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EARTH AND ENVIRONMENTAL SCIENCE
NEGATIVE-RESULT

Forage plant mixture type interacts with soil moisture to affect soil nutrient availability in the short term

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Abstract

Agricultural intensification within forage systems has reduced grassland floral diversity by promoting ryegrass (*Lolium spp.*), damaging soil functionality which underpins critical ecosystem services. Diverse forage mixtures may enhance environmental benefits of pastures by decreasing nutrient leaching, increasing soil carbon storage, and with legume inclusion, reduce nitrogen fertilizer input. This UK study reports on how species-rich forage mixtures affect soil carbon, phosphorus, and nitrogen at dry, medium and wet soil moisture sites, compared to ryegrass monoculture. Increasing forage mixture diversity (from 1 to 17 species) affected soil carbon at the dry site. No effect of forage mixture on soil phosphorus was found, while forage mixture and site did interact to affect soil nitrate/nitrite availability. Results suggest that forage mixtures could be used to improve soil function, but longer-term studies are needed to conclusively demonstrate environmental and production benefits of high-diversity forages.

Keywords: Forage mixtures; Soil carbon; Soil nitrogen

Introduction

The main function of agricultural soils is to support food production, but they also provide many other ecosystem services (Milne *et al.*, 2015). UK land use is dominated by grassland systems whose primary purpose is to provide feed for ruminants. Agricultural intensification has led to the dominance of ryegrass (*Lolium spp.*), which is considered the most profitable species in these systems (Hopkins & Wilkins, 2006). This simplification, however, comes at a cost of the reduction of many ecosystems functions (Cong *et al.*, 2014). Diverse forage mixtures may alleviate some issues as they contain species with varying phenology, root depth, and biomass when compared to lower diversity grassland mixtures (Skinner & Dell, 2016). Soil nutrient cycling and retention can be greatly influenced by species diversity, even in the short term of just two years (Steinbeiss *et al.*, 2008). Along with increasing human population and related demand for food, producers will also face climate change, with increased probability of extreme weather events such as droughts or flooding (Hopkins & Del Prado, 2007). Given their extent, forage systems must be able to maintain productivity, but should also contribute to climate change mitigation and soil sustainability. The objective of this paper is to evaluate potential benefits of forage mixtures other than ryegrass for soil health indicators along a water availability gradient. We hypothesized that forage mixture and prevailing soil moisture conditions interact to affect (H1) soil carbon content, (H2) nitrate/nitrite availability, (H3) soil ammonium content, and finally (H4) plant availability of soil phosphorus.

Materials and Methods

The experimental site is located at the University of Reading Farm in Sonning, Berkshire, UK (51°28’22.4”N 0°54’15.3”W). Three locations within the farm site were chosen for their varying soil moisture regime: dry (2%), medium (7%), and wet (14% soil moisture as of June 2018). Each of the sites was treated homogenously at least for 10 years prior to the experiment. At each location, four replicates of 4 forage mixtures in a 4x4 Latin square design were sown in September 2016. Each of the mixture plots was 4.2 x 5 m in size, the plots were a single species perennial ryegrass (R) and three forage mixtures: Smart Grass (6 species), Biomix (12 species), and Herbal (17 species, Table 1). Ten 2 cm diameter x 15 cm depth soil core samples were taken from each plot in June 2018, nearly two years after establishment and analysed for carbon, nitrogen and phosphorus content. Effects of forage mixture and site were evaluated using R studio (R Core Team, 2018) with plotrix package (Lemon, 2006). A detailed description of the field setup and analytical methodology is available at dx.doi.org/10.17504/protocols.io.bfrajm2e.

