Methodological Workshop in Evolutionary Biology – practical part

Ochotnica Górna field station

15th - 20th September 2016

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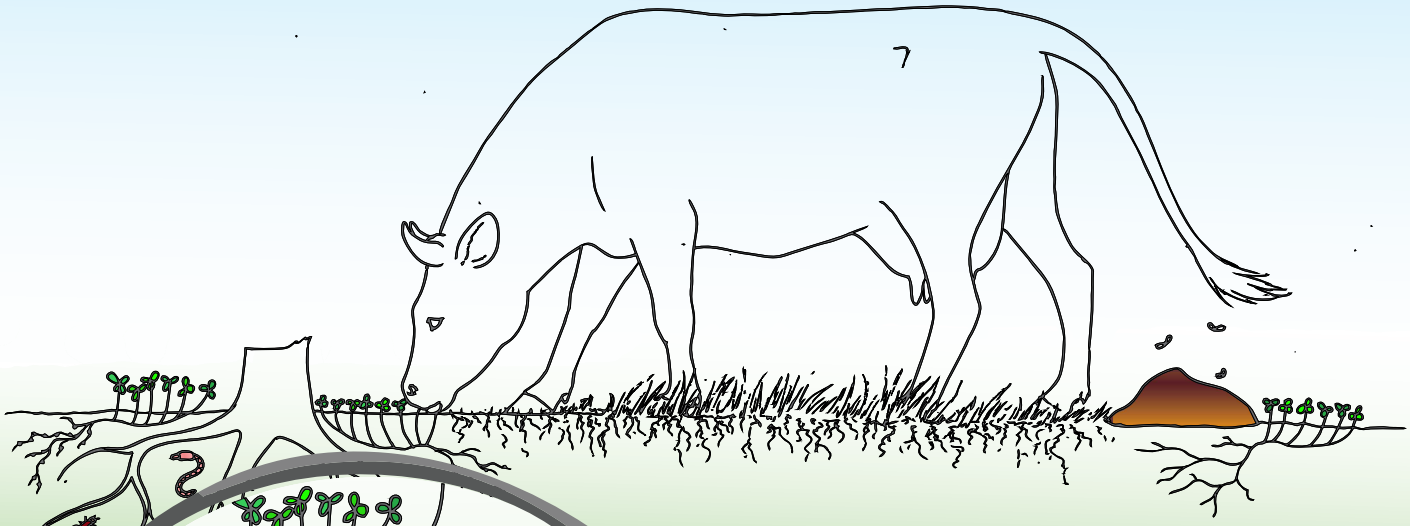

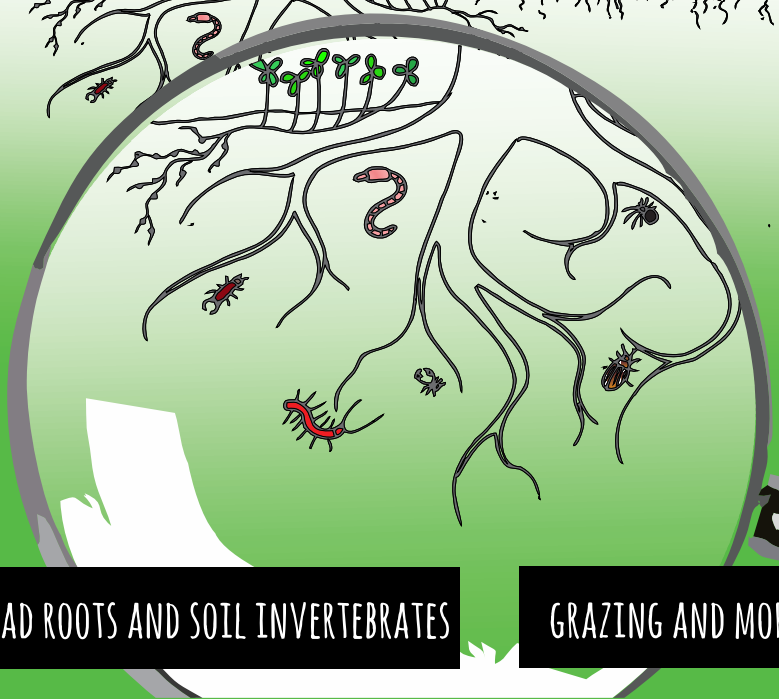

DEAD ROOTS AND SOIL INVERTEBRATES

GRAZING AND MORPHOLOGY OF PLANTS

MANURE AND ROOT NODULES

Contents:

Editors of the Report: Weronika Banot, Anna Giermek

First page project: Anna Giermek, Małgorzata Lipowska

Coordinators, reviewers and participants

Coordinator

Dr hab. Joanna Rutkowska, Institute of Environmental Sciences, Jagiellonian University

External reviewers

Prof. dr. Manfred Bauchinger, Institut für Strahlenbiologie, GSF-Forschungszentrum für Umwelt und Gesundheit, Germany

Prof. dr hab. Adam Łomnicki, Institute of Environmental Sciences, Jagiellonian University

Participants

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Dorota Lutyk, Institute of Environmental Sciences, Jagiellonian University

Jowita Niedojadło, Institute of Environmental Sciences, Jagiellonian University

Research topics suggested by participants

(**Bold –** topics chosen by voting)

