AGRICULTURAL AND FOOD SCIENCE

H. Li et al. (2019) 28: 176–189

Advantages of grass-legume mixture for improvement of crop growth and reducing potential nitrogen loss in a boreal climate

Honghong Li[1], Petri Penttinen[1,2], Hannu Mikkola[3] and Kristina Lindström[1,4]

[1]Ecosystems and Environment Research Programme, University of Helsinki, PO Box 65, FI-00014 Helsinki, Finland

[2]Zhejiang Provincial Key Laboratory of Carbon Cycling in Forest Ecosystems and Carbon Sequestration,
Zhejiang A&F University, Lin'an 311300, China

[3]Department of Agricultural Sciences, University of Helsinki, PO Box 28, FI-00014 Helsinki, Finland

[4]Helsinki Institute of Sustainability Science (HELSUS), University of Helsinki, Finland
e-mail: honghong.li@helsinki.fi

A three-year field experiment was established to assess intercropping for sustainable forage production in Finland. In split-plot design, fertilizer treatment with unfertilized control, organic fertilizer, and synthetic fertilizer was the main plot factor, and crop treatment with fallow, red clover (*Trifolium pratense*), timothy (*Phleum pratense*), and a mixture of red clover and timothy was the sub-plot factor. Dry matter, carbon and nitrogen yields in mixture plots were highest with relatively high N% and the optimum C:N ratio ($p < 0.05$). Fertilization increased annual yields of mixture and timothy but not that of red clover. Soil NO_3-N changed over time ($p < 0.05$) and was highest in fallow, followed by red clover, mixture, and timothy ($p < 0.05$), and the decrease during late growing season was smaller in the mixture and timothy plots. At the end of the experiment, soil C/NO_3-N ratio was higher in timothy and mixture while lower in red clover and fallow plots ($p < 0.05$), and the relationship between soil DNA and NO_3-N content may indicate that the potential nitrogen loss was lower in mixture and timothy than that in fallow and red clover plots.

Key words: sustainable agriculture, fertilizer-crop interaction, soil C/NO3-N ratio, soil DNA content

Introduction

Agriculture is the world's single largest driver of global environmental change (Rockström et al. 2017). The biogeochemical flows of nitrogen (N) challenge the resilience of the planet, especially in regions where industrial and intentional biological fixation of N is high (Steffen et al. 2015). Sustainable or ecological intensification of agriculture (Tittonell 2014, Mahon et al. 2017) is offered as one solution to this problem. On a field or farm scale it can mean production of more food or feed while reducing the potential negative environmental impacts and at the same time increasing contributions from natural capital and avoiding the unnecessary fertilizer inputs (Pretty et al. 2011).

Replacement of synthetic N fertilizer with biological N fixation (BNF) offers one important natural capital mean for achieving more sustainable food and feed production. Contrary to the industrial production of synthetic fertilizers, BNF relies on solar energy provided by the legume host to the bacterial nitrogenase, which reduces atmospheric N_2 to ammonia to be used by the plant (Franche et al. 2009). The symbiosis between legumes and rhizobia is an intricate system, the regulation of which is still only vaguely known. However, BNF seems usually to be sensitive to soil N. According to Adams et al. (2018), BNF was stimulated if the soil around the legume rhizosphere lacked N, while it was inhibited if the soil N was in surplus.

Timothy (*Phleum pratense*) is the main cattle fodder crop grown in Finland due to its high palatability and winter hardiness. The main legume fodder, red clover (*Trifolium pratense*) is normally grown as a mixture with other fodder crops in farmland, and BNF in Finnish forage legumes is well adapted to boreal conditions (Lindström 1984). Plant species or community composition play an important role for the N cycle and subsequent potential N loss in an intensified fertilization ecosystem (Scherer-Lorenzen et al. 2003, Abalos et al. 2018). For example, in a timothy-red clover mixture, the grass relies on soil N, whereas the legume is using biologically fixed N, and the uptake of rhizosphere N by the grass stimulates BNF of the legume (Nyfeler et al. 2011). After harvest, N rich root material is left in the soil and mineralized by soil microbes, thus releasing N to be used by the grass, especially in a perennial cropping system. Consequently, crop growth and dynamic change of the soil N pool (NH_4-N and NO_3-N) are closely related to fertilization managements and the choice of crop treatments. For example, the yield of grass-legume intercrop was greater than that of sole crop (Suter et al. 2015, Salehi et al. 2018); less soil N leached from mixture treatment than from monoculture treatment (Loiseau et al. 2001) but annual N_2O emissions were lower from grass sward than from grass-clover sward (Virkajärvi et al. 2010).

Manuscript received October 2018

Yokoyama et al. (2017) found that there was a strong positive relationship between soil DNA content and soil microbial biomass N, which indicates that DNA content could be treated as a proxy of microbial biomass. In addition, Ryden (1983) found that when soil NO_3-N content was higher than 5 µg N g^{-1}, denitrification responded rapidly if soil moisture was higher than 20% (w/w). Putz et al. (2018) found that a higher C/NO_3-N ratio favored dissimilatory nitrate reduction to ammonium (DNRA) over denitrification, therefore resulted in lower N_2O emission. According to the four general biological denitrification requirements by Philippot et al. (2007): 1) the presence of bacteria with denitrification capacity; 2) suitable electron donors such as organic carbon; 3) anaerobic conditions or restricted O_2 availability; and 4) presence of N-oxides (NO_3-N, NO_2-N, NO, or N_2O) as electron acceptors, we propose that exploring the relationship between soil DNA content and NO_3-N content could indicate potential N loss when the situation is prone to denitrification.

Our aim was to assess intercropping for improving forage production in a boreal climate and minimizing the potential N loss. A field experiment was established with pure timothy grass, pure red clover and their mixture fertilized with organic and synthetic N fertilizers. Crop growth, soil N pool dynamic change (NO_3-N and NH_4-N contents), total carbon, total N, C/NO_3-N ratio, pH, EC, moisture, and DNA content were monitored to reveal underlying soil mechanisms. We hypothesized that the mixture would be the most sustainable system in terms of forage crop growth and reducing potential N loss.

Materials and methods

Field management

The three-year field experiment was established in May 2013 at Viikki Experimental Farm, University of Helsinki, Finland (60˚13´42´´N 25˚ 2´34´´E). The pH in the clay loam was 6.41, electrical conductivity (EC) was 52.32 µS cm^{-1}, total C content was 25.32 g kg^{-1}, total N content was 1.68 g kg^{-1}, NO_3-N content was 5.42 mg kg^{-1}, and NH_4-N content was 4.51 mg kg^{-1}). The split-plot design had four 18 m × 8 m blocks, twelve 6 m × 8 m main plots, and forty-eight 6 m × 2 m sub-plots with no buffer spaces in-between. Fertilizer treatment with unfertilized control, organic fertilizer, and synthetic fertilizer was the main plot factor (Table 1), and the crop treatment with fallow, red clover, timothy, and a mixture of red clover and timothy was the sub-plot factor. After the field was harrowed, cow manure (1.2 kg t^{-1} soluble N, 0.96 kg t^{-1} P, 4.1 kg t^{-1} K) was applied to organic fertilizer plots at 40 t ha^{-1} and garden PK fertilizer (3.2% N (1.6% NO_3-N + 1.6% NH_4-N), 5% P, 20% K, Yara, Finland) was applied to synthetic fertilizer plots at 800 kg ha^{-1}. Red clover cv. Bjursele, timothy cv. Tuure, and a mixture with 25% red clover and 75% timothy were sown at the rate of 5 kg ha^{-1}, 9.3 kg ha^{-1}, and 8.7 kg ha^{-1}, respectively. Barley (*Hordeum vulgare*) cv. NFC Tipple was sown as a nurse crop at the rate of 196 kg ha^{-1} and harvested in August 2013.

