Impact of Crop Diversity, Fertilization and Legumes on Soil Organic Carbon in Grasslands

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Abstract

Agricultural soil quality is decreasing as a result of the expansion and intensification of agriculture. A key indicator of soil quality is soil organic carbon (SOC) which is degraded when land is converted from natural to cultivated systems and subject to intensive agricultural land use, reflecting reduced soil fertility and productivity. The aim of this study was to examine the effect of crop diversity, fertilizing levels and legumes on SOC accumulation in 3-year-old perennial production grasslands in Alnarp, Sweden. Since the soil was assumed to have a negligible content of inorganic (pH < 7), total carbon was used as an estimate of SOC. No significant differences in SOC depending on crop diversity, nitrogen fertilization treatment or presence of legumes were identified. The absence of significant differences was potentially due to local soil heterogeneity, where SOC was significantly dependent on the positioning within the researched blocks in the field, rather than on treatments. By examining how agricultural land management affect SOC contents in arable lands, methods enhancing soil C accumulation can be developed and improved, leading to extended agricultural soil quality and food production.

Keywords
Soil organic carbon, soil quality, agricultural productivity, perennial production grassland, fertilization, crop diversity, soil carbon

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List of abbreviations

Carbon = C
Soil organic carbon = SOC
Total carbon = TC
Nitrogen = N
Species composition = SC

Definitions

Crop diversity = the difference in species and cultivar characteristics of crops within cultivated systems

Soil quality = referring to quality in terms of enhanced crop productivity in an agricultural context, not including other ecosystem functions or processes of the soil

1. Introduction

Over the past decades, intensive soil cultivation and use of chemical fertilizers, pesticides, irrigation, high-yielding crop varieties and intensified soil tillage, as a way of expanding and intensifying agriculture, has led to a prodigious rise in crop yields. This profound change, known as “one of the most significant human alterations to the global environment”, has also had critical environmental consequences and has raised concerns regarding the long-term sustainability of agricultural systems (Matson et al., 1997). Degradation of both crop diversity and soil fertility due to land use change, from natural to cultivated systems, is widely recognized, whereas long-term ecological consequences remain poorly understood (De Snoo et al., 2012; McDaniel et al., 2014; Tilman, 1999). A consequence of decreasing crop diversity and intensive land use is the decline in carbon (C) content in soil, which has degrading effects on soil quality and is predicted to lead to alarming impacts on the productivity and long-term sustainability of agroecosystems (McDaniel et al., 2014; Matson et al., 1997).

Soil quality

In an agricultural context, the term soil quality is frequently used to determine the capacity of soil to sustain high plant productivity and to provide nutrient supply, water storage or structural stability (Ashman et al., 2002; Doran et al., 2000). The utilization of external inputs, such as fertilizers and pesticides has increased, which has enabled high productivity to be sustained in agricultural systems by compensating for, and simultaneously disguising, the losses in productivity caused by degrading soil quality (Reeves, 1997; van der Werf & Petit, 2001). The assessment of soil quality is thus not only important when estimating production levels of agricultural systems, but also when determining the effect of cultivation on soil, or to evaluate long-term sustainability of agricultural practices.

Therefore, adequate and quantifiable indicators of soil quality are urgently needed. These indicators should preferably be directly linked to biological, chemical and physical soil properties and be sensitive to changes in cultivation, in order to sufficiently describe soil quality changes for reliable soil quality monitoring (Doran et al., 2000). Soil organic C (SOC) is a keystone indicator of soil quality since it fulfils these criteria and is directly connected to other important soil quality indicators such as available water capacity, microbial activity, nutrient cycling and soil structure (McLauchlan, 2006; Karlen et al., 1997; Reeves, 1997; Lal, 2006).

Soil organic carbon

SOC and soil inorganic carbon constitute the total C (TC) in soil. SOC includes microbial, animal and plant residues at all stages of decomposition (Nelson & Sommers, 1996; Post & Kwon, 2000). The quantity of stored SOC is regulated by the equilibrium between C inputs and outputs from plant production, plant root exudates and plant decomposition. It also depends on abiotic factors like pH, temperature, precipitation and soil texture (Kell, 2012; Jobbágy & Jackson, 2000; Russell et al., 2009). SOC is heterogeneous, consisting of fractions with different turnover times, and is usually divided into two main components; a larger immobile or recalcitrant fraction and a smaller labile or active fraction (McLauchlan, 2006; Oades, 1988). The immobile component is mainly affected by soil type, prior land use and climate, whereas the labile fraction is affected by soil management (Lal, 2006). SOC can be further divided into a so-called light fraction, which consists of animal and plant residues, it is not bound to any mineral matter, and varies seasonally, depending on residue input (Post & Kwon, 2000). In cultivated soils, where plant residues are returned to the soil, for instance in permanent grasslands, the accumulation rate in the light fraction can be high, regardless of decomposition rates, and is to a large extent responsible for short-term shifts in SOC storage (Post & Kwon, 2000).

When natural vegetation is converted to cultivated systems, the total content of SOC decreases with around 60-75%, depending on the region, equivalent to a total C loss of 20 to 80 tons of C/ha soil (Lal, 2004), however with no given time range. Agricultural management degrades SOC since it alters the C equilibrium by causing physical disturbance of the soil, increasing SOC decomposition rates and reducing crop residues through harvesting (Tiessen et al., 1982; McLauchlan, 2006). Furthermore, increased rates of erosion due to land cultivation exposing the soil surface severely impact SOC, ultimately degrading soil quality and agricultural potential (Lal, 2003; Montgomery, 2007; Tiessen, 1982). These changes in SOC contents mainly affect the labile fraction of SOC, rather than the immobile fraction (Lal, 2006).