1. Why beech trees differ in number of galls? (WB)
2. Why renovation of the forest is spatially heterogeneous? (MC)
3. **How does the grazing affect morphology of plants? (SB)**
4. How plants benefits from flies as pollinators? (JN)
5. How buffer trees influence plants on the meadow? (AG)
6. **Why certain mushrooms are eaten only by snails or insects? (DL)**
7. How does human activity affects *Urtica* growth? (ML)
8. How do trees cope with the slope on which they grow? (ML)
9. Why do mushroom of the same species differ in shape? (DL)
10. How does distance from the water affects biomass of the plants? (AG)
11. How are plants affected by trees allelopathy? (JN)
12. **How does the presence of the dead roots shape the community of soil invertebrates? (SB)**
13. How are mushrooms affected by the presence of paths? (MC)
14. Why are some mushroom specimens more likely to be consumed by invertebrates? (WB)
15. **How cows' manure influences number of *Fabaceae* and number of their root nodules? (AG)**
16. How nicotine pollution affect ant communication? (DL)
17. How does age of beech trees affect its fertility? (ML)
18. Which factors determine the presence of *Campanulaceae* flowers? (WB)
19. Why the diversity of invertebrates is lower in the proximity of human habitats? (JN)
20. How does the density of the forest affect abundance of mushrooms? (SB)
21. How does the density of the forest affect abundance of the blackberries? (MC)

Chosen topics and research teams

How does the presence of the dead roots shape the community of soil invertebrates?

Stanisław Bury, Anna Giermek, Dorota Lutyk

Photo. Joanna Rutkowska

How does the grazing affect morphology of plants?

Michela Corsini, Małgorzata Lipowska

Photo. Joanna Rutkowska

How cows' manure influences number of *Fabaceae* and number of their root nodules?

Weronika Banot, Jowita Niedojadło

Photo. Joanna Rutkowska

How does the presence of the dead roots shape the community of soil invertebrates?

Project

Title: The importance of dead and alive tree roots in shaping soil invertebrate community Dead or alive? - how does the presence of the dead roots shape the community of soil invertebrates?

Authors: Stanisław Bury, Anna Giermek, Dorota Lutyk

Summary

Factors shaping the diversity and biomass of certain communities are among most important problems of ecology. They are also of special importance from the viewpoint of habitat management and biological conservation. The amount of dead wood is an outcome of forestry activities and it has been shown to constitute as a crucial component for forest biodiversity. However, so far most studies concerned the effect of decayed fallen trees on various aspects of surrounding communities. As most of the forests are managed and the amount of fallen trees is highly limited the only sources of dead wood are roots left after forestry clearance. Our project aims at exploring this problem.

Aim/Hypothesis:

1. Presence of the dead roots influence biodiversity of soil invertebrates

2. Presence of the dead roots influence biomass of soil invertebrates

Predictions:

1. In soil close to dead roots biodiversity of invertebrates is higher than close to roots of living trees

2. In soil close to dead roots biomass of invertebrates is higher than close to roots of living trees

Methods:

Soil samples 10x10x10cm

- 5x close to the dead roots
- 5x close to the roots of living trees

Invertebrates will be extracted from the soil using Tullgren funnels. Individuals will be assigned to taxonomic group (at least order). Out of this we will calculate Shannon-Wiener biodiversity index and Simpson dominance index. Whole individuals collected in one site will be weighed together to obtain total biomass per 1dm^3 of soil.

Impact of results

Results of our project will provide deeper insight into the role of dead wood, namely roots left after forest clearings. It will enable us to determine if such leavings increase species richness and biomass of soil invertebrates and thus if it compensate the lack of fallen trees in managed forest areas.

What features of invertebrate community is affected by the presence of fir snags?

Stanisław Bury, Anna Giermek*, Dorota Lutyk

Abstract

Dead wood has been proved to constitute as a crucial component for forest biodiversity and its amount is an outcome of forestry management. However, so far most studies concerned the effect of decayed fallen trees on various aspects of surrounding communities. As most of the forests are managed and the amount of fallen trees is highly limited the only sources of dead wood are snags left after forestry clearance. In our project we explored the role of fir snags in shaping diversity and biomass of soil invertebrates. We have found that both characters as well as Shannon-Wiener and Simpson indexes do not differ between snags and living trees. It seems that snags do not compensate for the lack of dead tree trunks and extensively managed forests are inevitably devoid of important component of soil invertebrate community.

Introduction

Factors shaping the diversity and biomass of certain communities are among most important problems of ecology. They are also of special importance from the viewpoint of habitat management and biological conservation. Dead wood at different stages of decay have an important ecological role in shaping the diversity of many different organisms. Dead trees are valuable habitats which providing for example food, shelter and breeding conditions for a large number of rare and threatened species: lichens, bryophytes, invertebrates, amphibians, birds and mammals (Jonnson et al 2005). Large quantities of deadwood also play a significant role in forest nutrient cycles, carbon budgets, soil morphology and natural regeneration. Unfortunately, only a low amount of dead wood is present in most managed Central European forests (1–3 m^3 /ha, Ammer 1991). In Central Europe only 0.2% of the deciduous forests are in a relatively natural state which includes a high amount of deadwood (50–200 m^3 /ha, Korpel 1995).