In the following years, organic and synthetic fertilizers were applied as described in Table 1. Cow urine served as the organic fertilizer in 2014. In 2015, since the soluble N level of cow urine was too low to meet the level of 75 kg N ha^{-1} in a reasonable volume, we used a slurry made of cow urine and cow manure.

Table 1. Nitrogen fertilization dates and rates, and crop harvest dates

Date of fertilizer applied	Fertilizer application rate (kg N ha^{-1})		Date of crop harvested
	Organic (Soluble N)	Synthetic ($Ca[NO_3]_2$)	
07 May 2014	35[a]	40	27 June 2014
08 July 2014	20[a]	20	24 September 2014
01 June 2015	75[b]	75	10 July 2015
16 July 2015	75[b]	75	14 September 2015

a = urine; b = slurry

The average monthly precipitation and temperature (Fig. 1) were collected from Finnish Meteorological Institute (http://en.ilmatieteenlaitos.fi/statistics-from-1961-onwards).

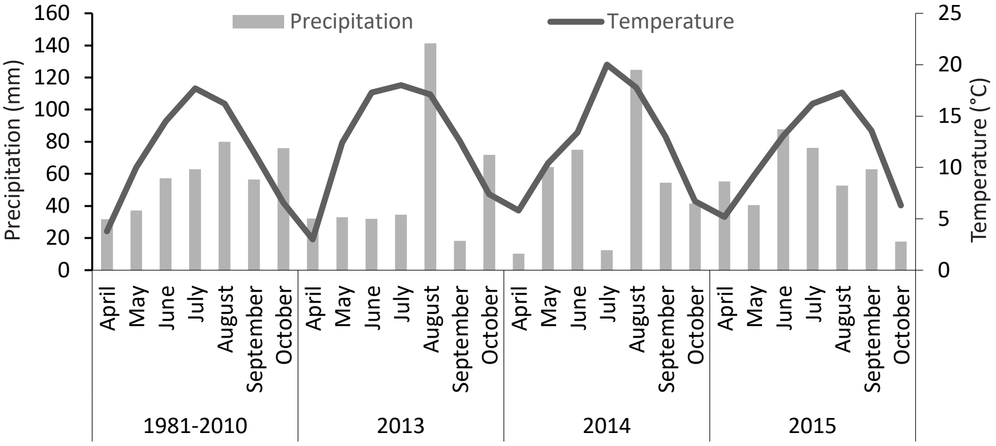

Fig. 1. Precipitation and temperature in the experiment site over the 1981–2010 and during the growing seasons 2013, 2014 and 2015

Crop harvesting and analysis

Crops were harvested two times per growing season from the middle of the plot with a 1.5 m wide combine harvester. After fresh weight (FW) had been measured on site, all the aboveground biomass was harvested and removed from the field. Subsamples were dried at 105 ˚C to determine water content (WC). The dry matter yield (DMY) was calculated as FW × (100%–WC) / (1.5 m × harvested length). Crop C% and N% were measured using Dumas combustion method with VarioMax CN analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) to determine C:N ratio, C yield (DMY × C%), and N yield (DMY × N%).

Soil sampling and physico-chemical analyses

From each plot, 16 subsamples taken with an Ø 2 cm auger from top soil (0–20 cm) were mixed to make a composite sample. Samples were passed through a 5 mm sieve to remove roots and stones, and stored at –20 °C. Altogether 348 soil samples were collected during 2013–2015. NO_3-N and NH_4-N were extracted from 20 g soil with 50 ml 2M KCl. NO_3-N and NH_4-N concentrations in the extracts were measured with Lachat QuickChem 8000 (Lachat Instruments, Milwaukee, USA) according to the manufacturer’s instructions. Soil pH and EC were measured in a 1:2.5 (w/w) soil-water mixture. Soil moisture was determined by drying at 105 °C until constant weight. To measure total C and N, soil was dried at 50 °C overnight, ground and analyzed by TRUSPEC elemental determinator (LECO, USA).

Soil DNA isolation and quantification

DNA was isolated from 0.25 g fresh soil with the Power Soil DNA Isolation Kit (MoBio, Carlsbad, USA) according to the manufacturer’s instructions. The quality of DNA was checked with electrophoresis in 1% agarose gel. DNA was quantified using PicoGreen dsDNA Quantification Reagent Kit (Molecular Probes, USA). Soil DNA content was calculated based on soil dry weight (Mikkonen et al. 2011).

Statistical analysis

The effects of fertilizer treatment, crop treatment, and fertilizer-crop interaction on crop growth and soil properties at separate sampling time points were analyzed by Univariate Analysis of Variance (UV-ANOVA), using the main-plot factor (fertilizer treatment) and sub-plot factor (crop treatment) as fixed factors, and block as the random factor. The variance of block and fertilizer was tested against the main plot variance (block × fertilizer), while the variances of crop treatment and fertilizer-crop interaction were tested against the subplot variance (Yan et al. 2015). To be approximately normal, the soil NO_3-N and NH_4-N were log transformed prior to analysis. Tukey HSD test was used as the post-hoc test. Differences were taken as statistically significant at $p < 0.05$. The relationship between soil DNA content and NO_3-N content were explored by using linear regression modelling. In the linear regression, soil NO_3-N content was the dependent variable, soil DNA content and crop factor were independent variables. In the crop factor, the fallow level was the reference level for the other three crop treatment levels. AIC (Akaike information criterion) value of the model was used to select the model and the model validations (homogeneity, influential values, independence, normality) were checked. Data were analyzed and visualized using packages “lattice” (Sarkar 2008), “ggplot2” (Wickham 2009), “ggpubr” (Kassambara 2017), “plyr” (Wickham 2011) and R scripts “HighstatLibV10.R” (Zuur et al. 2009) in RStudio Version 1.1.383 (RStudio Team 2016) based on R Version 3.5.0 (R Core Team 2018).

To test the within-subjects and between-subjects effects of repeated factor (sampling time points) on crop growth and soil properties (sphericity assumed), we used repeated measures analysis with sampling time as repeated factor and Bonferroni multiple comparisons as the post-hoc test (Yan et al. 2015). The repeated measures analysis was done in SPSS Statistics 24 (IBM, Armonk, NY, USA).

Results

The effects of fertilizer and crop treatments on crop growth

The dry matter, N and C yields of the mixture were higher than those of red clover and timothy in both 2014 and 2015 ($p < 0.05$) (Table 2, Table A.1). In 2014, when the fertilization rate was 55 kg N ha^{-1} year^{-1} in organic fertilizer plots and 60 kg N ha^{-1} year^{-1} in synthetic fertilizer plots, the annual yields of red clover were higher than that of timothy ($p < 0.05$), and there was no significant difference between fertilizer treatments (Table 2, Fig. 2a).

