mm sieve. Cleaned roots were

204 weighed, dried at 80°C for 48 h and weighed again. Standing root biomass in the topsoil (10 cm
205 depth) per ha was calculated by using estimated root biomass and the soil core volume. Root-to-
206 shoot biomass ratio was then calculated as the ratio of dry weight of roots (t ha^{-1}) and annual
207 yield (t ha^{-1}, average value from years 2008 through 2012).
208
209 *2.3. Determination of AM fungal colonization and extraradical hyphal length*
210 The extent of root colonization by AM fungi was determined in the roots from the composite soil
211 samples. Roots were soaked in 5% KOH at room temperature for two days, and stained using the
212 ink and vinegar technique (Vierheilig *et al.,* 1998). Stained roots were examined for mycorrhizal
213 colonization using the magnified intersection method, scoring 100 intersections per sample
214 (McGonigle *et al.,* 1990) at a magnification of 200×. Then we calculated percentages of root
215 length colonized by arbuscules, coils, vesicles, mycorrhizal hyphae, and the fraction of root
216 length devoid of any mycorrhizal structures.
217 Composite soil samples were thawed and a 5 g subsample was used for determination of
218 extraradical external hyphal length following the protocol described in Rillig *et al*. (1999) with
219 minor modifications. In short, soil was suspended in a solution containing 112 ml of deionized
220 water and 420 mg of $Na(PO_4)_6$, shaken for 30 s, allowed to settle for 30 min, and decanted into a
221 38 µm sieve. All material on the sieve was suspended in 200 ml of deionized water, shaken, and
222 3 ml was transferred on to a membrane filter with a pore size of 0.45 µm (GN-6 25mm; Pall Life
223 science, Mijdrecht, the Netherlands), placed in a vacuum manifold. Material on filters was
224 stained with 0.02% trypan blue in lactoglycerol for 5 min, washed with excess water and the
225 filters then mounted on microscopic slides. AM hyphae were distinguished from other hyphae
226 according to descriptions in Mosse (1959): "dark-to light-blue stained aseptate hyphae with
227 characteristic unilateral angular projections". Intersects in 30 random grids were counted with a
228 compound microscope at 200× magnification. Hyphal length was calculated per g soil dry weight
229 by estimating soil water loss after drying in an oven at 80 ºC for four days.
230
231 *2.4. Real-time PCR*
232 A subsample of each composite soil sample was dried at 80ºC for 48 h, homogenized, and sieved
233 through a 2 mm sieve. The soil DNA was extracted from 500 mg (dry weight) samples using the

Macherey-Nagel NucleoSpin Soil DNA kit (Macherey-Nagel GmBH & Co., Düren, Germany) following the standard protocol and stored at –20°C until use. Abundances of six AM fungal taxa were estimated using quantitative real-time polymerase chain reaction (qPCR) with specific AM fungal primers and hydrolysis (TaqMan) probes, using an AB StepOnePlus instrument (Applied Biosystems, Foster City CA, USA.). We employed taxon-specific markers targeting the nuclear large ribosomal subunit (nLSU) genes (Wagg *et al.,* 2011; Thonar *et al.,* 2012) for *Cetraspora pellucida, Claroideoglomus claroideum, Funneliformis mosseae, Diversispora celata, Gigaspora margarita,* and *Rhizophagus irregularis*. Additionally, for detection of *Rhizophagus* sp., we also used a marker targeting the mitochondrial large ribosomal subunit (mtLSU) gene (Kiers *et al.,* 2011; Couillerot *et al.,* 2012). Twenty billion copies of an internal standard (linearized plasmid carrying a fragment of cassava mosaic virus, GenBank accession AJ427910) were added into each sample before extraction, and quantified after the extraction, to estimate the DNA extraction efficiency and DNA extract quality, namely absence of PCR inhibitors (Thonar *et al.,* 2012; Janoušková *et al.*, 2015). Each qPCR reaction mixture contained 2 µl template (DNA extracts diluted 1:10), 0.4 µl forward primer (25 µM), 0.4 µl reverse primer (25 µM), 0.1 µl hydrolysis probe (25 µM), 4 µl of 5× HOT FIREPol® PROBE qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia), and 13.1 µl PCR-grade water.

Cycling conditions were as follows: initial DNA denaturation and DNA polymerase activation at 95°C for 15 min, then 50 cycles each with denaturation at 95°C for 10 s, annealing conditions specified in Table S1(electronic supplement), and elongation at 72°C for 10 s.