Soil invertebrates constitute as a group that is strongly dependent on dead and decaying wood. Invertebrates play also an important role in forest ecosystems by improving soil fertility, productivity and structure of the ecosystems and play a critical role in nutrient cycling through facilitating decomposition of organic matter and decaying roots (Gonzales and Seastedt 2001; Ca´rcamo et al. 2001). Soil invertebrates connect primary production to secondary consumers, influence vegetation species composition and constitute important links in detritus-based food chains (Brown and Gange 1990, Edwards and Bohlen 1996, Ponsard et al. 2000, Lavelle and Spain 2001). Therefore it is of special importance to get insight into the relationship between the presence and type of dead wood and the structure of soil fauna.

The amount of dead wood is highly variable and depends mostly on the intensiveness of forestry management. As mentioned in most constantly managed forests the availability of fallen tree trunks is scarce. Therefore in many cases the only sources of decaying wood are snags that remain after forest clearings. Surprisingly there are almost no studies that explore the effect of the presence of snags on the soil fauna. An understanding of how does the presence of the dead roots shape the community of soil invertebrates will not only allow us to predict the potential impacts of future environmental changes on soil invertebrates in the context of forest management (Blair et al. 2000), but will also allow us to evaluate if such leavings increase species richness and biomass of soil invertebrates.

In our study we investigated the diversity and biomass of soil invertebrates living on the forest floor of a managed beech–fir forest in which nature oriented forestry is practiced. The objective of this research was to study the impact of old tree snags on community of soil invertebrates. We addressed the following hypotheses:

1. Presence of snags influence biodiversity of soil invertebrates

2. Presence of snags influences biomass of soil invertebrates.

Material and Methods

Study area:

The project was performed in the managed beech-fir forest in Gorce Mountain. The study area was situated 2 km from the border of Gorczański National Park, on the altitude of

670–700 m a.s.l. The forest is dominated by beech (*Fagus sylvatica*), with significant part of silver fir (*Abies alba*).

Soil sampling:

To test whether invertebrates' biodiversity or biomass differ between soil close to the dead roots (snags) and next to the roots of living trees, five samples of soil (10x10x10cm) of each type were collected (Figure 1). Sampling sites were located not further than 10 cm distance from the tree trunk. All of the investigated trees were firs of similar age (with a diameter of 44 – 51cm), randomly chosen in the same type of forest.

Fig. 1. Example of sampling sites.

Invertebrates from each soil sample were blindly extracted by hand-sorting. Each found individual was gently transferred to the petri dish. Specimens were identified under binocular and assign to the order (according to Jura 2007). All invertebrates from one soil sample were weighted together to assess the biomass.

The biodiversity of each sample was calculated using Shannon–Wiener biodiversity index. To analyze community structure we also calculated Simpson dominance index.

Differences between groups in terms of biodiversity and biomass were analyzed using U Mann–Whitney test. Non-parametric test was chosen because of lack of normal distribution, caused by small sample size.

Results

Together we have extracted and identify 144 individuals (69 in samples from living roots group and 75 in samples from dead roots group) belonging to 15 different invertebrate orders (13 in samples from living roots group, 12 in samples from dead roots group, Table 1.).

Individuals from order Pseudoscorpionideae were found only in samples from dead roots group. All other order representatives were found in both groups. There were no differences between groups in terms of biomass (Mann–Whitney $U = 8.0$, $Z = -0.83$, $p = 0.4$, $N=10$, Figure 2.) nor biodiversity (Shannon – Wiener index: Mann–Whitney $U = 12.0$, $Z = 0.00$, $p = 1.0$, $N=10$; Simpson index: Mann – Whitney $U = 9.0$, $Z = -0.63$, $p = 0.53$, $N=10$).

Tab. 1. Number of taxa and individuals found in each sample site.

Order name	number of invertebrates											
	snag					living tree					sum	
	1	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	snag	tree
NEMATODA (Roundworms)	1		1	1		3			4		3	7
ANNELIDA (Segmented worms)												
Clitellata (earthworms)		1		1		1			3		2	4
ARTHROPODA												
1. ARACHINIDA (Arachnids)												
Araneae (spiders)	2	1		3	1			4	2	1	7	7
Acarina (mites, ticks)		10	3	1	2	1		2	3		16	6
Pseudoscorpionidae (pseudoscorpions)				1	6						7	0
2. INSECTA (Insects)												
Hemiptera (true bugs)										1	0	1
Coleoptera (beetles)	2			1	1						4	0
Diptera (true flies)				1						1	1	1
Lepidoptera (butterflies, moths)		1							2		1	2
Collembola (springtails)	3	2			9		4	2	8	1	14	15
3. MYRIAPODA												
Chilopoda (centipedes)	1	3	5	5	2	3	1		9		16	13
Diplopoda (millepedes)							2				0	2
ISOPODA (pillbugs, woodlice, isopods)	2		1			1	4	1	3		3	9
MOLLUSCA (Molluscs)												
slugs									1		0	1
snails					1		1				1	1