The total soil C pool holds twice as much C as the atmosphere, and approximately 10% of it is stored in agricultural soils (Kell, 2012; Kong, 2005). The loss of SOC, primarily through erosion and emitted as CO_2, does not only degrade soil quality and biomass productivity, but also affects the C balance in the atmosphere (Lal, 2004). Increased rates of C sequestration in soil would not only enhance productivity and food security, it could also offset 5-15% of the global emissions from fossil-fuels (Lal, 2004).

N fertilization

Nutrient and water availability are the main limiting factors of agricultural production. In order to overcome these restraints and enable high-yield production, the utilization of fertilizers and irrigation has increased tremendously (Matson et al., 1997; LeBauer & Treseder, 2008). Nitrogen (N) fertilization is widely believed to increase crop yields and SOC sequestration through enhanced above- and belowground plant growth. N fertilization leads to increased C inputs in plants (including crops) which cannot fixate N from the atmosphere and would otherwise be restrained by limited availability of N (Russell et al., 2009; Wilson & Al-Kaisi, 2008; Halvorson et al., 2002). Additionally, increased N fertilization can, lead to an increased amounts of plant residues returned to the soil. However, in order to increase the long-term accumulation of soil C, sufficiently high levels of N are required to trigger C input rates, through below- and aboveground plant growth, and to thus counteract decomposition rates leading to C output (Russell et al., 2009). A study by Johnson et al. (2007) showed that the level of available N enhances the decomposition rates for crops, which illustrates the complexity N and C interactions in soil. As reviewed by Conant et al. (2001), over 40 studies have detected an average 2.2% annual increase of C contents in grasslands in different climates, generally benefitting from higher levels of added fertilization. Long-term effects of increased rates of added N on SOC levels has also been detected in dryland annual cropping systems (Halvorson et al., 2002).

Crop diversity

Crop diversity influences numerous ecosystem processes, such as nutrient cycling and primary production, and can reduce insect, weed and pathogen pressure (McDaniel et al., 2014). As mentioned above, reduced crop diversity in agricultural lands has raised concerns regarding negative effects on ecosystem services in agricultural lands and reduction of long-term C storage in soil (Fornara & Tilman, 2008). Biodiversity can be enhanced in an agricultural context by combining two or more crops to gain biodiversity benefits and thereby secure and enhance crop productivity (Bilalis et al., 2005).

The advantages of high crop diversity and enhanced yield stability, which are very important in areas with limited crop production, can be maintained by either sequentially growing different crops, known as crop rotation, or by growing several crops simultaneously, known as intercropping (Bilalis et al., 2005; Liebman et al., 2004). Compared to aboveground benefits, relatively little is known about diversification effect on SOC dynamics (McDaniel et al., 2014). In comparison with monocultures, up to 500% higher TC soil content (20 cm soil depth) has been found in high-diversity mixtures (< 16 crop species) in grasslands during a 12-year study, where the C increase mainly occurred after 6 years and onwards (Fornara & Tilman, 2008). Further studies corroborate increased C storage in grasslands as an effect of increased crop diversity (< 60 crop species) after 2 years in the top 5 cm of soil (Steinbeiß et al., 2008). In contrast, C content below 10 cm decreased during the same period, possibly due to land use change - but this trend reversed after 4 years.

Plant functional traits

The potential for C accumulation in soil is not only regulated by abiotic soil factors such as soil texture, mineralogy and topography or by the level of crop diversity and fertilization, but also by plant traits altering C in- and outputs (De Deyn et al., 2008). It is important not only to mention the impact of crop diversity or species richness on accumulation of C in soil (Fornara & Tilman, 2008), but also potential effects of functional diversity and presence of certain species with important traits. As reviewed by Díaz & Cabido (2001), ecosystem processes are not only affected by the number of species, but also by the range and values of plant traits. Plant complementary functions, for instance the presence of N-fixing legumes together with grasses with high N use efficiency, is one of the most important factors enabling the improvement of C accumulation through crop diversity in agriculturally degraded soils (Fornara & Tilman, 2008). Species assemblages with different functional traits within an ecosystem are also affecting the accumulation and vertical distribution of SOC (Jobbágy & Jackson, 2000).

Legumes

The symbiotic plant trait of legumes enables atmospheric N_2 to be fixated and partly made available to subsequent crops, leading to increased soil quality and SOC (van Kessel & Hartley, 2000; Kong et al., 2005; Abberton, 2010). The connection between legumes and soil improvements such as increased yields was established by Hellriegel & Wilfarth (1888). Furthermore, the increased N made available to crops can lead to reduced needs of N fertilizers. The previously mentioned study by Fornara & Tilman (2008) identified a doubled soil C accumulation at one m depth in perennial grasslands where legume species are present, disregarding the level of diversity within the field after 12 years. The study also showed that legumes increased the total root biomass (0-60 cm) as well as the accumulation of N which, according to the authors, stimulates the storage of C in the soils by increasing above- and belowground biomass production and thereby enhancing C input. As reviewed by Conant et al. (2001), the annual increase of soil C due to the presence of legumes in grasslands averaged 2% in eight grasslands studies.

Perennial crops

Most of the C content in soil derives from plants, mainly from decaying plant tissue or from roots storing and exuding C (Kuzyakov & Domanski, 2000; De Deyn et al., 2008). It has been shown that perennial plants, with greater root biomass and root length, store more C in roots compared to annual plants (Warembourg & Estelrich, 2001) leading to a higher content of SOC, which accounts for the differences between perennial grasslands and annual croplands (DuPont et al., 2014). A significant effect of higher C accumulation in perennial grasslands depending on total below-ground biomass has also been detected (Fornara & Tilman, 2008). A study by DuPont et al. (2014) also identified significant correlations between the roots of perennial plants and improved soil properties, compared to annual grasslands. This illustrates the importance of perennial plants and their roots for maintenance of soil quality and productivity, combined with reduced requirements of external inputs. Further advantages of perennial crops with deeper roots are improved access to water during dry periods and less mobile phosphorus, in addition to their contribution to improved soil stability and reduced SOC losses through soil erosion (Kell, 2012; DuPont et al., 2014; Lal, 2004).