2.5. Soil properties

Soil moisture content was measured gravimetrically as the difference between the weight of fresh soil and that dried at 105°C for 24h. To measure soil pH, 30 g of dry soil was suspended in 30 ml deionized water for 20 minutes, centrifuged at 3044 g for 20 minutes and the supernatants (soil extracts) were filtered through a paper filter to remove floating particles. The pH was determined using a combination pH electrode G-P Combo w/RJ (Corning, USA). The concentration of mobile Ca^{2+} ions in the supernatants brought to pH 7.0±0.2 (using 1% HCl or KOH) was measured using a Ca-selective electrode (type 20-35, Monokrystaly, Turnov, Czech Republic) and corrected for acidic error using the calibration of the electrode response with

solutions of known Ca concentrations at different pH values. Soil organic C and total N concentrations were analyzed using a CN analyzer (Flash EA 2000, ThermoFisher Scientific, Waltham MA, USA). Soil available P was determined spectrophotometrically in aqueous soil extracts (3g dry soil and 30 ml of water) after shaking for 25 hours, subsequent centrifugation at 3044 g for 20 min and filtration through a 0.22µm filter (CME, Carl Roth, Karlsruhe, Germany). This approach to measure P availability was chosen because of large differences in soil pH between the different soil treatments. Soil pH can namely largely affect the estimates of P availability in soils when assessed by various extraction methods (Demaria *et al*. (2005).

2.6. Bulk density and soil carbon stocks

Bulk density was determined from the large soil cores (5 cm diameter). The entire core was weighted fresh, a subsample of the core was then taken, weighted, dried at 70°C for 3 days and weighted again. The ratio of fresh to dry weights of the soil subsample was used to calculate dry weight of the entire soil core of known dimensions. In order to determine the amount of soil C per ha, the volumetric organic C content of the topsoil was calculated by using gravimetric organic C concentrations measured on the composite soil samples (cores with 2.5 cm diameter, dried and sieved to pass 2mm sieve) and the soil density assessed on the large soil cores, and scaling up to 1×10^6L of soil (1ha surface, 10cm depth).

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2.7. Statistical analyses

Prior to analysis, root colonization data (percentage + 1) were square root-arcsine transformed (Zar, 1999). To evaluate differences between treatments, one-way ANOVA in STATISTICA 8.0 (Statsoft, Tulsa, OK, USA) and an additional post-hoc test using the least significant difference F-test ($p = 0.05$) was used. Control plots were considered as the fourth treatment in addition to the Ca, CaN, and CaNP for statistical comparisons, given the apparent absence of underlying soil gradients on the experimental grounds (see above). Redundancy Analysis (RDA) and Canonical Correspondence Analysis (CCA) in the CANOCO 4.5 program (terBraak & Šmilauer, 2002) was used to examine the relationships among AM fungal variables, soil properties, and hay yields. Further, a Monte Carlo permutation test with 499 permutations was used to reveal if the tested explanatory environmental variables (soil C, available soil P, total soil N, available soil Ca, soil

294 pH, and hay productivity at the plot level) had a significant impact on the AM fungal community
295 composition and abundance. Results of RDA and CCA were visualized in an ordination diagram
296 constructed by the CanoDraw program. Simple linear regression was used to examine the
297 relationships between the volumetric organic C content of the topsoil, density of AM fungal
298 hyphae in soil and hay production.
299

3. Results

3.1. Soil properties

Long-term fertilization and liming caused high variation in soil chemical properties across the different management treatments (Tables 1 and 2). Plant available P in soil was an order of magnitude higher in P fertilized plots compared to the other treatments (Table 1). Long-term liming, with or without fertilizer additiona, dramatically shifted soil pH. The fertilization had a profound effect on the total N and organic C concentrations in the soil, but neither of the practices did significantly change mobile Ca levels. Soil pH increased with liming and fertilizer from 5.32 in control plots to 7.14 in CaNP plots (Table 1). Soil bulk density varied between individual samples from 0.83 to 1.18 kg L^{-1}, and was significantly lower in the unfertilized control treatment compared to the CaNP treatment (Table 2).