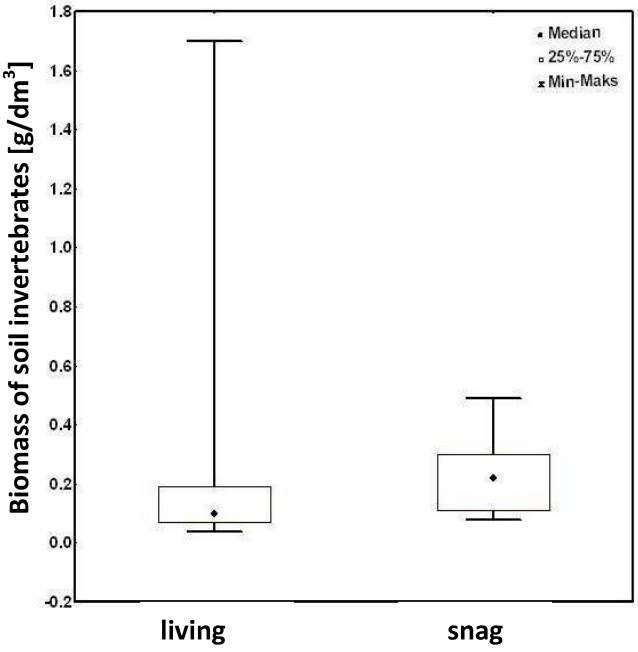

Fig. 2. Biomass of soil invertebrates in two study groups.

Our results are contrary to predictions and did not reveal any differences in the number of taxa nor the biomass of soil invertebrates with respect to the type of fir root. Both characteristics were highly similar which clearly indicates that dead roots do not enhance any measure of invertebrate community. Small differences in the composition have been noticed, for example pseudoscorpions were found only in surroundings of snags.

So far most studies have indicated strong and positive influence of decaying tree trunks on the diversity of various organisms including fungi, insects and even small vertebrates (Maguire 2002; Odor et al. 2006). Found by us lack of such effect of snags that are often the only decaying wood in managed forests can be explained by the fact that the largest part of the snag remains deeply underground and only small part of it is available to the soil organisms, since most studies reported the highest diversity of invertebrates up to 30 cm deep below the surface (Swift and Bignell 2001). Also the spatial structure of the forest may homogenize the impact of both living trees and snags, because they are not spatially divided.

Typically in non-managed forests the amount of dead wood is large which improves the abundance of invertebrates, hence positively affects the whole ecosystem. Our study indicates that snags do not enhance the diversity and biomass of soil fauna, therefore they do not compensate for the lack of decaying tree trunks. Soil invertebrates play an important role in the turnover of nutrients and organic matter and serve as a dietary component for small vertebrates (Gonzales and Seastedt 2001, Ca´rcamo et al. 2001). Thus the lack of

dead trunks may result in lower rate of renovation, lower diversity also in vertebrate community and disturbances in trophic relations among different groups of organisms in constantly and extensively managed forests that are devoid of decaying tree trunks.

Our research was conducted in small scale, on one species of tree and in one type of forest. The role of snags, however, is so far poorly understood which makes our findings a valuable contribution towards better understanding of this phenomenon and calls for further research. We postulate to investigate the role of snags in a wide scale and in a variety of environmental conditions. Additionally such knowledge may improve ecological bases of forest management plans.

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Letters to the editor

Dear Mark Adams

Editor in Chief

Forest Ecology and Management

On behalf of me and co-authors I am sending a manuscript concerning an important aspect of forest ecology, namely the role of tree snags on characteristics of soil invertebrate fauna. Our project explored the role of dead pieces of trees that remains after forest clearings often being the only source of decaying wood in forest under constant management. So far most studies neglected this aspect focusing only on dead tree trunks. We have found that snags does not enhance species richness or the biomass of soil fauna, hence they do not fulfill the function of dead wood decaying on the surface of forest bottom.

We believe that our findings have important implications for forest management. Therefore I consider our manuscript as a valuable contribution towards better understanding of the role of dead wood in shaping the structure and dynamics of forest ecosystems

Yours sincerely,

Stanislaw Bury

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18.09.2016

The Editor of
Biological Conservation

Dear Sir or Madame,

We would be grateful if you could consider our manuscript entitled “What features of invertebrate community are affected by the presence of fir snags?” to accept to publication in the Biological Conservation.

We have carried out research showing that tree snags do not compensate for the lack of dead tree trunks, and extensively managed forests are inevitably devoid of important component of soil invertebrate community. The role of snags, however, is so far poorly understood which makes our findings a valuable contribution towards better understanding of this phenomenon and calls for further research. Additionally such knowledge may improve ecological bases of forest management plans.

We would like to inform you, that our paper is not under consideration of any other journal, data was not published in the past and the manuscript has been accepted by all of the authors.

Yours sincerely,

Anna Giermek

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18 September 2016

The Editor
Elsevier: Forest Ecology and Management

Dear Sir or Madame,

Please find enclosed manuscript entitled “What features of invertebrate community is affected by the presence of fir snags?”. I would be grateful if you could kindly consider it for publication in Forest Ecology and Management.

The manuscript concerns dead wood as a crucial component for soil invertebrates biodiversity shaping and its role in forestry management. In contrast to previous studies we focused on the snags. Our aim was to test whether presence of snags positively influence soil invertebrates. We have found that both biomass and biodiversity of invertebrates do not differ between soil close to snags and living trees, which indicate that dead roots cannot compensate the lack of fallen trees in the managed forest. In our opinion this result is very interesting and meaningful for the field of forest management.