1.1 Aim of the study

The development of agricultural systems meeting requirements both for environmental sustainability and for efficient food production is one of the great challenges of our time. One way to achieve high-yield and reduced environmental impact is to change the way agricultural systems are managed. Improved agricultural management which efficiently increase SOC accumulation and improves nutrient cycling via crop rotation or intercropping may be a route towards sustainable agriculture.

The aim of this study was to investigate how agricultural management practises affect SOC contents, as a soil quality indicator, in perennial production grasslands, and ultimately determine optimal treatments leading to high SOC accumulation rates. This was investigated by measuring whether *i)* crop diversity and/or *ii)* level of N fertilization and/or *iii)* presence of legumes affect the content of SOC. The research field consisted of perennial grasses and herbs, legumes and meadow species.

According to Olsson et al. (2009), the content of soil inorganic carbon, bound as $CaCO_3$, is negligible at soil $pH < 7$. The pH in the research field is 6.3, as measured in 2011 (Mårtensson, pers. comm.). Therefore, the content of inorganic carbon was disregarded in this study and the measured TC was assumed to be equal to SOC.

The hypotheses were:
H_1 Increased crop diversity results in higher SOC contents.
H_2 N fertilization results in higher SOC contents.
H_3 Presence of N-fixing legumes results in higher SOC contents.

2. Methods

In order to investigate whether the content of SOC, measured as TC, was influenced by crop diversity, the inflow of fertilizers and/or legumes, soil samples were taken from plots with varying crop diversity, fertilization levels and either with or without legumes. Four different perennial species compositions (SCs) were chosen, sown 3 years (2012) before the soil samples were taken:

SC1 → One grass species: *Festuca* x *Lolium* ‘Hykor’
SC2 → Mixture of three grass species: *Phalaris arundinacea* ‘Bamse’, *Festuca* x *Lolium* ‘Hykor’ and *Dactylis glomerata* ‘Donata’
SC3 → Mixture of three grass species and legumes: SC 2 and *Medicago sativa, Trifolium hybridum, Trifolium repens* and *Galega orientalis*
SC4 → Native meadow seed mixture: SC 3 and various other meadow species (see Appendix I)

The research field was divided into four larger blocks/replicates where each of the four SCs occurred three times in three separate plots (6 × 6 m) within each block (1-4). The three plots of one SC within a block received different levels of fertilization (0, 60 and 120 kg added N per hectare and year) (figure 1). Legumes were present in SC3 and SC4 plots. Each block consisted of 27 plots, arranged in 3 rows (3 × 9 plots), from which 12 were part of the experiment.

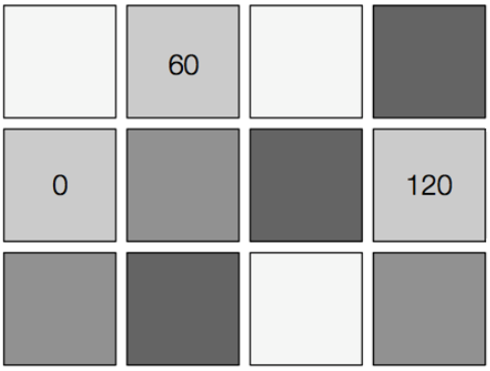

Figure 1. The figure shows a simplified version of one block with four species compositions (SCs) marked in different grey shades. Each SC occurs three times within one block with different fertilizing levels (0, 60 and 120 kg of N ha^{-1} yr^{-1}).

The soil sampling was made at 0-10 cm depth with a soil corer (2.5 cm in diameter). In order to reduce the edge effects influencing the results, the samples were taken 1 m from the borders and inwards. Within each plot, 5 subsamples were taken and combined to one pooled sample. A more detailed view of the positioning of each subsample within one plot is shown in figure 2.

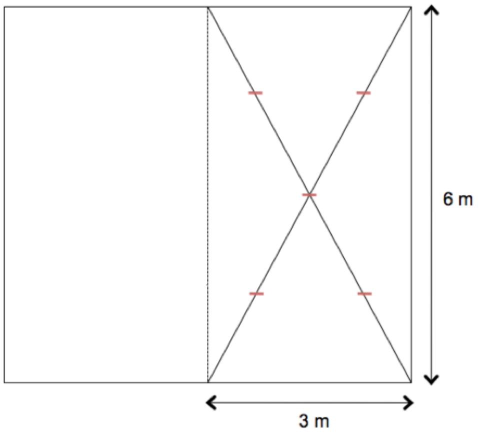

Figure 2. Position of 5 subsamples taken within one experimental plot. Subsamples were at least 1 m from the border, preventing edge effects.

Four pooled samples from a neighbouring agricultural field located around 10 m from the blocks were collected. Each group of 5 subsamples were taken alongside one block (one pooled sample from the agricultural field for each of the four blocks) and were treated as an additional row belonging to the blocks when being statistically processed. In total, 48 pooled samples and four reference samples were collected in sealed plastic bags and kept cold with freezer packs during soil sampling in order to minimize the effect of respiration. All samples were stored during 5 d, at -18 °C, until analysis.