3.2. Plant responses to lime and fertilizers

Hay yield was significantly increased by application of lime and the N and P fertilizers ($F = 118.8$, $p < 0.001$; Fig 1a). The standing biomass of roots in October 2011 tended to have lower values in the CaN treatment compared to the other treatments, yet the differences remained insignificant ($F = 2.06$, $p = 0.123$; Fig 1b). Root biomass in June 2012 showed similar pattern as in October 2011, with no significant differences between treatments ($F=1.58$, $p=0.21$), but the values were about 3 times lower than those in October 2011 (data not shown). Root-to-shoot biomass ratio was higher in the unfertilized control and in the limed treatments than in the CaN and CaNP treatments ($F = 13.8$, $p < 0.001$; Fig. 1c). Plant species richness decreased with the complexity of soil amendments, with significantly fewer species being found in the CaNP plots than the other treatments ($F = 11.1$, $p < 0.001$; Fig. 1d); relative cover of herbs tended to decrease ($F = 3.16$, $p = 0.0535$; Fig. 1e), grass cover increased ($F = 5.22$, $p = 0.011$; Fig. 1f), and cover of legumes decreased ($F = 5.53$, $p = 0.015$, data not shown) with increasing complexity of soil amendments.

3.3. Mycorrhizal responses to soil amendments

329 The percentage of root length colonized by any AM fungal structures varied significantly among
330 the treatments (F=3.18, p=0.036; Fig. 2a) and was lower in the CaNP treatment than in the
331 unfertilized control. The colonization of roots in the CaN treatment showed similarly high values
332 as in the unfertilized control plots (Fig. 2a). Similar picture was observed when scrutinizing the
333 impact of the management practices on the rates of root colonization by specific AM fungal
334 structures (Fig. 2 b – d), but only the coils (Fig 2d) showed statistically significant (F = 3.64, p =
335 0.022) decrease in abundance in the CaNP as compared to the other treatments. . The density of
336 extraradical AM fungal hyphae in the soil was significantly higher in the CaN plots , as
337 compared to the control and the CaNP treatments, with the latter having the lowest hyphal
338 density among all the treatments (F = 5.91, p = 0.002; Fig 2e). According to the CCA (Monte
339 Carlo permutation test p = 0.018; Fig. S1), root colonization and density of AM fungal hyphae in
340 the soil were negatively related to available soil P and hay yield and positively related to the N
341 and C concentrations in the soil.

342 The qPCR profiling of individual AM fungal taxa abundance in soil yielded positive detection
343 between 30% (*Claroideoglomus*) and nil (*Cetraspora*) of the analyzed samples (details not
344 shown). There were either no effects of soil management on the abundance of the tested AM
345 fungal taxa or the abundance in the limed and/or fertilized treatment higher than in the control
346 plots (Fig 3). Additionally, there was an obvious difference in the response to P fertilizer
347 between *Claroideoglomus*, whose abundance was decreased by P fertilization as compared to N
348 fertilization only, and *Diversispora*, which was not significantly affected by P fertilization (Fig.
349 S2a, b). Multivariate analysis (Fig. 3) showed that the abundances of the tested AM fungal taxa
350 were significantly affected by soil properties (Monte Carlo permutation test p=0.02) and mostly
351 driven by soil amendments, increasing with pH (*Rhizophagus, Claroideoglomus, Diversispora*),
352 yields and/or soil P (*Funneliformis*).

353 *3.4. Soil C stock*

354 The amount of soil organic C in the top 10 cm was significantly increased by a sole lime
355 amendment and significantly decreased by simultaneous addition of lime, N and P as compared
356 to the unfertilized controls (F = 12.7, p < 0.001; Fig. 2f). There was also a strong decrease in the
357 soil C/N ratio with increasing complexity of amendments (Table 2). Soil C/N ratios were higher
358 in the control and limed treatments as compared to the other treatments, with the values being
359 still significantly higher in the CaN than in the CaNP treatments (Table 2). Volumetric organic C
360 content of topsoil had a negative relationship with hay yield (R^2 = 0.21, p = 0.003) and a positive
361 relationship with the density of AM fungal hyphae in the soil (R^2 = 0.22, p = 0.002; Fig. 4),

whereas it showed no significant relationship with any of the AM fungal abundance estimates in the roots (analyses not shown).