Results

A long-term effect of site on soil carbon concentration was found ($P < 0.001$; Figure 1), but there was no effect of forage mixture (reject H1). At the dry site, soil carbon content was greater in the R treatment compared to the Smartgrass and Herbal mixtures ($P < 0.01$ for both). There was an interactive effect of

Table 1. Forage mixture species selection list (R: Ryegrass; SG: Smart Grass; B: Biomix; H: Herbal)

Species	Latin	R	SG	B	H
Perennial Ryegrass	*Lolium perenne* L.	✓	✓	✓	✓
Timothy	*Phleum pratense* L.		✓	✓	✓
Cocksfoot	*Dactylis glomerata* L.			✓	✓
Festulolium	-			✓	✓
Tall Fescue	*Festuca arundinacea* Schreb.				✓
Meadow Fescue	*Festuca pratensis* Huds.			✓	✓
Red Clover	*Trifolium pratense* L.		✓	✓	✓
White Clover	*Trifolium repens* L.		✓	✓	✓
Alsike Clover	*Trifolium hybridum* L.			✓	✓
Sweet Clover	*Melilotus* spp.				✓
Black Medick	*Medicago lupulina* L.			✓	
Lucerne	*Medicago sativa* L.			✓	
Sainfoin	*Onobrychis spp.*				✓
Birdsfoot Trefoil	*Lotus corniculatus* L.				✓
Plantain	*Plantago lanceolata* L.		✓	✓	✓
Chicory	*Cichorium intybus* L.		✓	✓	✓
Yarrow	*Achillea millefolium* L.				✓
Burnet	*Sanguisorba minor* Scop.				✓
Sheep’s Parsley	*Petroselinum crispum* Mill.				✓

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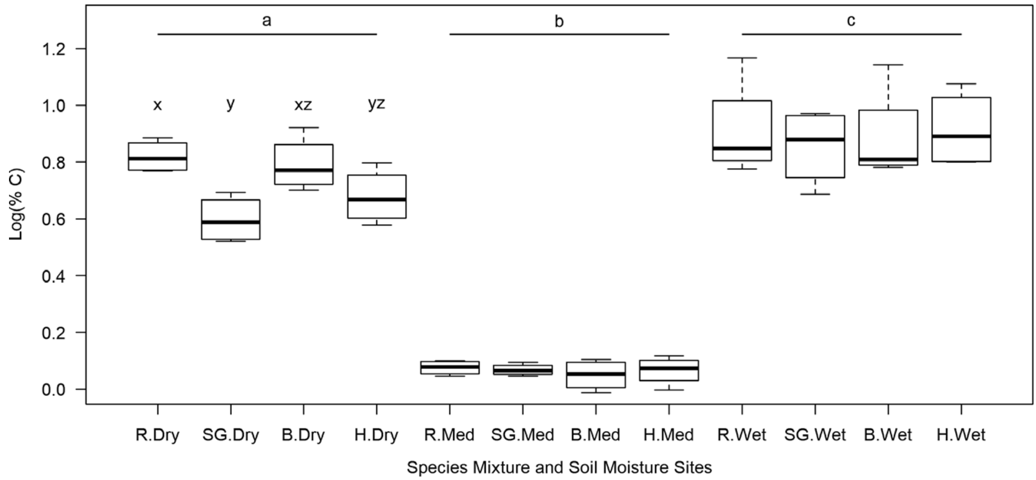

Fig. 1. Boxplot of log soil carbon (C) content in dry, medium soil moisture and wet sites as affected by plant species mixtures: R – Ryegrass (1 species); SG – Smart Grass (6 species); B – Biomix (12 species); and H – Herbal (17 species). Boxes show median, middle 50% of data and upper and lower quartile data range. Letters denote significant difference between treatments.