Table 2. Crop growth under different fertilizer and crop treatments

Treatment		Crop dry matter yield (Mg ha^{-1})				Crop N%			
		2014		2015		2014		2015	
		28 June	24 Sep.	10 July	14 Sep.	28 June	24 Sep.	10 July	14 Sep.
Tests of Between-Subjects effects									
Treatment	df	Significance level							
FT	2	ns	ns	**	***	*	**	ns	ns
CT	2	***	***	***	***	***	***	ns	ns
FT× CT	4	ns	ns	**	***	*	ns	ns	ns
Tests of Within-Subjects effects (sphericity assumed)									
Source	df	Significance level							
Time	3	***				***			
Time×FT	6	***				ns			
Time×CT	6	***				**			
Time ×FT×CT	12	ns				ns			
Treatment		Crop N Yield (kg ha^{-1})				Crop C:N Ratio			
		2014		2015		2014		2015	
		28 June	24 Sep.	10 July	14 Sep.	28 June	24 Sep.	10 July	14 Sep.
Tests of Between-Subjects effects									
Treatment	df	Significance level							
FT	2	ns	ns	ns	**	ns	*	ns	ns
CT	2	***	***	***	*	***	***	ns	*
FT×CT	4	*	ns	ns	*	ns	ns	ns	ns
Tests of Within-Subjects effects (sphericity assumed)									
Source	df	Significance level							
Time	3	***				***			
Time×FT	6	***				**			
Time×CT	6	***				**			
Time ×FT×CT	12	ns				ns			

FT = fertilizer treatment; CT = crop treatment; Time = sampling time points; df = degrees of freedom; ns = not significant; * when $p < 0.05$, ** when $p < 0.01$, *** when $p < 0.001$

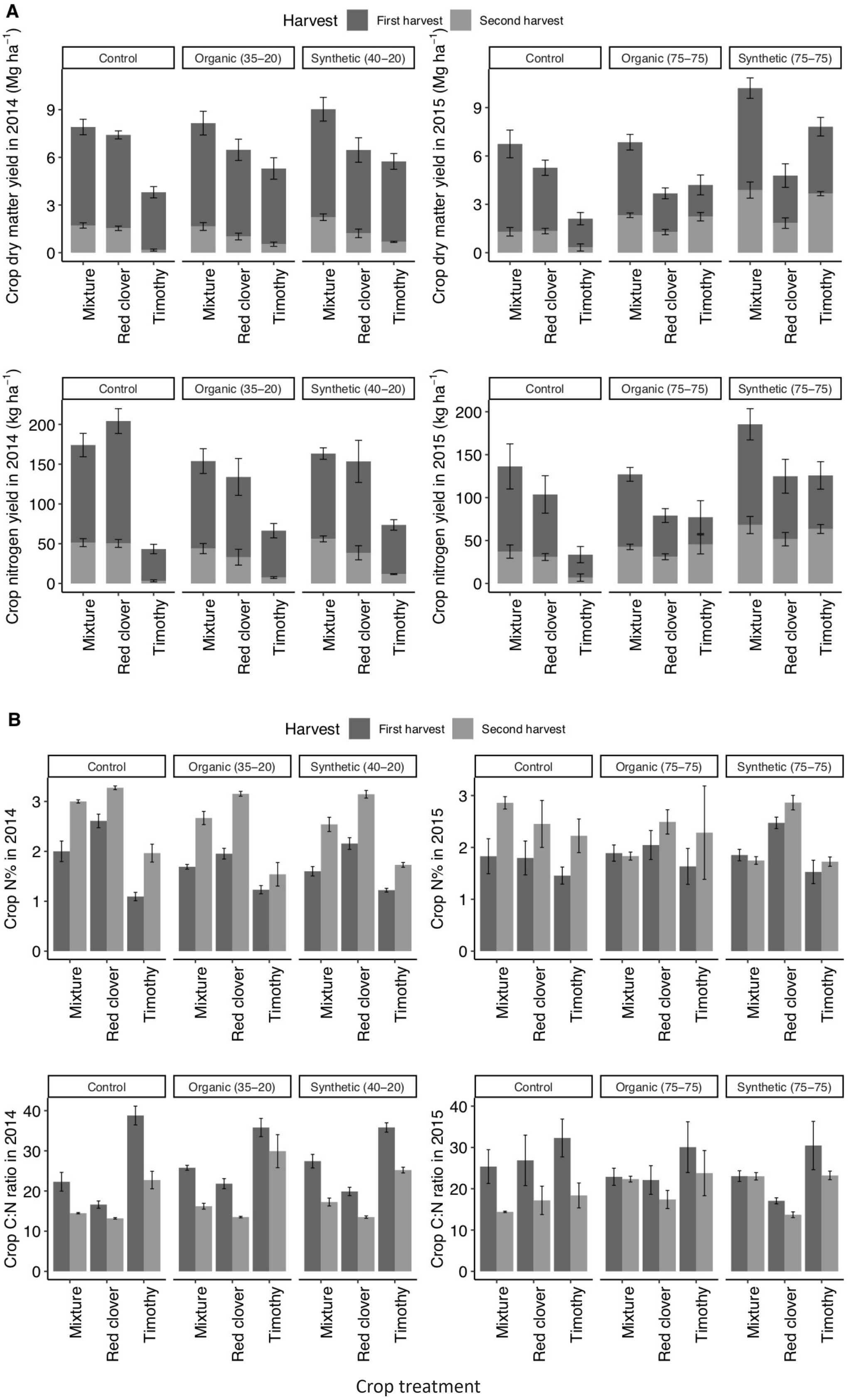

Fig. 2. Crop dry matter and nitrogen yields (A) and crop N% and C:N ratio (B) under fertilizer-crop interaction. The fertilizer application rates (kg N ha^{-1}) are indicated in brackets, the error bars represent the standard error of mean.

In 2015, when the fertilization rate was 150 kg N ha^{-1} year^{-1}, the dry matter yield was different between fertilizer and fertilizer × crop interaction ($p < 0.05$) (Table 2). In the unfertilized control, the yield of red clover was higher than that of timothy, whereas with synthetic fertilizer, the yield of timothy was higher than that of red clover (Fig. 2a). Compared to the unfertilized control, fertilization increased the annual yield of mixture and timothy but not that of red clover (Fig. 2a). The N% of red clover was highest, and the N% was higher in the mixture than in timothy in 2014 ($p < 0.05$) but not in 2015 ($p > 0.05$) (Table 2). Compared to the unfertilized control, the N% and N yield of fertilized mixture and red clover were lower while that of fertilized timothy was higher in the first harvest of 2014 (Fig. 2). The C:N ratio of mixture was approximately 25:1 in the first harvest both in 2014 and 2015 (Fig. 2b). Compared to the first harvest, the crop yield and C:N ratio was lower, while the N% was higher in the second harvest (Fig. 2).