4. Discussion

Our results show that seven decades of yield-promoting measures, particularly the N and P fertilization dramatically reduced soil C stocks in a temperate grassland. This reduction in the top 10 cm is about 6 tons C ha^{-1} when comparing the fully fertilized treatment with the unmanaged control. There is even a higher reduction of 11 tons C ha^{-1} comparing the fully fertilized treatment to the lime-only treated control. This latter comparison represents a reduction of 20.4% of the C stocks in the topsoil of the limed control, which is more than a half of the figure to be expected due to land use change from grassland to cropland (-36%, Poeplau *et al*., 2011). The C loss from the topsoil of the CaNP treatment over the last 70 years outweighed the C contained in the increased annual hay yield by 6- and 12-fold when compared to the control with no inputs and to the limed treatment, respectively. This comparison takes into account average hay yields over the last fifteen years, assuming 45% C content of hay. Due to lack of records, we are unable to draw a full C balance of the system over the entire 70 years duration of the experiment or for soil depths greater than 10 cm. Nevertheless, the magnitude of the observed effect calls for a very careful consideration of the design of grassland management practices that aim to both maximize hay yield and enhance belowground C sequestration. There is an urgent need to better understand the mechanisms that control the accrual of soil C stocks and, based on this information, to design highly productive agrosystems with high C sequestration potential.

Photosynthesis is the source of the vast majority of C in grassland soils, and thus one would assume that the greater the productivity, the greater the C remaining in the system. There are, however, many complicating factors: the processes of decomposition and mineralization determine the ultimate fate of soil organic C; and the stoichiometry of C, N, and P within the system has a strong influence on the complex interactions involved in these processes. Organic C enters the soil as root or shoot debris (necromass), root exudates, AM fungal biomass (trophically completely dependent on host plants) or AM fungal necromass. Thereafter, organic C enters the belowground food web involving soil animals, fungi and prokaryotes. At every trophic step, a portion of organic C is lost as CO_2 and a part is stabilized in the soil over shorter or longer periods of time. As a result, realized soil C stocks depend on plant productivity (i.e., input quantity), biomass partitioning between roots and shoots, degree of association of plants

with AM fungi, degradability of plant and/or AM fungal necromass (i.e., input quality), activity of soil microbes, and competition among plants, AM fungi, and soil saprobes for essential resources such as N and P, as well as fluctuations of environmental properties such as temperature and moisture (Cotrufo *et al.*, 2013). Theoretically, plants and associated AM fungi could be seen as competitors of soil saprobes for available P and/or N thus suppressing their decomposition activity (Leifheit *et al.*, 2015). Alternatively, AM fungi and fine roots could prime soil saprotrophic life with fresh C from aboveground (Murphy *et al.*, 2015), thus increasing activity of soil saprobes and reducing soil C stocks. We will describe three non-mutually exclusive mechanisms that may explain the decline of soil C that was associated with P fertilization in our study: 1) allocation to roots and AM symbioses, 2) degradability of litter inputs, and 3) stoichiometric control of soil microbial activity.

4.1. Allocation to roots and AM symbioses

The functional equilibrium model predicts that plant allocation to roots will decrease when they are not limited by mineral nutrients (Brouwer, 1983; Nielsen *et al.*, 2001; Hermans *et al.*, 2006). Here we showed that the root-to-shoot biomass ratio significantly decreased in the CaN and CaNP treatments, corroborating the idea that plants allocate biomass to the organs required to gain access to the most limited resources. Enrichment with N or with N and P together increased relative allocation aboveground to shoots and reduced allocation to roots (Fig. 1). The functional equilibrium model applies to plant allocation to AM symbioses as well as roots (Johnson *et al.*, 2003; Johnson *et al.*, 2013). It is interesting to note that in our study allocation to roots was reduced in both the CaN and CaNP treatments, while allocation to AM fungi (soil hyphae) was only reduced in the CaNP treatment (Fig. 2e). This strongly suggests that AM symbioses are critical for the P nutrition while roots are critical for the N nutrition of plants.

The opposite relationships of hay yield and AM hyphae with soil C suggests that plant allocation to shoots (hay) and away from AM symbioses may have a large impact on soil C storage (Fig. 4). The positive correlation between soil C and AM fungal hyphae could arise from increased C inputs via hyphal biomass and necromass as well as increased C storage through enhanced soil aggregation and hence protection against degradation (Zhu & Miller, 2003; Rillig, 2004; Leifheit *et al.*, 2015), as well as from lower microbial activity in the unfertilized soil due to