We declare that the manuscript consist of our original work and contain only unpublished data. It was not under consideration of any other journal. All the authors approved the manuscript and this submission.

Yours sincerely,
Dorota Lutyk

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Reviews

Manfred Bauchinger

The paper fulfills all the formal requirements for publication in the indicated Journal.

The Abstract is informative. Material and Methods give reliable information on the research work. The presentation of the results is convincing. Discussion is convincing as well and conclusions are justified.

To avoid too much explanations a series of editorial changes and comments are directly included in the Word file.

On page 4 the designation of the ordinate in Fig. 4 must be changed into the right order.

Magnitude of biomass should be indicated in Fig. 2 (see also file)

In Table 1 name of the groups: snags and living trees

Weronika Banot

I find the article interesting, not only to specialists, as the topic concerns a general environmental issue and does not require detailed knowledge to be understood. However, I have some suggestions that you could take into consideration.

Title

The title gives proper information about the study; however, I find it not fully grammatically correct and would suggest e.g. 'Which features (...) are affected (...)'.

Abstract

In my opinion it summarizes the study well. However, it is not clear for me what are the 'both characters' mentioned in line 14 because above them you mention also the two biodiversity indices which makes in total 4 'characters' and your study tested two features (biodiversity and biomass).

Introduction

For me it clearly describes the context and reasons of the study. However, there is one misleading sentence in lines 45-49 – I do not understand why are two opposed sentences (connected with 'but') because for me both mean the same implication.

Materials and methods

I appreciate that you gave detailed information about study location. I have one doubt relating to the method of data analysis. From line 76 it can be concluded that the taxa analysed will be orders. However, in Table 1 with results although heading of the first column states that there are order names presented, data are in fact not always assigned to the order but in some cases to the higher taxon: type (Nematoda) or class (Clitellata). How does it affect calculations of biodiversity indices?

Results

Results are presented clearly, although you omitted some information. In lines 88-89 you state that all order representatives except Pseudoscorpionidae were present in both groups but you do not mention that, as shown in Table 1, also Hemiptera, Coleoptera, Diploptera and slugs were present in only one of the groups. Moreover, you could explain what each of the biodiversity indices informs about and discuss it.

Discussion

The discussion is interesting and logically explains the results with relation to other studies. Contribution of your study to its field is convincing. I suggest only a correction in line 106, where you state about no differences in the *number of taxa* while in fact you compared not species richness but other biodiversity indices: Shannon-Wiener and Simpson.

References

The list of references should be unified.

There are also some linguistic, grammar or spelling comments.

-line 24: 'which providing' -> 'providing' or 'which provide'

-line 32: unnecessary 'as'

-line 54: should be ‘influences’

-line 74: ‘blindly extracted’ is misleading, suggests extracting animals without looking at the sample.

-line 76: should be ‘assigned’

-line 85: I would change ‘together’ for ‘in total’; identify -> ‘identified’

-Table 1- column heading: I suggest ‘individuals’ instead of ‘invertebrates’ because it seems to me more precise as ‘invertebrates’ could be mistaken for the number of species.

-line 106: use consequently one tense in the sentence

-line 124: I found this sentence difficult to understand, try to reformulate it or divide into shorter sentences.

Małgorzata Lipowska

The title of the report is slightly misleading, suggesting a much wider and more detailed study than one that was performed. It would be more accurate if it referred to the features that were the object of study, that is biodiversity and biomass of invertebrate communities. Moreover, the title omits the important fact that the objective of the study was to evaluate the presence of decaying roots – the word “snag” may refer to a dead tree part of any height and state of decomposition. In this case the studied “snags” were as short as stumps due to being cut rather than broken, and has been dead for a long time. Therefore I suggest that the title should be changed to better fit the subject of the study.

The abstract is clear and compact, introducing the reader to the role of dead wood in forest biology. It presents both results and discussion in a brief yet accurate manner, however the concluding remark feels too broad for the study that did not involve comparison of dead roots with dead tree trunks.

The introduction is broad and supported by satisfactory number of quotations. The only thing it seems to lack of is the information of what amount of a tree stays underground – and, as a result, what percentage of total wood mass is being lost due to forest management. What would also be worth discussing is the size of the area affected by decaying tree, and proportion of snag density between wild and managed forests.

From the technical point of view the importance of Figure 1 is questionable – especially since publishing it would require color printing. The figure is not essential for the work, as it only illustrates the sampling site which is already described in the text. Other than that the paper fits in the publisher's criteria for word count and structure, is well-written and easy to read, with only a few grammar errors.

I find the work interesting and novel. The topic could be easily expanded by introducing additional factors, and can be a subject of further study. In the future, it would be interesting for example to compare the soil around dead roots, dead tree trunks and lacking any supply of dead wood. Therefore this paper can get other researchers interested in this field. The lack of statistical significance can be due to the limited scope of study, so the authors should be encouraged to perform more research on the topic they present.

Diversity and biomass of soil invertebrates are not affected by the presence of fir stumps

Stanisław Bury, Anna Giermek*, Dorota Lutyk

Abstract

Dead wood has been proved to constitute as a crucial component for forest biodiversity and its amount is an outcome of forestry management. So far most studies concerned the effect of decayed fallen trees on various aspects of surrounding communities. As most of the forests are managed and the amount of fallen trees is highly limited the only sources of dead wood are stumps left after forestry clearance. In our project we explored the role of fir stumps in shaping diversity and biomass of soil invertebrates. We have found that both biomass and biodiversity do not differ between stumps and living trees. It seems that stumps do not compensate for the lack of dead tree trunks and extensively managed forests are inevitably devoid of important component of soil invertebrate community.