The effects of fertilizer and crop treatments on soil properties

Soil NO_3-N and NH_4-N content

The soil N content (NO_3-N and NH_4-N) were measured at seven time points which were classified into three periods according to fertilizer and crop managements. The soil NO_3-N and NH_4-N contents changed over time and were different between fertilizer treatment at the beginning of the periods ($p < 0.05$) (Table 3) and were generally highest in the synthetic fertilizer plots (Fig. 3). The NO_3-N content was highest in fallow ($p < 0.05$), followed by red clover, mixture, and timothy (Table 3, Table A.2). The soil NH_4-N differed between crop treatments at several time points, and was generally lowest in timothy plots ($p < 0.05$) (Table 3, Table A.2). Fertilizer-crop interaction was significant regarding soil N content at several time points and the effects from crop, fertilizer, and fertilizer-crop interaction changed over time ($p < 0.05$) (Table 3). Soil N content decreased during the second and third periods (Fig. 3b, 3c). Compared to fallow and red clover plots, the decrease was smaller in mixture and timothy plots (Fig. 3b, 3c).

Table 3. Soil NO_3-N and NH_4-N contents under fertilizer and crop treatment

		First period(A)			Second period(B)		Third period(C)	
Soil NO_3-N (mg kg^{-1})		2014			2015		2015	
		23 May	06 June	28 June	16 Aug.	22 Sep.	08 July	24 Sep.
Tests of Between-Subjects effects								
Treatment	df	Significance level						
FT	2	*	*	ns	**	ns	***	**
CT	3	***	***	***	***	***	***	***
FT× CT	6	ns	ns	ns	ns	ns	*	*
Tests of Within-Subjects effects (sphericity assumed)								
Source	df	Significance level						
Time	6	***						
Time × FT	12	***						
Time × CT	18	***						
Time × FT × CT	36	***						
		First period(A)			Second period(B)		Third period(C)	
Soil NH_4-N (mg kg^{-1})		2014			2014		2015	
		23 May	06 June	28 June	16 Aug.	22 Sep.	08 July	24 Sep.
Tests of Between-Subjects effects								
Treatment	df	Significance level						
FT	2	ns	ns	ns	***	ns	**	ns
CT	3	ns	***	***	**	ns	**	ns
FT× CT	6	ns	*	ns	ns	ns	*	ns
Tests of Within-Subjects effects (sphericity assumed)								
Source	df	Significance level						
Time	6	***						
Time × FT	12	***						
Time × CT	18	***						
Time × FT × CT	36	*						

FT = fertilizer treatment; CT = crop treatment; Time = sampling time points; df = degrees of freedom; ns = not significant; * when $p < 0.05$, ** when $p < 0.01$, *** when $p < 0.001$

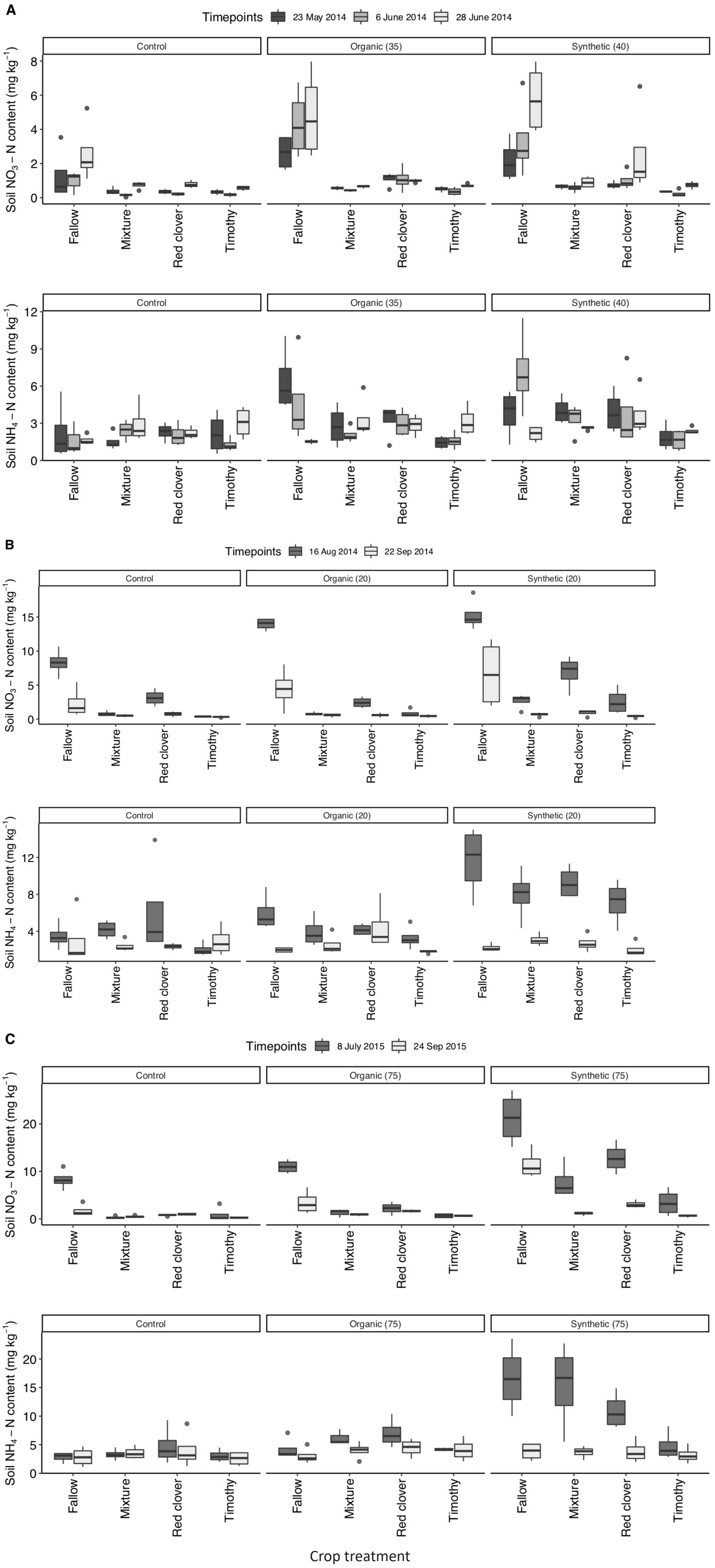

Fig. 3. Box plots (n=4) of soil nitrogen pool dynamic change during the first period (A), second period (B), and third period (C) regarding fertilizer-crop interaction. The fertilizer application rates (kg N ha^{-1}) were indicated in brackets. The individual points are the data points outside of 1.5 times of the inter-quantile range.

Soil total C, total N, C:N ratio and C:NO$_3$-N ratio

The soil total C and N contents were generally lower in the control plots than in the fertilized plots, and generally higher in the red clover plots than in the other crop treatments (Table 4). The soil C/NO$_3$-N ratio was higher in timothy and mixture plots than in other crop treatments ($p < 0.05$) (Table 4), and generally followed the order fallow < red clover < mixture < timothy within each fertilizer treatment (Fig. 4).