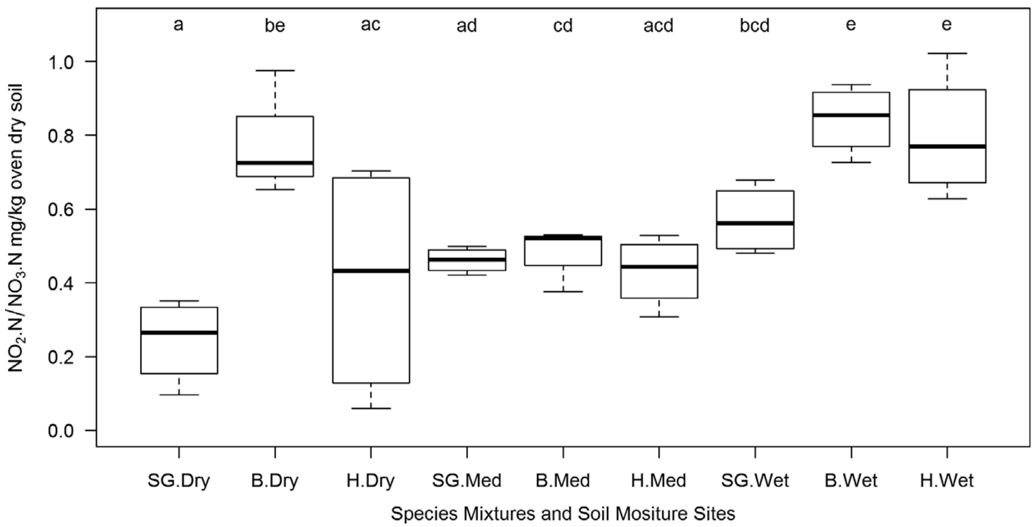

Fig. 2. Boxplot of soil nitrate/nitrite (NO_2.N/NO_3.N) content in the dry, medium soil moisture and wet sites as affected by plant species mixture: SG – Smart Grass (6 species); B – Biomix (12 species); and H – Herbal (17 species). Ryegrass data were not considered due to nitrogen application in that treatment. Boxes show median, middle 50% of data and upper and lower quartile data range. Letters denote significant difference between treatments.

forage mixture and site on soil nitrogen from nitrate/nitrite ($P < 0.05$; Figure 2). More nitrate/nitrite accumulated in the soil under Smartgrass and Herbal mixtures as soil moisture increased. There were significant differences in soil ammonium availability between sites ($P < 0.001$; Figure 3). But no interaction between species and site on soil ammonium concentration (reject H3). Soil phosphorus concentrations differed significantly between sites ($P < 0.001$), the dry site had the highest concentration of soil phosphorus, while the medium soil moisture site had the lowest. No interactive effect of forage mixture and site on soil phosphorus concentration was found (reject H4) (see Table 2 for data).

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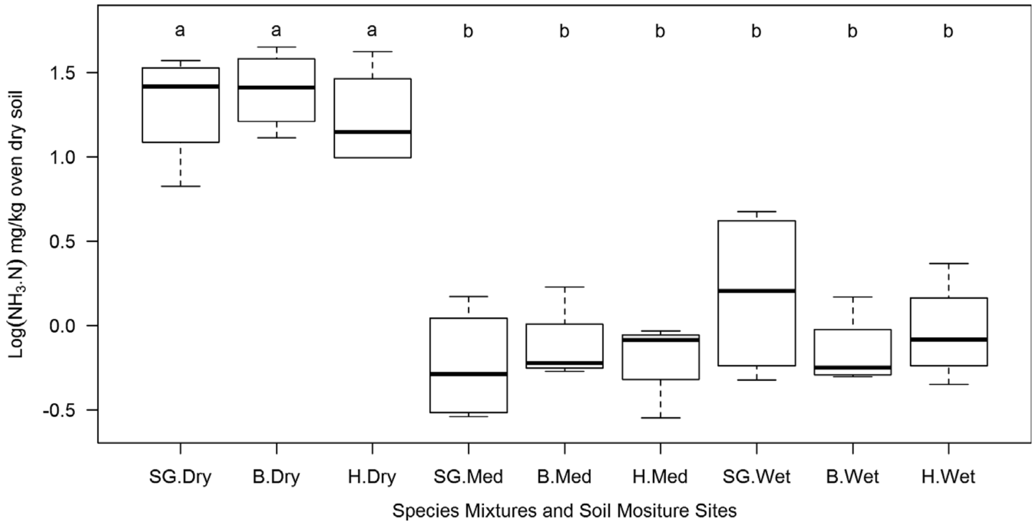

Fig. 3. Boxplot of log nitrogen from ammonium (NH_3.N) in the dry, medium soil moisture and wet sites as affected by plant species mixture: SG – Smart Grass (6 species); B – Biomix (12 species); H – Herbal (17 species). Ryegrass data were not considered due to nitrogen application in that treatment. Boxes show median, middle 50% of data and upper and lower quartile data range. Letters denote significant difference between treatments.