Introduction

Dead wood at different stages of decay has an important ecological role in shaping the diversity of many different organisms in forest ecosystems. Dead trees are valuable habitats providing for example food, shelter and breeding conditions for a large number of rare and threatened plant and animal species: lichens, bryophytes, invertebrates, amphibians, birds and mammals (Jonnson et al 2005). Large quantities of dead wood also play a significant role in forest nutrient cycles, carbon budgets, soil morphology and natural regeneration. The amount of dead wood is highly variable and depends mostly on the intensiveness of forestry management. Unfortunately, only a low amount of dead wood is present in most managed Central European forests (1–3 m^3 /ha, Ammer 1991). In Central Europe only 0.2% of the deciduous forests are in a relatively natural state which includes a high amount of dead wood (50–200 m^3 /ha, Korpel 1995).

Soil invertebrates constitute as a group that is strongly dependent on dead and decaying wood. Invertebrates play also an important role in forest ecosystems by improving soil fertility, productivity and structure of the ecosystems and play a critical role in nutrient cycling through facilitating decomposition of organic matter and decaying roots

(Gonzales and Seastedt 2001; Ca´rcamo et al. 2001). Soil invertebrates connect primary production to secondary consumers, influence vegetation species composition and constitute important links in detritus-based food chains (Brown and Gange 1990, Edwards and Bohlen 1996, Ponsard et al. 2000, Lavelle and Spain 2001). Therefore it is of special importance to get insight into the relationship between the presence and type of dead wood and the structure of soil fauna.

So far most studies explored the effect of dead tree trunks on the soil fauna. However in most managed forest the only sources of decaying wood are stumps that remain after forest clearings. Surprisingly, there are almost no studies that explore the effect of the presence of stumps on the soil fauna. Understanding of how does the presence of the dead roots shape the community of soil invertebrates will allow us to evaluate if such leavings increase species richness and biomass of soil invertebrates, hence to predict the potential impacts of future environmental changes on soil invertebrates in the context of forest management.

In our study we investigated the diversity and biomass of soil invertebrates living on the forest floor of a managed beech–fir forest in which nature oriented forestry is practiced. The objective of this research was to study the impact of old tree stumps on community of soil invertebrates. We addressed the following hypotheses:

1. Presence of stumps influences biodiversity of soil invertebrates
2. Presence of stumps influences biomass of soil invertebrates.

For both of them we predict positive effect of stumps.

Material and Methods

Study area:

The project was performed in the managed beech-fir forest in Gorce Mountain. The study area was situated 2 km from the border of Gorczański National Park, on the altitude of 670–700 m a.s.l. The forest is dominated by beech (*Fagus sylvatica*), with significant share of silver fir (*Abies alba*).

Soil sampling:

To test whether invertebrates' biodiversity or biomass differ between soil close to the dead roots (stumps) and next to the roots of living trees, five samples of soil (10x10x10 cm) of

each site type were collected. Sampling sites were located not further than 10 cm distance from the tree trunk. All of the investigated trees were firs of similar age (with a diameter of 44–51 cm), randomly chosen in the same type of forest.

Invertebrates (see Table 1.) from each soil sample were extracted by hand-sorting and each individual was gently transferred to a petri dish. Specimens were identified under binocular and assigned to the order (according to Jura 2007). All invertebrates from one soil sample were weighed on the electronic balance (KERN, Germany, d=0.01g) to assess the biomass [g/dm^3].

The biodiversity of each sample was assessed using Shannon–Wiener biodiversity index. To analyze community structure we also calculated Simpson dominance index. Both indexes were calculated according to Keylock (2005). Differences between groups in terms of biodiversity and biomass were analyzed using Mann–Whitney U test. Non-parametric test was chosen because of lack of normal distribution, caused by small sample size.

Results

In total we have extracted and identified 144 individuals (69 in samples from living tree and 75 in samples from stump groups) belonging to 15 different invertebrate orders (13 in samples from living tree and 12 in samples from stump groups, Table 1.).

Pseudoscorpionideae and Coleoptera were found only in samples from the stump group. Slugs, millipedes and hemiptera were found only in the samples from the living tree group. All other order representatives were found in both groups. There were no differences between invertebrates in soil collected near stumps and living trees in terms of biomass (medians of living trees: 0.10, stumps: 0.22; Mann–Whitney: U=8.0, p=0.4, N=10, Figure 2.). Biodiversity also did not differ between groups (Shannon–Wiener index: medians of living trees: 1.51, stumps: 1.53; Mann–Whitney: U=12.0, p=1.0, N=10; Simpson index: medians of living trees: 0.25, stumps: 0.28; Mann–Whitney: U=9.0, Z = 0.63, p=0.53, N=10).

Tab. 1. Number of taxa and individuals found in each sample site.