Table 4. Soil total C, total N, C/N ratio and C/NO$_3$-N ratio under different fertilizer and crop treatments

		Total C (% dw)		Total N (% dw)		C/N ratio		C/NO$_3$-N ratio (×10^4)	
		28 June 2014	24 Sep. 2015	28 June 2014	24 Sep. 2015	28 June 2014	24 Sep 2015	28 June 2014	24 Sep. 2015
Control		2.268a	2.433a	0.165a	0.167a	13.83a	14.64a	2.89a	4.82a
Organic		2.602a	2.762a	0.188a	0.193a	13.91a	14.33a	2.76a	2.67a
Synthetic		2.644a	2.698a	0.191a	0.189a	13.92a	14.29a	2.31a	2.33a
SEM		0.042	0.057	0.004	0.004	0.13	0.16	0.20	0.49
Fallow		2.443a	2.602a	0.178a	0.179a	13.84a	14.63a	0.73c	1.05c
Mixture		2.512a	2.575a	0.181a	0.179a	13.86a	14.42a	3.54a	4.04a
Red clover		2.543a	2.691a	0.183a	0.189a	13.97a	14.24a	2.45b	1.77b
Timothy		2.521a	2.656a	0.182a	0.185a	13.86a	14.40a	3.89a	6.23a
SEM		0.049	0.065	0.004	0.005	0.15	0.18	0.22	0.50
Tests of Between-Subjects effects									
Treatment	df	Significance level							
FT	2	ns	ns	ns	ns	ns	ns	ns	ns
CT	3	ns	ns	ns	ns	ns	ns	***	***
FT×CT	6	ns	ns	ns	ns	ns	ns	ns	ns
Tests of Within-Subjects effects (sphericity assumed)									
Source	df	Significance level							
Time	1	***		ns		***		***	
Time×FT	2	ns		ns		ns		*	
Time×CT	3	ns		ns		ns		*	
Time ×FT×CT	6	ns		ns		ns		ns	

SEM = standard error of the means; FT = fertilizer treatment; CT = crop treatment; Time = sampling time points; df = degrees of freedom; ns = not significant; * when $p < 0.05$, ** when $p < 0.01$, *** when $p < 0.001$. Different letters in a column indicates significant differences.

Soil pH, EC and moisture

Soil pH was generally lowest in the synthetic fertilizer plots ($p < 0.05$) and pH in timothy plots was higher than that in red clover plots ($p < 0.05$) on 28 June 2014 and 24 September 2015 (Table 5). EC was generally highest in the organic fertilizer plots, especially when compared to the control (Table 6). In crop treatment, EC was generally highest in fallow plots, followed by red clover, mixture and timothy ($p < 0.05$). Soil moisture was lower in the fallow plots than in the planted plots ($p < 0.05$) on 16 August 2014 and 22 September 2014 (Table A.3).

Soil DNA content and its relationship with soil NO$_3$-N

Soil DNA content changed over time from June 2014 to July 2015 ($p < 0.05$) (Table A. 4) and was generally highest in the organic fertilizer plots (Table A.4). In September 2015 with high soil NO$_3$-N and moisture, a linear relationship between soil DNA and NO$_3$-N content was found. According to the linear regression model (Fig. 5, Table 7), as the soil DNA content increased, the soil NO$_3$-N decreased in fallow and red clover plots while it increased in the mixture and timothy plots.

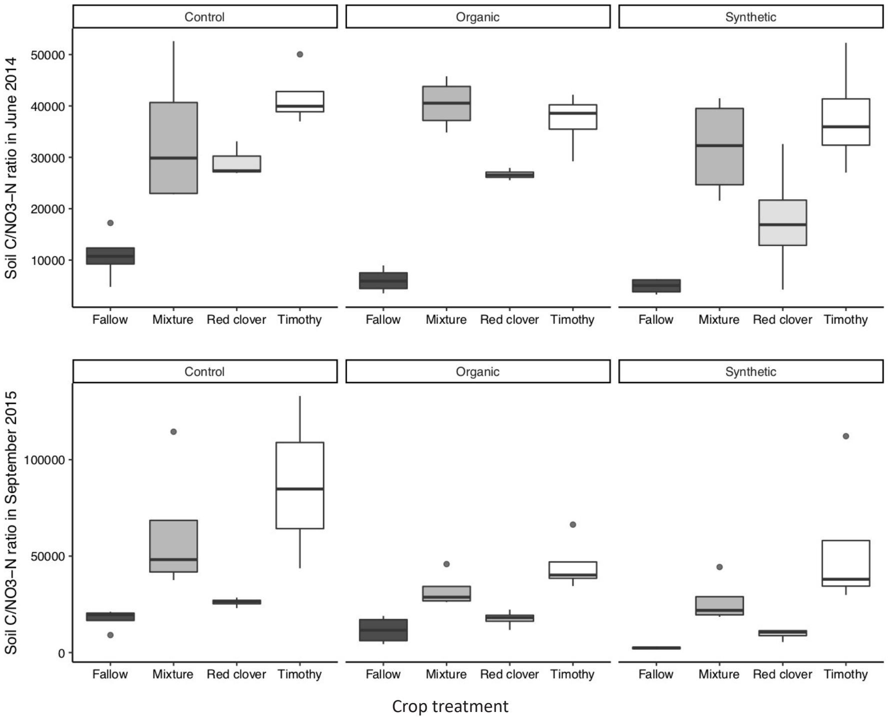

Fig. 4. Box plots (n=4) of soil C/NO$_3$ ratio regarding fertilizer-crop interaction in June 2014 and September 2015. The individual points in the figure are data points which are outside of 3/2 times of inter-quantile range.

Table 5. Soil pH under different fertilizer and crop treatments

Treatment		pH						
		2014					2015	
		23 May	06 June	28 June	16 Aug.	22 Sep.	08 July	24 Sep.
Control		6.20a	6.23a	6.35a	6.22a	6.33a	6.31a	6.37b
Organic		6.21a	6.28a	6.32a	6.21a	6.31a	6.38a	6.43a
Synthetic		6.11b	6.16a	6.22a	6.09b	6.24b	6.19b	6.19c
	SEM	0.02	0.03	0.02	0.02	0.02	0.02	0.02
Fallow		6.15a	6.17a	6.29b	6.06b	6.29a	6.30ab	6.28c
Mixture		6.18a	6.22a	6.31ab	6.20a	6.30a	6.29b	6.35ab
Red clover		6.17a	6.21a	6.25b	6.20a	6.24a	6.25b	6.30bc
Timothy		6.19a	6.29a	6.36a	6.24a	6.33a	6.35a	6.39a
	SEM	0.03	0.03	0.02	0.02	0.03	0.02	0.02
Tests of Between-Subjects effects								
Treatment	df	Significance level						
FT	2	**	ns	ns	*	*	**	***
CT	3	ns	ns	*	***	ns	*	**
FT×CT	6	ns	ns	ns	ns	ns	ns	ns
Tests of Within-Subjects effects (sphericity assumed)								
Source	df	Significance level						
Time	6	***						
Time×FT	12	***						
Time×CT	18	***						
Time×FT×CT	36	ns						

SEM = standard error of the means; FT = fertilizer treatment; CT = crop treatment; Time = sampling time points; df = degrees of freedom; ns = not significant; * when $p < 0.05$, ** when $p < 0.01$, *** when $p < 0.001$. Different letters in a column indicate significant differences.