Table 2. Ammonium (NH_3), nitrite (NO_2), nitrate (NO_3) and phosphorus (P) concentration (mg/kg,) and carbon (C, %) in oven dried soil (mean ± standard error). Forage mixtures used in this experiment were R – Ryegrass (1 species); SG – Smart Grass (6 species); B – Biomix (12 species); H – Herbal (17 species).

Site	Mix	NH_3	NO_2/ NO_3	P	C
Wet	R	1.00 (± 0.106)	2.79 (± 0.325)	8.65 (± 1.599)	2.51 (± 0.237)
Wet	SG	1.61 (± 0.381)	2.15 (± 0.177)	7.83 (± 0.867)	2.37 (± 0.156)
Wet	B	1.06 (± 0.128)	3.18 (± 0.175)	9.75 (± 2.544)	2.45 (± 0.228)
Wet	H	1.21 (± 0.193)	3.01 (± 0.321)	8.76 (± 2.067)	2.51 (± 0.173)
Med	R	5.16 (± 0.331)	4.82 (± 0.589)	3.04 (± 0.167)	1.08 (± 0.014)
Med	SG	1.00 (± 0.173)	1.74 (± 0.065)	3.21 (± 0.096)	1.07 (± 0.011)
Med	B	1.10 (± 0.143)	1.83 (± 0.139)	3.22 (± 0.159)	1.05 (± 0.028)
Med	H	1.03 (± 0.109)	1.63 (± 0.180)	3.22 (± 0.117)	1.07 (± 0.027)
Dry	R	70.18 (± 15.737)	85.64 (± 18.182)	22.29 (± 1.332)	2.27 (± 0.065)
Dry	SG	4.66 (± 0.674)	0.92 (± 0.217)	23.21 (± 1.382)	1.82 (± 0.076)
Dry	B	5.00 (± 0.576)	2.90 (± 0.266)	24.08 (± 2.275)	2.21 (± 0.109)
Dry	H	4.30 (± 0.682)	1.53 (± 0.617)	25.56 (± 0.971)	1.98 (± 0.096)

Discussion

The lack of any significant effect of species mixture on soil carbon content found in the present study conflicts with previous work showing that increasing species diversity positively affects soil carbon accumulation (Skinner & Dell, 2016), possibly due to the relatively short-term nature of the current study. Skinner and Dell (2016) measured soil carbon around a decade after experimental treatment establishment. However, increased carbon accumulation in the top 5 cm soil profile can occur after just

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2 years; and in the top 20 cm within 4 years (Steinbeiss *et al.*, 2008). Had the current samples been split into three 5 cm sampling depths, carbon accumulation may have been observed in the surface layer.

The wet site had a significantly higher soil carbon content than the medium or dry sites (Figure 1), likely due to higher water availability stimulating primary productivity and thus carbon deposition. With the predicted increase in temperature in parts of the UK, the dry site could give us an insight into future abilities of pastures to store soil carbon. In this instance, the 12 species forage mixture accumulated significantly more soil carbon than the 6 species mixture (Figure 1).

Forage mixture and soil moisture availability showed an interactive effect on soil nitrate/nitrite concentrations (Figure 2). More soil nitrate/nitrite was found under the Biomix and Herbal mixtures compared to the Smartgrass mixture at the wet site. This could be attributed to the higher proportion of sown legumes in these two mixtures (Biomix 220 g/kg; Herbal 370 g/kg) compared to the Smartgrass mixture (80 g/kg), confirming earlier studies showing effects of legume inclusion on soil nutrient concentrations (Cong *et al.*, 2014).

Forage mixture had no significant effect on the concentration of nitrate/nitrite at the medium site. The contrast between the medium and the other two sites alludes to the fact that mixtures may require more time to establish full functionality and to demonstrate any significant changes in soil chemistry from the addition of diverse forage species such as legumes under non-stressed environments (Cong *et al.*, 2014).