Soil samples were sieved (2 mm) to separate most of the plant residues and stones. Screened samples were put in aluminium trays and dried in an oven at 70 °C during 70 hours. After the samples cooled off, still kept inside the oven to prevent absorption of moisture from the outside air, seven g of each dried soil sample (Sartorius TE412; ± 0.01 g) were homogenized and milled with a ball mill (Retsch MM400) for two minutes at a frequency of 30 shakes per second. Milled samples were placed in sealed plastic capsules, in order to prevent the samples from absorbing moisture. Between 49 and 51 mg from each milled samples were weighed (Mettler Toledo Excellence Plus; ± 0.01 mg) and placed in tin capsules (5 × 8 mm), carefully sealed with a pair of tweezers in order to keep air out. As external standards, 2-5 mg samples of acetanilide (N-phenylacetamide, 71.09% C) and 50 mg samples of standard soil (Thermo Scientific GNC, 2.29% C), were placed and sealed in tin capsules. Between weighing, the soil samples were dried at 70 °C for approx. 1 h to evaporate water.

Scale, tools and workspace were cleaned with ethanol (70 %) and plastic gloves were used to limit contamination of samples. Each tin capsule was sealed and weighed before placed in Flash 2000 (± 1 %) for elemental analysis to determine TC (%). For every 30 samples of soil, three samples of acetanilide were used as references to create a standard curve. Additionally, one sample of standard soil with known C concentration was used to control the validity of each standard curve. Finally, obtained results were analysed with a two-way ANOVA using GLM univariate Analysis of Variance (with Tukey's Post Hoc, $p \leq 0.05$), using SPSS Statistics 22.

A pilot study was performed one week before the actual study to determine the accuracy and reliability of the sampling method. In the pilot study, 10 subsamples from two chosen plots in two different blocks were analysed separately. A separate analysis of the subsamples from one plot gave an indication of the small-scale variation within each plot. This was an opportunity to test if the sampling method was adequately precise, and if five subsamples could be combined without compromising the result. In addition, all methods and instruments were checked during the pilot study. Subsamples with SOC contents diverging > 1% from the mean value of the plot were reanalysed in order to detect measurement errors, accordingly with the quality assurance arrangements of the analysis. The plots measured in the pilot study were chosen due to location, each situated in the middle of two blocks, without taking management treatment into account.

3. Results

3.1 Main study

3.1.1 Crop diversity, N fertilization and legumes

The SOC content, measured as TC, was not affected by the level of crop diversification or the level of N fertilization. There was no interaction between crop diversification and fertilization, as revealed by the two-way ANOVA (two-way ANOVA using GLM univariate analysis of variance, Tukey's Post Hoc, $p \leq 0.05$); SC: $p = 0.826$, N: $p = 0.372$, SC×N: $p = 0.781$. A table with all results from the statistical processing is to be found in Appendix II. The measured TC was within the range of the C content in the standard soil samples with known C concentrations. In order to quantify the variation within the blocks and within the agricultural field, the standard deviation was calculated (figure 3). A complete table with all results from the elemental analysis is shown in Appendix III.

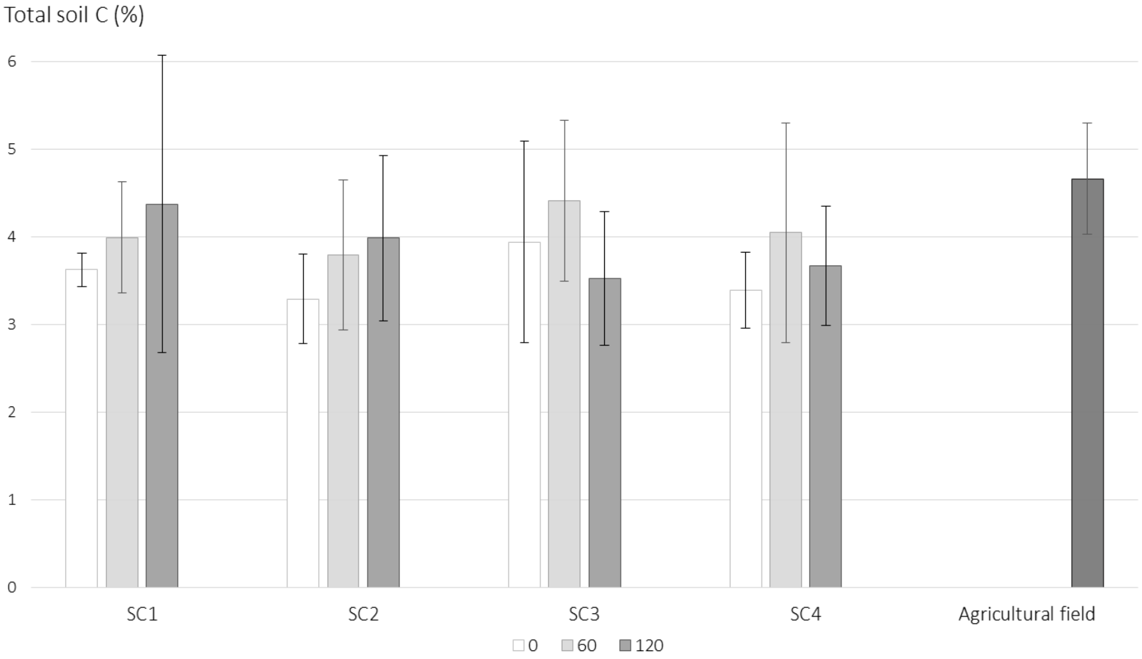

Figure 3. Total soil C (%) (TC) under four species compositions (1) one grass; (2) three grasses; (3) three grasses and legumes, and (4) grasses, legumes and native meadow seed mixture, at three fertilization levels (0, 60 and 120 kg N ha^{-1} yr^{-1}). SOC differences between the factors SC, N or SC×N are not significant ($p > 0.05$).