Taxa	number of invertebrates											
	stumps						living trees					
	1	**2**	**3**	**4**	**5**	sum	**1**	**2**	**3**	**4**	**5**	sum
NEMATODA (Roundworms)	1		1	1		3	3			4		7
ANNELIDA (Segmented worms)												
Clitellata (earthworms)		1		1		2	1			3		4
ARTHROPODA												
1. ARACHINIDA (Arachnids)												
Araneae (spiders)	2	1		3	1	7			4	2	1	7
Acarina (mites, ticks)		10	3	1	2	16	1		2	3		6
Pseudoscorpionidae (pseudoscorpions)				1	6	7						0
2. INSECTA (Insects)												
Hemiptera (true bugs)						0					1	1
Coleoptera (beetles)	2			1	1	4						0
Diptera (true flies)				1		1					1	1
Lepidoptera (butterflies, moths)		1				1				2		2
Collembola (springtails)	3	2			9	14		4	2	8	1	15
3. MYRIAPODA												
Chilopoda (centipedes)	1	3	5	5	2	16	3	1		9		13
Diplopoda (millepedes)						0		2				2
ISOPODA (pillbugs, woodlice, isopods)	2		1			3	1	4	1	3		9
MOLLUSCA (Molluscs)												
slugs						0				1		1
snails					1	1		1				1

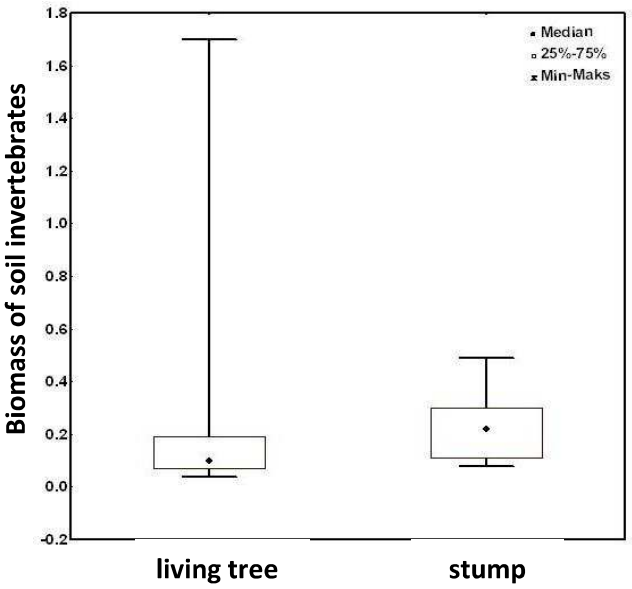

Fig. 2. Biomass of soil invertebrates in two studied groups.

Discussion

Our results are contrary to predictions and do not reveal any significant differences in biodiversity and the biomass of soil invertebrates between the stump and living trees group. It clearly indicates that dead roots do not enhance any measure of invertebrate community. Small differences in the composition have been noticed for pseudoscorpions, beetles, slugs, millipedes and true bugs, which were found only in the soil from one site. However, due the low numbers making any strong statements is far premature.

In the studies of Maguire (2002) and Odor et al. (2006) a strong and positive influence of decaying tree trunks on the diversity of various organisms such as fungi, insects and even small vertebrates has been reported. This contrasts with the present findings in stumps that are often the only decaying wood in managed forests. This may be explained by the fact that the largest part of the stump remains deeply underground whereas only small part of it is easily available to the soil organisms. Actually Swift and Bignell (2001) reported the highest diversity of invertebrates up to 30 cm depth below surface. Also the spatial structure of the forest may homogenize the impact of both living trees and stumps, because they are not spatially separated.

Typically in non-managed forests the amount of dead wood is high which increases the abundance of invertebrates, hence positively affecting the whole ecosystem. Our study indicates that stumps do not enhance the diversity and biomass of soil fauna and therefore, do not compensate for the lack of decaying tree trunks. Soil invertebrates play an important role in the turnover of nutrients and organic matter and serve as a dietary component for small vertebrates (Gonzales and Seastedt 2001, Carcamo et al. 2001). Thus the lack of dead trunks may result in lower rate of renovation, lower diversity also in vertebrate community and disturbances in trophic relations among different groups of organisms in constantly and extensively managed forests that are devoid of decaying tree trunks.

Our study was conducted in small scale, on only one species of tree in a managed beech–fir forest. Since so far, the role of stumps, is poorly understood we feel that our findings are a valuable contribution towards better understanding of this phenomenon and calls for extended research. We, therefore, postulate to investigate the role of stumps in a wider scale and in a variety of environmental conditions. Additionally such knowledge may improve ecological bases of forest management plans.

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How does the grazing affect morphology of plants?

Project

Title: How does the grazing affect morphology of plants?

Authors: Michela Corsini, Małgorzata Lipowska

Summary: We want to investigate the shift of life history traits in plants inhabiting areas subjected to intense cattle grazing. We predict a morphological adaptation due to the increased risk of being consumed. We are going to study morphological traits of a common meadow plant species, white clover (*Trifolium repens*). We expect a reduction in terms of plant size on cattle pastures, that would benefit the plant by making it harder to graze on.

Aim/Hypothesis: The leaves and stems of white clover (*Trifolium repens*) are reduced in size as a response to cattle grazing.