Soil ammonium concentration was lower at the wet and medium sites compared to the dry site (Figure 3). Biological reasons for the low ammonium concentration at the medium and wet sites may include: (i) water not a limiting factor for plant growth, stimulating ammonium uptake; or (ii) denitrification and/or volatilization being higher in the wetter soils so more nitrogen is lost to the atmosphere than at the dry site.

There was no significant effect of plant species mixture on soil phosphorus availability. The dry site had higher soil phosphorus availability than the medium and wet sites. Years of arable cropping and fertilizer application at the dry site may explain such high phosphorus concentrations compared to the other two sites, known as the legacy effect (Van der Putten *et al.*, 2013).

Conclusion

The current short-term findings suggest that there is a significant interactive effect of forage mixture and inherent site soil moisture content on soil nitrate/nitrite concentration. Timing of sampling, both in terms of elapsed time since sowing of mixtures and seasonal sample collection, may affect the observations. Farmer adoption of forage mixtures may be an important measure to realise environmental improvements such as increased carbon sequestration or decreased phosphorus and nitrogen leaching. Further studies are therefore required to define which forage mixtures encourage longer-term pasture sustainability, with environmental benefits both above and below ground, in addition to economic and social benefits.

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Author Contributions. AT, DB, TM, HJ, CR and ML conceived and designed the study. SS conducted data gathering, performed statistical analyses and wrote the article with edit inputs from AT, DB, TM, HJ, CR and ML.

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Conflicts of Interest. The authors declare there are no conflicts of interest.

Ethical Standards. Not applicable.

Data availability. Raw data were generated at the University of Readings Crops Research Unit.

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Peer Reviews

Reviewing editor: Dr. Takashi Toyofuku[1,2]

[1]JAMSTEC, ASTER/X-star, Natsushima-cho 2-15, Kanagawa, Yokosuka, Japan, 237-0061
[2]Tokyo University of Marine Science and Technology, Minato-ku, Japan, 108-8477

This article has been accepted because it is deemed to be scientifically sound, has the correct controls, has appropriate methodology and is statistically valid, and met required revisions.

doi:10.1017/exp.2020.47.pr1

Review 1: Forage plant mixture type interacts with soil moisture to affect soil nutrient availability

Reviewer: Dr. Tõnu Tõnutare

Estonian University of Life Sciences, Soil Science Chair, Kreutzwaldi 5, Tartu, Estonia, 51014

Date of review: 23 June 2020

Conflict of interest statement. Reviewer declares none

Comments to the Author: The topic of manuscript is interesting and has importance from point of ecology. Unfortunately there are large shortcomings. The period of research is obviously too short for making conclusions about changes in soil carbon contents. The conclusions are made on the basis of soil C, NO2/NO3, NH3 and P contents. If the research was to investigate the effect of plants and various sites on changes of contents of this species, then a comparison of initial and final status is needed. At moment there is no information about the initial contents of C, NO2/NO3, NH3 and P in the sites. Also no information about methods used for nitrate, nitrite, ammonium and phosphorus determination. Also the reviewer can't understand the reason for using logarithmic scales for C and ammonium graphs. Given address in manuscript (doi.org/10.17504) didn't help in understanding of research because reviewer can't find any information there.

At the moment it is not clear what are the differences in soils caused by plants or soils were different already in the beginning of the project. Therefore this manuscript is not ready for publication.

Score Card

Presentation

2.9 /5

Is the article written in clear and proper English? (30%)	5/5
Is the data presented in the most useful manner? (40%)	2/5
Does the paper cite relevant and related articles appropriately? (30%)	2/5

Context

3.3 /5

Does the title suitably represent the article? (25%)	2/5
Does the abstract correctly embody the content of the article? (25%)	4/5
Does the introduction give appropriate context? (25%)	3/5
Is the objective of the experiment clearly defined? (25%)	4/5

Analysis

2.4 /5

Does the discussion adequately interpret the results presented? (40%)	2/5
Is the conclusion consistent with the results and discussion? (40%)	2/5
Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)	4/5

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doi:10.1017/exp.2020.47.pr2

Review 2: Forage plant mixture type interacts with soil moisture to affect soil nutrient availability

Reviewer: Dr. Krishna Bhandari

Texas Tech University System, Lubbock, United States, 79409-5025

Date of review: 09 July 2020

Conflict of interest statement. Reviewer declares none

Comments to the Author: Although I selected "Accept after minor revision", it needs substantial revision. Please see my attached file with inserted comments.