Table 6. Soil EC under different fertilizer and crop treatments

Treatment		EC µS cm^{-1}						
		2014					2015	
		23 May	6 June	28 June	16 Aug.	22 Sep.	08 July	24 Sep.
Control		42.9a	44.2b	29.7a	70.3c	53.0c	54.2c	34.4b
Organic		54.2a	66.5a	35.3a	113.0a	75.2a	82.2b	57.1a
Synthetic		64.7a	59.4a	36.0a	96.1b	59.8b	96.1a	57.3a
	SEM	10.2	3.5	2.0	4.5	1.6	3.3	2.5
Fallow		57.8a	77.1a	43.0a	147.4a	79.3a	90.0a	58.3a
Mixture		62.9a	47.6b	30.9b	71.0c	54.4c	73.1bc	46.6b
Red clover		48.8a	55.6b	31.5b	86.9b	61.2b	81.9ab	50.1ab
Timothy		44.9a	46.6b	29.3b	67.2c	55.7c	65.1c	43.4b
	SEM	11.7	4.0	2.2	5.2	1.8	3.8	2.9
Tests of Between-Subjects effects								
Treatment	df	Significance level						
FT	2	ns	*	ns	**	***	***	**
CT	3	ns	**	**	***	***	**	**
FT×CT	6	ns	ns	ns	ns	ns	*	*
Tests of Within-Subjects effects (sphericity assumed)								
Source	df	Significance level						
Time	6	***						
Time×FT	12	***						
Time×CT	18	***						
Time×FT×CT	36	ns						

SEM = standard error of the means; FT = fertilizer treatment; CT = crop treatment; Time = sampling time points; df = degrees of freedom; ns = not significant; * when $p < 0.05$, ** when $p < 0.01$, *** when $p < 0.001$. Different letters in a column indicate significant differences.

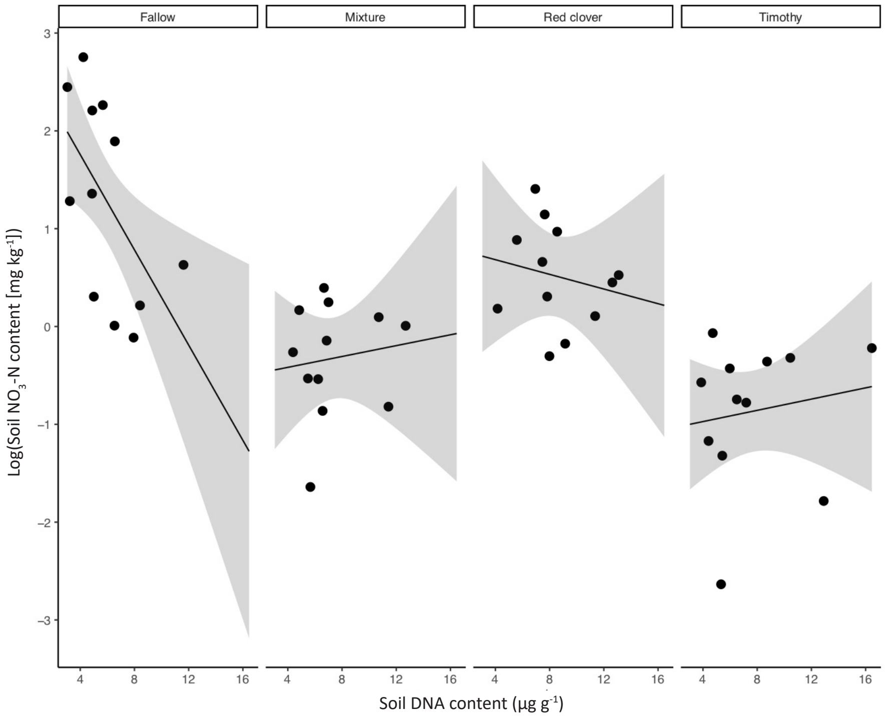

Fig. 5. Linear regression model between soil DNA content and NO$_3$-N content. The grey areas indicate the fitted values ± 2 standard error.

Table 7. Estimated regression parameters, standard errors, t-values and *p*-values

	Estimate	Std. Error	t-value	*p*-value
Intercept	2.73	0.57	4.76	2.53e-5***
DNA	-0.24	0.09	-2.73	0.00939**
Crop Mixture	-3.26	0.85	-3.84	0.00043***
Crop Red clover	-1.90	0.92	-2.06	0.04570*
Crop Timothy	-3.82	0.75	-5.11	8.40e-6***
DNA: Crop Mixture	0.27	0.12	2.26	0.02914*
DNA: Crop Red clover	0.21	0.12	1.71	0.09470
DNA: Crop Timothy	0.27	0.10	2.58	0.01369*

The adjusted R-squared equals 0.5712

Discussion

We established a three-year field study to assess sustainable forage production in a boreal climate. Both the crop growth characters and soil properties were investigated to know which fertilization treatment (control, organic, synthetic) and crop management (bare fallow, pure red clover, pure timothy, mix of red clover and timothy) could yield higher with minimal negative environmental effects.

As predicted, we found that the grass-legume mixture was the most sustainable crop management, which yielded higher and was less prone to potential N loss. When not fertilized, the dry matter and N yields of red clover and clover-timothy mixture were higher than those of timothy. The yield of the mixture was higher than that of red clover, possibly due to the niche complementarity of clover and timothy (Nyfeler et al. 2009) and the stimulation of BNF due to the uptake of N by grass (Nyfeler et al. 2011). When fertilized at 150 kg N ha^{-1} in 2015, the dry matter yields of mixture and timothy were increased. However, high fertilization rate did not increase the dry matter yield of red clover, possibly explained by inhibition of nodulation of rhizobia due to surplus soil N (Streeter and Wong 1988), which results in a decrease in BNF (Adams et al. 2018). Interestingly, the yields of mixture plots in the unfertilized control were higher than those of red clover and timothy in fertilized treatments in 2014, which indicated the advantages of mixture in increasing contributions from BNF. Additionally, as presented by Peoples et al. (2004), the lower crop C:N ratio (16–25:1) of mixture than that of timothy (22–36:1) may better balance the microorganisms dietary requirements and promote mineralization by microbes.

In the plant treatments, NO_3-N content was highest in red clover and lowest in timothy, possibly resulting from BNF and the subsequent nitrification in red clover and mixture treatments. High soil NO_3-N in fallow and red clover may result in high risk of potential N loss because of leaching and N_2O emission from denitrification (Meng et al. 2005, Ju et al. 2006), especially with legumes (Scherer-Lorenzen et al. 2003). Both soil NO_3-N and NH_4-N decreased during late growing season and the extent of the decrease was different among fertilizer and crop treatments. In line with findings that higher fertilization induced excessive nitrate leaching (Eriksen et al. 2015, Karimi et al. 2017), the decrease of NO_3-N and NH_4-N within synthetic fertilized treatments was stronger than that of the organic fertilized treatment. However, considering the low dry matter yield in the organic fertilizer treatment, it is not justified to conclude that the N loss when using organic fertilizer were smaller than using synthetic fertilizer. Additionally, in the light of the stringency criteria from Kirchmann et al. (2016), concerning N input source, application time, and application rate, it is more scientific to assess yield per input and N loss per yield or per input when comparing the sustainability of organic and synthetic fertilizer treatments in our case.