low P availability for the microbes, which could further be enhanced by direct competition for resources like available P between AM fungi and other soil biota (van Groeningen *et al*., 2006; Welc *et al*., 2010). Other studies have also reported a positive correlation between AM fungal abundance and soil C levels (Treseder & Allen, 2000; Johnson *et al*., 2006; Wilson *et al.,* 2009). Future research is needed to determine the mechanisms generating these correlations. The degree to which AM fungi and their associated microbes increase soil C stocks though direct input of recalcitrant forms of C versus their influence on soil aggregate structure (and protection from decomposition) is currently unknown. It is important to recognize that in some systems, AM fungi might actually reduce soil C levels by stimulating organic matter decomposition by other soil microbes (Hodge *et al*., 2001; Cheng *et al.*, 2012). This idea is also supported by the view that most C allocated to the AM fungi flushes through the ecosystem and is respired back to the atmosphere within few days (Johnson *et al*., 2002; Lendenmann *et al*., 2011). Depending on the importance of either of these processes (increased C inputs and stabilization or stimulation of microbial decomposition) the effects of AM fungi on C stocks may be positive, negative or neutral (Verbruggen *et al.,* 2013). Further issue to consider for interpretation of our results is the fact that they are only correlative and may simply mean that AM fungi thrive in more organic soil.

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The community composition of plants and AM fungi was strongly influenced by the different fertilizer treatments. Apart from stimulating mycorrhizal fungi in general (Fig. S1), application of lime together with N increased the abundance of AM fungal taxa *Claroideoglomus* and *Diversispora* (Fig. S2). In contrast, P enrichment drove a sharp decrease of AM fungal hyphae density in soil, although the specific response of the individual fungi to P application differed, with some taxa such as *Diversispora celata* not responding at all to addition of P (Fig. S2). This suggests that plants either selectively down-regulate certain fungal taxa in response to high P, or that high P-levels directly inhibit some AM fungal taxa. However, it should be noted that observed changes in the fungal community composition could only roughly explain changes in AM fungal hyphal density in soil. Partly, this is certainly due to the method used to screen for the AM fungal taxa abundances, which misses many taxa for which the quantitative molecular markers are not yet available. This was the case of the control treatment that showed a complete absence of both *Diversispora* and *Claroideoglomus*, although uncharacterized AM fungi were

obviously present in great abundance (Fig. S2). These values, although correct, do not signal lower activity of AM fungi in general, but only the particular focal species (Jansa *et al*., 2014; Ohsowski *et al*., 2014). Finally, reduced mycorrhization in the CaNP treatment could have arisen because of selection of a plant community with reduced mycorrhizal dependency over the course of the experiment. The shift from a diverse dicot-rich community to a less diverse grass-dominated community (Fig. 1) can be expected to generate many changes that could influence soil C dynamics.

4.2. Degradability of litter inputs

Litter quality is likely to vary across the different fertilizer treatments and this may have a strong influence on decomposition. Previous research at the Rengen Grassland Experiment has shown that P concentration in harvested forage from the CaNP plots was about twice as high as in the other plots, with most of the other parameters remaining the same (Schellberg *et al*., 1999; Hejcman *et al*., 2010). Concentration of N in the harvested forage from the CaN plots was higher than in the CaNP plots, but only slightly and insignificantly elevated as compared to control and the limed controls (Hejcman *et al*., 2010). This resulted in much lower N/P ratio of the harvested forage in the CaNP treatment (4.8) than in the other three treatments (where the mean ranged between 13.6 and 17.7, Hejcman *et al*., 2010). The P-rich biomass in the CaNP treatment is likely to be degraded faster than if P in the biomass is low (Cornelissen & Thompson, 1997; Freschet *et al.,* 2013), resulting in more C to be removed from the P-rich organic inputs than if the inputs are of low quality.

In addition to the direct influence of chemical fertilizers on forage quality, the indirect effect through altered plant community composition is also likely to be important. We observed the highest soil C storage in the treatments with the highest plant diversity, the unfertilized control and the limed plots (Fig. 1), in agreement with other observations from the Jena and Wageningen biodiversity experiments, where plant diversity correlated positively with belowground C storage (Steinbeiss *et al*., 2008; Cong *et al*., 2014). This correlation could be explained either through production of more degradation-recalcitrant litter by the plants adapted to nutrient-limited environments (Pastor & Cohen, 1997), or through changes in the composition and/or activity of soil microbial communities (including the AM fungi) in response to the

differential management. It has been demonstrated that manipulation of the soil microbial diversity could have dramatic consequences on multiple ecosystem functions including litter decomposition, nutrient cycling and C sequestration (Wagg *et al*., 2014).