Possible SOC differences, measured as TC, between SC without legumes (SC1 and 2) and SC with legumes (SC3 and 4) were tested individually as well as with different fertilization levels (0, 60 and 120 kg N ha^{-1} yr^{-1}) in order to detect potential effects of SC, N or SC×N. No significant differences were detected, neither for the plots with legumes (SC: $p = 0.503$, N: $p = 0.341$, SC×N: $p = 0.742$) or the plots without (SC: $p = 0.521$, N: $p = 0.350$, SC×N: $p = 0.935$). The mean values for SC with and without legumes were 3.83% (with) and 3.82% (without) SOC with standard deviations 0.88 and 0.92. Trends of SOC depending on SC, N or SC×N without and with legumes were also tested for each block and each row, but no significant effects were identified. A table with all results from the statistical processing is to be found in Appendix II.

3.1.2 Neighbouring agricultural field

SOC contents, measured as TC, in the neighbouring agricultural field were 4.00% (block 1), 4.24% (block 2), 5.26% (block 3) and 5.16% next to block 4 (statistics were not calculated).

3.1.3 Field variation

Since no statistically supported differences were identified in the study, the field variation was examined *ad hoc* and an underlying gradient in the field was detected. The locations of peaks detected in block 3 (TC ≥ 5.0 %) were assessed (figure 4).

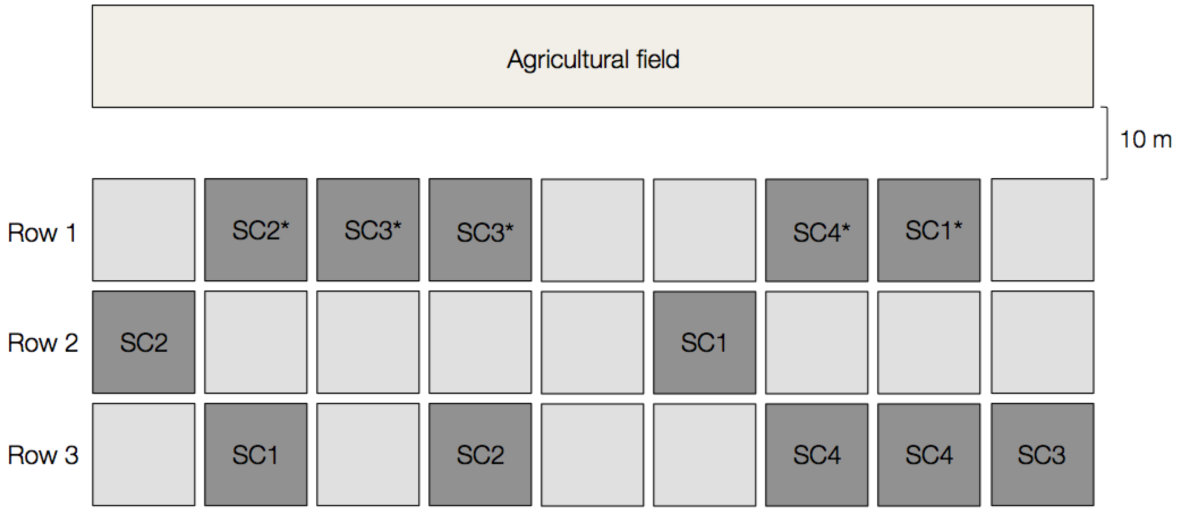

Figure 4. A schematic view of block 3 showing the position of four different species compositions (SC) within one block, and the neighbouring agricultural field (SC1: one grass species, SC2: three grass species, SC3: three grass species and legumes, SC4: mixture of meadow species, grass species and legumes). Asterisks: total C contents ≥ 5.0 %. The light-shaded plots were not included in the study.

Mean values of TC for each row within the 4 blocks as well as from the neighbouring agricultural field are shown in table 1. TC differences depending on row were found to be significantly decreasing from the upper parts to the lower parts of the blocks (p = 0.000; two-way ANOVA using GLM univariate analysis of variance). A table with all results from the statistical processing is to be found in Appendix II.

Table 1. Total soil C (%) (TC) averages in rows and a neighbouring agricultural field (AF).

TC (%)	**Block** 1	**Block** 2	**Block** 3	**Block** 4	**Mean** (AF or row)	**N samples** (per row)
AF	4.00	4.24	5.26	5.16	4.66	4
Row 1	3.28	4.29	5.69	4.01	4.32	15
Row 2	3.27	4.29	4.86	3.47	4.00	13
Row 3	3.23	3.67	3.34	3.00	3.31	20

Additionally, SOC differences depending on row with the neighbouring agricultural field included as an additional row was also significantly increasing for each row with the highest contents in the agricultural field (p = 0.000; two-way ANOVA using GLM univariate analysis of variance). The differences of SOC depending on block were tested the same way, and a significant difference is found (p = 0.000). In addition, the correlation between SOC depending on block for each of the three rows (split file: row) was tested and significant trends for row 1 (p = 0.000) and row 2 (p = 0.002) were found, whereas no significant trend was identified for row 3 (p = 0.171). SOC depending on SC, N or SC×N for each row (split file: row) was tested, but no significant trends were found. A table with all results from the statistical processing is to be found in Appendix II.

3.2 Pilot study

The TC ranged from 3.69 and 4.79% in plot A and B (table 2). According to the standard procedure, subsamples 3 (plot A) and 1 (plot B) were reanalysed because of the divergent values (> 1 %) in comparison with the other values, and were found to be higher than initially measured. TC in the standard soil samples confirmed validity of the known C concentration.

Table 2. Total soil C (%) TC measured in 10 subsamples from 2 different plots (A and B) in the pilot study. Two divergent subsamples, A3 and B1, were re-analysed and the values were averaged and the standard deviation (SD) was calculated.