Less soil N was found to be leached from mixture than from clover monoculture (Loiseau et al. 2001, Saarijärvi et al. 2007), which may explain why the N loss in soil over time was smaller in the mixture than in red clover plots when concerning the higher N yields in mixture plots than that in red clover plots. Virkajärvi et al. (2010) found that N_2O emissions were lower in a grass sward than in a legume-grass mixture, and as suggested by Putz et al. (2018), the high C/NO_3-N ratio in timothy may have resulted in lower N_2O emission than in the mixture. High soil moisture and NO_3-N content enhance denitrification (Ryden 1983, Dobbie et al. 1999). In our case in September 2015 the soil NO_3-N content and moisture were both high, which may trigger biological denitrification. In this study, the soil DNA content was treated as a proxy for soil microbial biomass, and the relationship between DNA content and NO_3-N content may indicate that the potential N loss in the mixture and timothy plots were lower than in the fallow and red clover plots. As suggested by Herai et al. (2006), the microbial biomass N formation decreased NO_3-N leaching, and this could possibly explain why high DNA content was accompanied by high soil NO_3-N content in mixture and timothy. In addition, as Zechmeister-Boltenstern et al. (2002) suggested that substantial NO_3-N leaching was accompanied by the highest N_2O emission, less leaching in mixture and timothy plots may also contribute to decreased N loss through denitrification.

Long term N fertilization may result in soil acidification (Barak et al. 1997, Rice and Herman 2012). Accordingly, pH was lowest in synthetic fertilizer plots after two-year intensive fertilizer application. The acidification may induce more N loss through N_2O emission (Barak et al. 1997, Šlmek and Cooper 2002, Rice and Herman 2012). Therefore, N supplied as organic fertilizer or by BNF may be considered more stable and less prone to N losses, suggesting that these N sources may be considered more sustainable in forage production.

As climate changes, the growing season in boreal areas might be prolonged as presented in Peltonen-Sainio et al. (2009), and the increasing needs for N fertilization may challenge the sustainable intensification system. Therefore, the complex interaction between fertilizer management, cropping management, and local weather need to be further studied.

Acknowledgements

This work was supported by the Ministry of Agriculture and Forestry of Finland (KESTE project) and the Magnus Ehrnrooth Foundation. We appreciate the helpful comments from Frederick Stoddard, Mervi Seppänen, Laura Alakukku and N_2 group members on improving the manuscript. We also appreciate M.Sc. Vesa Luukkonen for helping in field work. Honghong Li acknowledges China Scholarship Council for a four-year scholarship covering the stipend of her PhD study at University of Helsinki.

References

Abalos, D., Groenigen, J.W. & De Deyn, G.B. 2018. What plant functional traits can reduce nitrous oxide emissions from intensively managed grasslands? Global Change Biology 24: 248–258. https://doi.org/10.1111/gcb.13827

Adams, M.A., Buchmann, N., Sprent, J., Buckley, T.N. & Turnbull, T.L. 2018. Crops, Nitrogen, Water: Are Legumes Friend, Foe, or Misunderstood Ally? Trends in Plant Science 23: 539–550. https://doi.org/10.1016/j.tplants.2018.02.009

Barak, P., Jobe, B.O., Krueger, A.R., Peterson, L.A. & Laird, D.A. 1997. Effects of long-term soil acidification due to nitrogen fertilizer inputs in Wisconsin. Plant and Soil 197: 61–69. https://doi.org/10.1023/A:1004297607070

Dobbie, K.E., McTaggart, I.P. & Smith, K.A. 1999. Nitrous oxide emissions from intensive agricultural systems: Variations between crops and seasons, key driving variables, and mean emission factors. Journal of Geophysical Research 104: 26891–26899. https://doi.org/10.1029/1999JD900378

Eriksen, J., Askegaard, M., Rasmussen, J. & Søegaard, K. 2015. Nitrate leaching and residual effect in dairy crop rotations with grass-clover leys as influenced by sward age, grazing, cutting and fertilizer regimes. Agriculture, Ecosystems & Environment 212: 75–84. https://doi.org/10.1016/j.agee.2015.07.001

Franche, C., Lindström, K. & Elmerich, C. 2009. Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. Plant and Soil 321: 35–59. https://doi.org/10.1007/s11104-008-9833-8

Herai, Y., Kouno, K., Hashimoto, M. & Nagaoka, T. 2006. Relationships between microbial biomass nitrogen, nitrate leaching and nitrogen uptake by corn in a compost and chemical fertilizer-amended regosol. Soil Science and Plant Nutrition 52: 186–194. https://doi.org/10.1111/j.1747-0765.2006.00031.x

Ju, X.T., Kou, C.L., Zhang, F.S. & Christie, P. 2006. Nitrogen balance and groundwater nitrate contamination: comparison among three intensive cropping systems on the North China Plain. Environmental Pollution 143: 117–125.https://doi.org/10.1016/j.envpol.2005.11.005

Karimi, R., Akinremi, W. & Flaten, D. 2017. Cropping system and type of pig manure affect nitrate-nitrogen leaching in a sandy loam soil. Journal of Environmental Quality 46: 785–792. https://doi.org/10.2134/jeq2017.04.0158

Kassambara, A. 2017. ggpubr: ‘ggplot2’ Based Publication Ready Plots. R package version 0.1.6. https://CRAN.R-project.org/package=ggpubr.

Kirchmann, H., Kätterer, T., Bergström, L., Börjesson, G. & Bolinder, M.A. 2016. Flaws and criteria for design and evaluation of comparative organic and conventional cropping systems. Field Crops Research 186: 99–106. https://doi.org/10.1016/j.fcr.2015.11.006

Lindström, K. 1984. Analysis of factors affectingin situ nitrogenase (C_2H_2) activity of *Galega orientalis*, *Trifolium pratense* and *Medicago sativa* in temperate conditions. Plant and Soil 79: 329. https://doi.org/10.1007/BF02184326

Loiseau, P., Carrere, P., Lafarge, M., Delpy, R. & Dublanchet, J. 2001. Effect of soil-N and urine-N on nitrate leaching under pure grass, pure clover and mixed grass/clover swards. European Journal of Agronomy 14: 113–121. https://doi.org/10.1016/S1161-0301(00)00084-8

Mahon, N., Crute, I., Simmons, E. & Islam, M.M. 2017. Sustainable intensification - “oxymoron” or “third-way”? A systematic review. Ecological Indicators 74: 73–97. https://doi.org/10.1016/j.ecolind.2016.11.001

Meng, L., Ding, W. & Cai, Z. 2005. Long-term application of organic manure and nitrogen fertilizer on N_2O emissions, soil quality and crop production in a sandy loam soil. Soil Biology and Biochemistry 37: 2037–2045. https://doi.org/10.1016/j.soilbio.2005.03.007

Mikkonen, A., Kondo, E., Lappi, K., Wallenius, K., Lindström, K., Hartikainen, H. & Suominen, L. 2011. Contaminant and plant-derived changes in soil chemical and microbiological indicators during fuel oil rhizoremediation with *Galega orientalis*. Geoderma 160: 336–346. https://doi.org/10.1016/j.geoderma.2010.10.001

Nyfeler, D., Huguenin-Elie, O., Suter, M., Frossard, E., Connolly, J. & Lüscher, A. 2009. Strong mixture effects among four species in fertilized agricultural grassland led to persistent and consistent transgressive overyielding. Journal of Applied Ecology 46: 683–691. https://doi.org/10.1111/j.1365-2664.2009.01653.x

Nyfeler, D., Huguenin-Elie, O., Suter, M., Frossard, E. & Lüscher, A. 2011. Grass-legume mixtures can yield more nitrogen than legume pure stands due to mutual stimulation of nitrogen uptake from symbiotic and non-symbiotic sources. Agriculture, Ecosystems & Environment 140: 155–163. https://doi.org/10.1016/j.agee.2010.11.022