4.3. Stoichiometric control of soil microbial activity

Decomposer microorganisms require a certain balance of essential nutrients and the most limiting nutrient may control the decomposition process (Sinsabaugh *et al*., 2013). The processes of mineralization and immobilization are well known to be sensitive to the C/N ratio of the substrate and surrounding environment; however, C/N/P ratios can also be very important (Elser *et al*., 2000). Many grassland systems, particularly those on highly weathered or carbonate rich soils are P limited and N enrichment of these systems may exacerbate P limitation and immobilize organic substrates. For instance, in a laboratory based decomposition study Craine *et al*. (2007) found that increasing N reduces litter decomposition, potentially leading to higher soil C accumulation. Also, van Groenigen *et al*. (2006) found a strong positive effect of N availability on build-up of the soil C reservoir. Looking at actual C stocks in grasslands, Fornara & Tilman (2012) and Fornara *et al*., (2013) report significantly higher C under only-N fertilization in studies lasting for 27 and 19 years, respectively. In the latter study, simultaneous addition of N and P entirely negated this effect. These observations were more recently confirmed by both laboratory and field studies, indicating that both C mineralization and gaseous N emissions are promoted by P additions to soil (He & Dijkstra, 2015; Fisk *et al.*, 2015). This broadly agrees with our results: when P limitation is removed, soil C storage decreases. However in our work, N-only addition did not increase soil C compared to the control, even after 70 years of fertilization (Fig. 2f).

Concluding remarks

The Rengen Grassland Experiment provides an unprecedented opportunity to examine how long-term management influences community structure and ecosystem processes such as C sequestration. Our study provides correlative evidence that AM symbioses contribute to soil C stocks and that this C pool may be diminished if AM fungal biomass is reduced in response to P fertilization. Future experimental work is needed to more thoroughly understand the mechanisms

causing these correlations and to provide more direct proof of the principle. Isotopes can be used to measure C pools and track C fluxes through plants, mycorrhizas and decomposer food-webs. Also, stoichiometric models should be tested to elucidate the relative importance of C/N/P ratios in soil C dynamics. Although we acknowledge a number of limitations of our study, such as lack of records on C stocks in deeper soil horizons and limited data on AM fungal biomass and community composition, our results convey a very clear message - that designing future agroecosystems to simultaneously maximize aboveground yields and maximize belowground C storage may be a difficult or even an impossible goal to achieve.

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Figure legends:

Fig. 1. Annual hay yield (a), root standing biomass (b), root-to-shoot biomass ratio (c), plant species richness (d), cover of dicots (e), and cover of grasses (f) as affected by the management treatments (Control – no amendments, Ca – lime added, CaN – lime and nitrogen added, CaNP – limed and nitrogen and phosphorus fertilizers added). Bars represent means +1 standard errors (n=10 for a-c and n=5 for d-f). Different letters indicate significant differences between treatment means (least significant difference F-test, $p < 0.05$). Biomass of roots has been estimated only for the topsoil (0-10cm depth) in October 2011, and upscaled to 1 ha surface to compare with hay yields. Average yields of seasons 2008 – 2012 are shown.

Fig. 2. Extent of root length colonized by any of the arbuscular mycorrhizal (AM) fungal structures (a), and that colonized specifically by arbuscules (b), vesicles (c), AM fungal coils (d), the density of AM fungal hyphae in the soil (e) and the concentration of organic carbon (C) in the topsoil (f) as affected by the management treatments. Bars represent means +1 standard errors (n=10). Different letters indicate significant differences between treatment means (least significant difference F-test, $p < 0.05$). The percentage values were arcsin-square root transformed for the statistical comparisons so as not to violate ANOVA assumptions. Soil C stocks have been estimated only for the topsoil (0-10 cm depth) and upscaled to 1 ha surface.

Fig. 3. Ordination diagram showing results of redundancy analysis of abundance of individual arbuscular mycorrhizal fungal taxa in soil in relation to soil chemical properties and hay yield. *Rhizophagus irregularis* abundance was assessed with quantitative PCR marker targeting mitochondrial large ribosomal subunit (LSU) gene whereas the other AMF taxa with markers for nuclear LSU genes. Environmental variables: Soil N and C – total nitrogen and organic carbon concentrations in the soil, respectively. Soil P and Ca – available phosphorus and mobile calcium concentrations in the soil, respectively. Yield – hay productivity per year (average of 2008-2012 seasons). First and second axes explain 62.8% and 25.1% of the mycorrhizal fungal community and environmental data relationship, respectively.

Fig. 4.
Negative relationship between volumetric soil carbon (C) concentration and hay yields (a) and a positive relationship between the volumetric soil C concentration and the length density of extraradical mycelium (ERM, log transformed) of the arbuscular mycorrhizal fungi in the soil (b). Every dot represents a separate experimental plot. Both regression lines $p < 0.01$.