Subsample (square, number)	**TC** (%) (1st measure)	**TC** (%) (2nd measure)	**Mean ± SD** (1st)	**Mean ± SD** (2nd)
A1	3.97	-		
A2	4.44	-		
A3	3.00	3.92	4.10 ± 0.68	4.29 ± 0.36
A4	4.32	-		
A5	4.79	-		
B1	3.00	3.64		
B2	4.74	-		
B3	4.34	-	4.22 ± 0.72	4.35 ± 045
B4	4.27	-		
B5	4.75	-		

4. Discussion

4.1 Effects of crop diversity, N fertilization and legumes

No significant effects of SC, N or SC×N on the content of SOC, measured as TC, were identified for all blocks together. Due to time and budget constraints, TC was chosen as an indicator of SOC since SOC measurements would have required more profound analytical methods. The absence of significant differences in SOC raises the question whether TC adequately reflects SOC, and furthermore whether the total SOC pool adequately reflects possible changes appearing within the fractions of SOC. As stated, changes in land management mainly causes differences in the labile fraction (Lal, 2006), which does motivate more precise measurements of SOC, e.g. by analysing changes in different fractions of SOC. A more elaborate analysis may have provided more appropriate data to reveal subtle changes caused by crop diversity, fertilization or legumes, and may have facilitated detection of differences due to short-term land use changes. Apart from the labile fraction, it would have been interesting to further investigate the light fraction since it responds more rapidly to seasonal fluctuations. However, assuming that SOC equals TC, the conclusion is that SOC is not significantly affected by SC, fertilization treatment or legumes in the field after 3 years.

An aspect worth considering is the number of crop species in the plots with high diversity (3 grass species, 4 legumes and meadow species) compared to < 60 crop species part of the studies determining significant increases due to crop diversity (Fornara & Tilman, 2008; Steinbeiß et al., 2009). It is possible that diversity effects could not be shown in this study is because crop diversity was not high enough to sufficiently affect SOC.

It is surprising that presence of legumes did not have any effect on SOC in the investigated field. As mentioned in the introduction, N-fixation by legumes is thought to play an important role in increasing soil fertility and productivity by enhancing plant growth through improved plant N availability, leading to greater allocation of SOC. It was therefore expected to find increased SOC contents in plots with legumes, at least when combined with certain fertilization treatments. N-fixation is generally believed to improve conditions for plant growth and thereby enhancing SOC accumulation (Russell et al., 2009; Wilson & Al-Kaisi, 2008; Halvorson et al., 2002). The corresponding patterns between SC1-SC2 (without legumes) and SC3-SC4 (with legumes) in figure 3, indicates that the plots without legumes benefit from fertilizers. Although not supported by the statistical tests, it is an interesting preliminary finding, since plots without N-fixing legumes would have been expected to be more dependent on added fertilizers. One possible reason for the absence of significant differences could be abundance of legumes in the different plots. Legumes were sown 3 years ago, but may not grow sufficiently in these plots today. Perhaps the actual effect of legumes is negligible since they are outgrown by other species. With this in mind, it would have been interesting to not only measure SOC, but to also make an inventory of plant species within the plots.

As a matter of fact, SOC could be affected by crop diversity, fertilization treatment or legumes, but other factors prevalent in the field can further contribute SOC and thereby override the investigated parameters. In this context, it is important to keep in mind that these factors can both be hiding expected differences, but also opposite differences showing a decrease of SOC due to crop diversity, fertilization levels or legumes. When no significant differences are identified, the hypotheses can neither be regarded nor disregarded.

4.2 Soil depth and time scale

Studies have found significant increases of soil C related to crop diversity at 20 cm soil depth in grasslands (Steinbeiß et al., 2008; Jobbágy & Jackson, 2000; Fornara & Tilman, 2008), while the measurements in this study were made at 10 cm. Since the root structures of perennial crops affect the vertical SOC distribution and enables C storage on greater soil depth in comparison to annual crops (Jobbágy & Jackson, 2000), it is possible that the effect of varying crop diversification, fertilization or legumes primarily appears on a greater soil depth. This could be one reason why no significant differences were identified. Therefore, it would have been interesting to take additional samples on 20-30 cm depth to determine the vertical distribution of SOC. On the other hand, since land management primarily affects labile fractions of SOC which mainly occurs in the topsoil (Lal, 2006), potential differences in SOC due to the treatments investigated in the study could also become more apparent at the surface, advocating soil sampling at shallower depth.

Temporal variation is another aspect that should be considered. The crops in the field investigated in this study were sown 3 years ago, and it is possible that the actual effects of different levels of crop diversity, fertilization treatments or legumes in the plots cannot be identified until after a longer period of time. Other factors not investigated in the study can possibly influence SOC and outweigh smaller C fluctuations occurring during the first years. The accumulation of C in soil is a slow process and studies of diversity effects must be of sufficient duration towards a better understanding of the dynamics of this process (Fornara & Tilman, 2008). The patterns seen in figure 3 are interesting, even though not supported by the statistical tests ($p > 0.05$), and could potentially turn into significant trends if the same measurements would have been done in 5-10 years from now. This is accentuated by Fornara & Tilman (2008), where the increase of C contents in grasslands due to crop diversity was first detected after 6 years and onwards. These findings, as well as the study by Steinbeiß et al. (2001), identifying a C increase in the top 5 cm of soil but a decrease at greater soil depth during the first 2 years, illustrates the importance of sufficient numbers of measurements of C when conducting this type of study, covering both temporal and vertical changes of the C pool.