Peltonen-Sainio, P., Jauhiainen, L., Hakala, K. & Ojanen, H. 2009. Climate change and prolongation of growing season: changes in regional potential for field crop production in Finland. Agricultural and Food Science 18: 171–190. https://doi.org/10.2137/145960609790059479

Peoples, M.B., Angus, J.F., Swan, A.D., Dear, B.S., Haugaard-Nielsen, H., Jensen, E.S., Ryan, M.H. & Virgona, J.M. 2004. Nitrogen dynamics in legume-based pasture systems. In: Mosier, A.R., Sayers, J.K. & Freney, J.R. (eds). Agriculture and the Nitrogen Cycle: Assessing the Impacts of Fertilizer Use on Food Production and the Environment. SCOPE 65. Island Press. p. 103–114. http://hdl.handle.net/102.100.100/184371?index=1

Philippot, L., Hallin, S. & Schloter, M. 2007. Ecology of Denitrifying Prokaryotes in Agricultural Soil. Advances in Agronomy 96: 249–305. https://doi.org/10.1016/S0065-2113(07)96003-4

Pretty, J., Toulmin, C. & Williams, S. 2011. Sustainable intensification in African agriculture. International Journal of Agricultural Sustainnability 9: 5–24. https://doi.org/10.3763/ijas.2010.0583

Putz, M., Schleusner, P., Rütting, T. & Hallin, S. 2018. Relative abundance of denitrifying and DNRA bacteria and their activity determine nitrogen retention or loss in agricultural soil. Soil Biology and Biochemistry 123: 97–104.https://doi.org/10.1016/j.soilbio.2018.05.006

R Core Team 2018. R: A language and environment for statistical computing 2018. R Foundation for Statistical Computing, Vienna, Austria.

Rice, K.C. & Herman, J.S. 2012. Acidification of Earth: An assessment across mechanisms and scales. Applied Geochemistry 27: 1–14. https://doi.org/10.1016/j.apgeochem.2011.09.001

Rockström, J., Williams, J., Daily, G., Noble, A., Matthews, N., Gordon, L., Wetterstrand, H., DeClerck, F., Shah, M., Steduto, P., de Fraiture, C., Hatibu, N., Unver, O., Bird, J., Sibanda, L. & Smith, J. 2017. Sustainable intensification of agriculture for human prosperity and global sustainability. Ambio 46: 4–17. https://doi.org/10.1007/s13280-016-0793-6

RStudio Team 2016. RStudio: Integrated Development Environment for R. RStudio, Inc., Boston, MA.

Ryden, J.C. 1983. Denitrification loss from a grassland soil in the field receiving different rates of nitrogen as ammonium-nitrate. Journal of Soil Science 34: 355–365. https://doi.org/10.1111/j.1365-2389.1983.tb01041.x

Saarijärvi, K., Virkajärvi, P. & Heinonen-Tanski, H. 2007. Nitrogen leaching and herbage production on intensively managed grass and grass-clover pastures on sandy soil in Finland. European Journal of Soil Science 58: 1382–1392. https://doi.org/10.1111/j.1365-2389.2007.00940.x

Salehi, A., Mehdi, B., Fallah, S., Kaul, H.P. & Neugschwandtner, R.W. 2018. Productivity and nutrient use efficiency with integrated fertilization of buckwheat-fenugreek intercrops. Nutrient Cycling in Agroecosystems 110: 407–425.https://doi.org/10.1007/s10705-018-9906-x

Sarkar, D. 2008. Lattice: Multivariate Data Visualization with R. Springer, New York. ISBN 978-0-387-75968-5.

Scherer-Lorenzen, M., Palmborg, C., Prinz, A. & Schulze, E.D. 2003. The role of plant diversity and composition for nitrate leaching in grasslands. Ecology 84: 1539–1552. https://doi.org/10.1890/0012-9658(2003)084[1539:TROPDA]2.0.CO;2

ŠImek, M. & Cooper, J.E. 2002. The influence of soil pH on denitrification: progress towards the understanding of this interaction over the last 50 years. European Journal of Soil Science 53: 345–354. https://doi.org/10.1046/j.1365-2389.2002.00461.x

Steffen, W., Richardson, K., Rockström, J., Cornell, S.E., Fetzer, I., Bennett, E.M., Biggs, R., Carpenter, S.R., de Vries, W., de Wit, C.A., Folke, C., Gerten, D., Heinke, J., Mace, G.M., Persson, L.M., Ramanathan, V., Reyers, B. & Sorlin, S. 2015. Planetary boundaries: Guiding human development on a changing planet. Science 347. https://doi.org/10.1126/science.1259855

Streeter, J. & Wong, P.P. 1988. Inhibition of legume nodule formation and N_2 fixation by nitrate. Critical Reviews in Plant Sciences 7: 1–23. https://doi.org/10.1080/07352688809382257

Suter, M., Connolly, J., Finn, J.A., Loges, R., Kirwan, L., Sebastià, M. & Lüscher, A. 2015. Nitrogen yield advantage from grass-legume mixtures is robust over a wide range of legume proportions and environmental conditions. Global Change Biology 21: 2424–2438. https://doi.org/10.1111/gcb.12880

Tittonell, P. 2014. Ecological intensification of agriculture-sustainable by nature. Current Opinion in Environmental Sustainability 8: 53–61. https://doi.org/10.1016/j.cosust.2014.08.006

Wickham, H. 2009. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York. https://doi.org/10.1007/978-0-387-98141-3

Wickham, H. 2011. The Split-Apply-Combine Strategy for Data Analysis. Journal of Statistical Software 40: 1–29. https://doi.org/10.18637/jss.v040.i01

Virkajärvi, P., Maljanen, M., Saarijärvi, K., Haapala, J. & Martikainen, P.J. 2010. N_2O emissions from boreal grass and grass-clover pasture soils. Agriculture Ecosystems & Environment 137: 59–67. https://doi.org/10.1016/j.agee.2009.12.015

Yan, L.J., Penttinen, P., Simojoki, A., Stoddard, F.L. & Lindström, K. 2015. Perennial crop growth in oil-contaminated soil in a boreal climate. Science of the Total Environment 532: 752–761. https://doi.org/10.1016/j.scitotenv.2015.06.052

Yokoyama, S., Yuri, K., Nomi, T., Komine, M., Nakamura, S.-i., Hattori, H. & Rai, H. 2017. The high correlation between DNA and chloroform-labile N in various types of soil. Applied Soil Ecology 117: 1–9. https://doi.org/10.1016/j.apsoil.2017.04.002

Zechmeister-Boltenstern, S., Hahn, M., Meger, S. & Jandl, R. 2002. Nitrous oxide emissions and nitrate leaching in relation to microbial biomass dynamics in a beech forest soil. Soil Biology and Biochemistry 34: 823–832. https://doi.org/10.1016/S0038-0717(02)00012-3

Zuur, A.F., Ieno, E.N., Walker, N.J., Saveliev, A.A. & Smith, G.M. 2009. Mixed effects models and extensions in Ecology with R. Springer, New York. https://doi.org/10.1007/978-0-387-87458-6