Score Card

Presentation

3.6 /5

Is the article written in clear and proper English? (30%)	4/5
Is the data presented in the most useful manner? (40%)	3/5
Does the paper cite relevant and related articles appropriately? (30%)	4/5

Context

3.5 /5

Does the title suitably represent the article? (25%)	4/5
Does the abstract correctly embody the content of the article? (25%)	4/5
Does the introduction give appropriate context? (25%)	2/5
Is the objective of the experiment clearly defined? (25%)	4/5

Analysis

2.6 /5

Does the discussion adequately interpret the results presented? (40%)	2/5
Is the conclusion consistent with the results and discussion? (40%)	3/5
Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)	3/5

doi:10.1017/exp.2020.47.pr3

Review 3: Forage plant mixture type interacts with soil moisture to affect soil nutrient availability

Reviewer: Dr. Simone Ravetto Enri

University of Turin, Agricultural, Forest and Food Sciences, Torino, Italy, 10124

Date of review: 16 July 2020

Conflict of interest statement. Reviewer declares none

Comments to the Author: Abstract: Add UK to specify the environment spp. not in italics

L24 How does increasing human populations interacts with producers?

Forage systems/ mixture are not appropriate terms. Use 'grassland' instead (or in addition). But see comments below.

How was ammonium measured? soil-extracted? In water or KCL?

explain the reason for choosing NO2/NO3 as soil health indicator. Two indicators related to N are expected to react fast to plant species, while soil C is not. unclear whether differences depend on species after only 2 years.

L29-30 ecosystem services are not just linked to the extension of a habitat

L38-39 define dry-medium-wet conditions in terms of soil moisture.

Was statistical analysis performed on raw or transformed variables? Why? If the 3 sites were located separately at the experimental farm, shouldn't the statistical analysis consider a random effect?

L45 and followings. Not 'site effect' -> 'soil moisture'

L47 Please check doi.

Table 1: Petroselenium -> Petroselinum

Remove the abbreviation PRG, confusing.

L53 A two-years experiment is a SHORT- not long-term experiment!! Moreover, a two-years old grassland is a Temporary Grassland. The ecosystem services provided by and expected from a TG are completely different from Permanent Grassland ones… The extension of these TG in UK is minimal… the introduction has to consider this point. Additionally, is it common a two-years temporary grassland? Usually one-year herbages or 3-to-4-years TG are found. Your situation appears unusual and hampers the interest for a wide audience.

Fig2-3 Why data on Ryegrass are not presented?

specify the reason for presenting C and ammonium as logarithm in Fig. 1-3.

Fig.3 no interaction site- mixture, why do you present the differences for each histogram…?Why data on phosphorous are not presented?

Order Letters in Fig.s 1-2 (a>b>c…).

Discussion Too short and based almost only on guesses, rewrite basing on the obtained results.

You analysed a single soil depth, while various depths could be considered. add this limitation.

L72-73 Was the site treated homogeneously before your trial? specify.

L83 'pasture' could be inappropriate since ryegrass TG are rarely grazed.

Score Card

Presentation

2.9 /5

Is the article written in clear and proper English? (30%)	5/5
Is the data presented in the most useful manner? (40%)	2/5
Does the paper cite relevant and related articles appropriately? (30%)	2/5

Context

3.2 /5

Does the title suitably represent the article? (25%)	3/5
Does the abstract correctly embody the content of the article? (25%)	4/5
Does the introduction give appropriate context? (25%)	1/5
Is the objective of the experiment clearly defined? (25%)	5/5

Analysis

2.6 /5

Does the discussion adequately interpret the results presented? (40%)	2/5
Is the conclusion consistent with the results and discussion? (40%)	3/5
Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)	3/5

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