4.3 Field variation

Since the neighbouring agricultural field is managed consistently, containing the same crop species throughout, similar contents of SOC were expected within the field. However, the measurements differed between 4 and 5 %. This indicates that SOC variation over the field is not only dependent of current agricultural practice. Possible reasons behind these differences in the field could be local abiotic factors such as varying sand or clay content, other geological properties of the soil or effects from previous agricultural practices, which still affect the area.

Samples taken in block 3 with prominently high contents of SOC (≥ 5.0 %) were all located at the edge of the block, adjacent to the agricultural field. The mutual positioning and corresponding SOC contents of the plots indicates a geographic factor impacting the plots positioned in one part of the block rather than the plots in other parts of the block. This positional effect between the rows is confirmed by the significant differences in SOC depending on row, and could possibly outweigh crop diversity, fertilization or legumes effects. One explanation for this geographical variation, where SOC decreases with distance from the adjoining agricultural field, could be edge effects originating from the management of the agricultural field. The agricultural field is treated with fertilizers and the topography of the area could lead to fertilization and nutrient leakage from the agricultural field affecting the upper rows of the blocks rather than the lower ones. Higher contents of available nutrients through fertilization are expected to increase SOC contents due to a stimulating effect on biomass production, corroborating the discovered differences.

Sand gatherings on top of the milled samples and soil gatherings at the bottom were acknowledged and experimental errors due to inhomogeneous samples must be taken into account. This could, for instance, cause the variation between the subsamples analysed in the pilot study. The relatively large variation between the SOC content in subsamples, taken within the same plot during the pilot study, must be considered when analysing the results of the main study. Mixing varying subsamples instead of analysing them separately may not be correct since important results could be lost. At the same time, the pooled samples should be considered to be mean values; variation of SOC in the same plot must be averaged in order to gain an overview of the whole field.

4.4 For future studies

The accumulation process of SOC is, as discussed, impacted by several factors and not necessarily temporarily or vertically linear. This is challenging when conducting studies investigating agricultural management effects on SOC. In order to adequately identify SOC changes due to crop diversity, fertilization and legumes, the study first of all needs to be of *sufficient duration* to capture year-to-year changes. Although this particular study did not identify significant effects in the test field, it is important to repeat measurements in order assess possible temporal changes in SOC over several years. Secondly, completing measurements covering *vertical SOC changes* in the field would be interesting to do in future studies, since it enables a better understanding of the vertical distribution of SOC content and changes, but this was not within the time frame of this study. The mentioned studies identifying diversity, fertilization and legume effects at various soil depths emphasize the difficulty of sufficiently capturing SOC changes by just measuring at one soil depth, and motivates a more detailed vertical profiling of SOC. Thirdly, the detected variation in the field illustrates the importance of assessing the *level of soil variations* in experimental fields when conducting similar studies, or when planning the design of experimental fields. For instance, the plots within the blocks could have been arranged accordingly to a ‘latin square’ (Winer, 1962), where each treatment occurs once in each row and column, instead of just within every block. This would allow for variation to be disregarded when statistically processing the data and analysing the results. Another option for future studies would be to measure the variation beforehand by taking more reference samples alongside with the blocks.

5. Conclusion

The experimental data collected in this study did not allow verifying or falsifying the hypotheses. A positional variation of SOC between samples possibly prevented any positive or negative effects of crop diversity, fertilization and legumes on SOC to be confirmed, leading to the hypotheses neither being regarded nor disregarded. Although the main questions have not been answered, this study impressively illustrates the difficulties and challenges of determining the impact of agricultural management on SOC. Furthermore, this study should be seen as a first attempt, and will hopefully provide a bases for more, detailed, studies in the future.

5.1 Outlook

Over the past decades, agricultural intensification has had severe environmental consequences and degrading effects on soil quality and fertility, and is a serious threat for the long-term productivity of agroecosystems. The anthropogenic inputs are continuously increasing, leading to tremendously high productivity levels, which at the same time disguise the losses of environmental quality caused by intense land use. Whether the agricultural systems will remain sustainable in terms of production capacity on a long-term basis raises concerns. This illustrates the need for deeper understanding of the consequences of agricultural soil management. Understanding how and to what extent agricultural management, including crop diversity, fertilization treatments and legumes, influence accumulation of SOC, enables a development of agricultural methods that reduce degrading soil effects and this is crucial for enhancing agricultural sustainability.

The storage of C in soil should be seen in a wider perspective, not only addressing its importance for ecosystem processes, soil quality or soil productivity. Soil management provides an opportunity to sequester anthropogenic C and to stabilize our climate. The global CO_2 emissions from combustion of fossil fuels are widely recognized, whereas the soil emissions from land use change or soil cultivation remain uncertain. This gap of knowledge emphasizes the need of a greater understanding of the C pathways in terrestrial systems, which enables more precise determinations of sources and sinks. From a greenhouse gas perspective, it is highly relevant to understand how and to what extent changes in agricultural management influences the outputs and inputs of C in soil. This will enable a development of agricultural management causing reduced soil C losses to the atmosphere. This is important, not only for enhancing agricultural productivity and sustainability, but also for our ability to predict the magnitude of climate change.

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Appendix

Appendix I - Species included in SC4 (native meadow seed mixture)

Plant species	**Latin name**	**Variety**	**Share** (%)	**RSR*** (kg/ha)	**Seeding** (kg/ha)
Reed canary grass	*Phalaris arundinacea*	BAMSE	2	15	0,3
Tall fescue	*Festololium*	HYKOR	2	25	0,5
Cocksfoot grass	*Dactylis glomerata*	DONATA	2	20	0,4
Timothy	*Phleum pratense*	RAGNAR	2	12	0,24
Lucerne (Alfalfa)	*Medicago sativa*	CRENO	2	15	0,3
Alsike clover	*Trifolium hybridum*	FRIDA	2	10	0,2
White clover	*Trifolium repens*	HEBE	2	5	0,1
Eastern galega	*Galega orientalis*	GALE	2	20	0,4
Yellow sweetclover	*Melilotus officialis*	-	2	20	0,4
Red clover	*Trifolium pratense*	TITUS	2	12	0,24
Hairy vetch	*Vicia villosa*	-	2	40	0,8
Black medic	*Medicago lupulina*	VIRGO PAJBJERG	2	12	0,24
'Pratensis diversity mix'		-	76	30	22,8

* RSR = recommended seeding rate

Appendix II – Complete results from statistical processing of data in SPSS

Total soil C depending on species composition (SC), fertilization treatment (N) and on both factors (SC×N) (two-way ANOVA using GLM univariate analysis of variance).

Sources	**df**	**SS**	**MS**	**F-value**	**p-value** (sig.)
SC	3	0.795	0.265	0.299	0.826
N	2	1.805	0.903	1.018	0.372
SC×N	6	2.822	0.470	0.530	0.781
Residual	36	31.926	0.887		

Total soil C in plots either with or without legumes depending on species composition (SC), fertilization treatment (N) and on both factors (SC×N) (two-way ANOVA using GLM univariate analysis of variance).

Legumes	**Sources**	**df**	**SS**	**MS**	**F-value**	**p-value** (sig.)
With legumes	SC	1	0.390	0.390	0.467	0.503
	N	2	1.905	0.953	1.142	0.341
	SC×N	2	0.506	0.253	0.303	0.742
	Residual	18	15.020	0.834		
Without legumes	SC	1	0.403	0.403	0.43	0.521
	N	2	2.090	1.045	1.112	0.350
	SC×N	2	0.126	0.063	0.067	0.935
	Residual	18	16.906	0.939		

Total soil C depending on row, both including and excluding data from the agricultural field (AF) (two-way ANOVA using GLM univariate analysis of variance).

	df	**SS**	**MS**	**F-value**	**p-value** (sig.)
Without AF	2	11.977	5.988	11.084	0.001
Residual	20	10.805	0.54		
With AF	2	11.977	5.988	11.461	0.000
Residual	23	12.017	0.522		

Total soil C depending species composition (SC), fertilization treatment (N) and on both factors (SC×N) for each of the three rows (two-way ANOVA using GLM univariate analysis of variance).

Row no.	**Sources**	**df**	**SS**	**MS**	**F-value**	**p-value** (sig.)
1	SC	3	0.676	0.225	0.222	0.879
	N	2	2.165	1.082	1.082	0.395
	SC×N	2	2.945	1.473	1.473	0.298
	Residual	7	7.117	1.017		
2	SC	3	1.784	0.595	0.986	0.484
	N	2	1.528	0.764	1.267	0.375
	SC×N	3	0.835	0.278	0.462	0.724
	Residual	4	2.412	0.603		
3	SC	3	1.434	0.478	3.372	0.068
	N	2	0.047	0.023	0.164	0.851
	SC×N	5	2.176	0.435	3.07	0.069
	Residual	9	1.276	0.142		

Appendix III – Complete table of results from the elemental analysis for all plots and the neighbouring agricultural field.

Block.SC.N	Weight (mg)	TC (%)	Block.SC.N	Weight (mg)	TC (%)
B1:SC1:0	50.453	3.534269094	B3:SC1:120	50.295	6.662854820
B1:SC1:60	49.899	3.277437449	B3:SC2:0	50.833	3.650492668
B1:SC1:120	50.429	3.251415730	B3:SC2:60	50.542	4.830135822
B1:SC2:0	50.531	3.209416866	B3:SC2:120	50.529	5.173720837
B1:SC2:60	50.751	2.616544962	B3:SC3:0	50.123	5.666152954
B1:SC2:120	50.758	3.638872147	B3:SC3:60	50.796	6.019147873
B1:SC3:0	50.701	3.310989380	B3:SC3:120	50.522	3.040561676
B1:SC3:60	49.439	3.018060207	B3:SC4:0	50.216	3.186139822
B1:SC3:120	50.548	3.436880112	B3:SC4:60	50.704	4.947741508
B1:SC4:0	50.88	3.337858915	B3:SC4:120	50.202	3.344860554
B1:SC4:60	50.948	2.722234726	B4:SC1:0	50.392	3.606578588
B1:SC4:120	50.593	3.632591963	B4:SC1:60	50.886	3.040762901
B2:SC1:0	49.800	3.903963327	B4:SC1:120	50.094	2.941349268
B2:SC1:60	49.522	4.211947441	B4:SC2:0	49.895	2.606085300
B2:SC1:120	50.971	4.652363300	B4:SC2:60	49.460	3.709577322
B2:SC2:0	49.357	3.708436966	B4:SC2:120	50.525	4.206120014
B2:SC2:60	50.427	4.030059338	B4:SC3:0	49.340	3.334557533
B2:SC2:120	49.844	2.936388493	B4:SC3:60	50.294	4.025633335
B2:SC3:0	49.704	3.465946436	B4:SC3:120	50.553	3.009884596
B2:SC3:60	49.490	4.575687885	B4:SC4:0	50.698	3.046500444
B2:SC3:120	49.579	4.632330418	B4:SC4:60	50.385	4.300461769
B2:SC4:0	49.942	4.018646240	B4:SC4:120	50.606	3.072501183
B2:SC4:60	49.981	4.333310223	AF* 1	50.239	4.003705978
B2:SC4:120	49.834	4.635048389	AF* 2	50.029	4.239787579
B3:SC1:0	50.243	3.461656570	AF* 3	50.465	5.258509159
B3:SC1:60	49.916	4.882868767	AF* 4	50.065	5.156890869

* AF = agricultural field