Methods: We will identify specific areas: three cattle pastures and three cattle-free meadows. We will pick sites characterized by similar local climatic and environmental conditions. Within each site we will set up three 3-m transects. We will collect white clover (*Trifolium repens*) plants at 0, 10, 20 etc. cm along each transect. We will measure each specimen in terms of stem length as well as width and length of individual leaves. This data will allow us to calculate the area of each leaf and total leaf area per plant. Finally we will perform a nested ANOVA analyses of each parameter in order to test clover morphology differences in relation to cattle grazing events.

Impact of results: The result of this study will give us insight into clover's adaptation ability to environmental challenges imposed by intense grazing habits of domestic animals. This will allow us to understand whether life history traits of white clover get shifted by high risk of being consumed.

The impact of cattle grazing on morphology of meadow flora – a study on white clover (*Trifolium repens*)

Summary

Grazing cattle is one of the main factors affecting meadow ecosystems. Numerous studies have reported changes and adaptations of local flora to grazing activity in pastures. Our study focused on a common herbaceous meadow species, white clover (*Trifoluim repens*) which is characterized by high palatability index and thus being commonly consumed by cattle. We expected to observe a reduction in plant size on pastures, decreasing the risk of being consumed. The measurements of stem and leaf length of plants sampled on cattle pastures and cattle-free meadows have partially proven the thesis of size reduction on pastures. However, the observed relation may also be explained by a shift in recruitment, leading to lower average age of plants growing on cattle-grazed sites. A more in-depth study eliminating the factor of age difference between the sites should be performed. This would allow to understand the observed trend towards shortening of stem in white clover plants growing on pastures.

Introduction

The variation of plants attributes is driven by a complex combination of different, simultaneously acting factors and their interactions. Among them livestock grazing is one of the strongest, being able to produce a shift in plants' life-history traits. The identification of traits which explain and predict the response of plant communities to intense cattle grazing is one of the main tools in management of grazing systems (Coley et al, 1985). Furthermore, previous studies explained how grazing ungulates have generally shaped ecosystems around the world at local and global scale, especially because of a profound impact on plant communities. The main reason of this alteration is correlated to the nature of plants response in grazed areas (Acosta et al., 1989). Among all these studies, a general decrease in terms of plant height and leaf size as result of cattle grazing has been widely documented (Sala et al. 1986; Ellison, 1960; Diaz et al., 1992). However, all of these studies lead to different outcomes at single species level. Diaz classified species by single life-history trait (annual or pluriannual species), plant height and grazing response; this study pointed out that grazing-resistant species were shorter in height with smaller and more tender leaves, while grazing-susceptible species disappear from pastures rather than

adapt to them (Diaz et al., 2001). The experiment also showed how leaf resistance is associated with low palatability and grazing avoidance. The pressure given by cattle grazing on plant traits may have important consequences at latest stage: Austrheim observed lower recruitment in plants characterized by a higher specific leaf area on disturbed-pasture areas. The opposite has been observed in *Silene dioica*: the same experiment proved that this species does not have a significant reaction in terms of recruitment capacity, probably because of its low palatability index.

Understanding the impact of grazing on grassland biology is of fundamental importance for both their conservation and management. However, the knowledge of this phenomenon remains insufficient and more empirical data on plants' responses to grazing activity are needed. In this study we focused our attention on traits response of a herbaceous perennial plant, white clover (*Trifolium repens*), which is native to Europe and central Asia. This species is commonly found on both wild meadows and pastures, and is characterized by a high palatability index (Hovelqnd, 2014). Based on the results of previous studies, we hypothesized that the plant of interest will reduce its size as a consequence of cattle grazing. Such adaptation would aid the plant by making it more difficult to be grazed on. We investigated the response in terms of traits variability within different sites which are characterized by the presence or absence of cattle activity.

Materials and methods

The study was performed in Ochotnica Górna (Poland), in the Jaszcze valley. For the purpose of the study three meadows being utilized as cattle pastures and three meadows beyond cattle's reach were identified in the area. On each meadow three 3-m transects were set up and white clover plants were collected at 0, 30, 60 etc. cm along the transect. The samples were then measured by observers blind to their origin. The parameters measured were stem length, length of individual leaves and leaf damage. In the preliminary study the total leaf area was proven to be correlated with mean leaf length.

The data was analyzed with MIXED procedure offered by SAS 9.4 software. The influence of grazing on stem length and mean leaf length was analyzed with nested ANOVA including factors of grazing exposure, individual meadow nested within grazing exposure, transect number within each meadow and a random factor of observer. In order to test the uniformity of plant size difference between pastures and cattle-free meadows an ANCOVA analyses was performed expanding the ANOVA model used on stem length by adding

mean leaf length as a cofactor. A similar ANCOVA model was used to analyze the effect of grazing on the number of damaged clover leaves.

Results

The sampling procedure has provided measurements of 156 white clover plants sampled in the field. The nested ANOVA analyses has proven that both stems and leaves of white clover are shorter in areas subjected to grazing ($p=0.043$ and $p=0.022$ respectively, Fig. 1). After the expansion of the model by a covariate of mean leaf length, the influence of grazing on stem length was found to be weaker. However, the trend for stems being shorter on pastures than on graze-free areas persisted ($p=0.075$). The model has also proven a strong correlation between leaf and stem length ($p<0.001$, Fig. 2). The influence of cattle on leaf damage was not statistically significant ($p=0.448$).

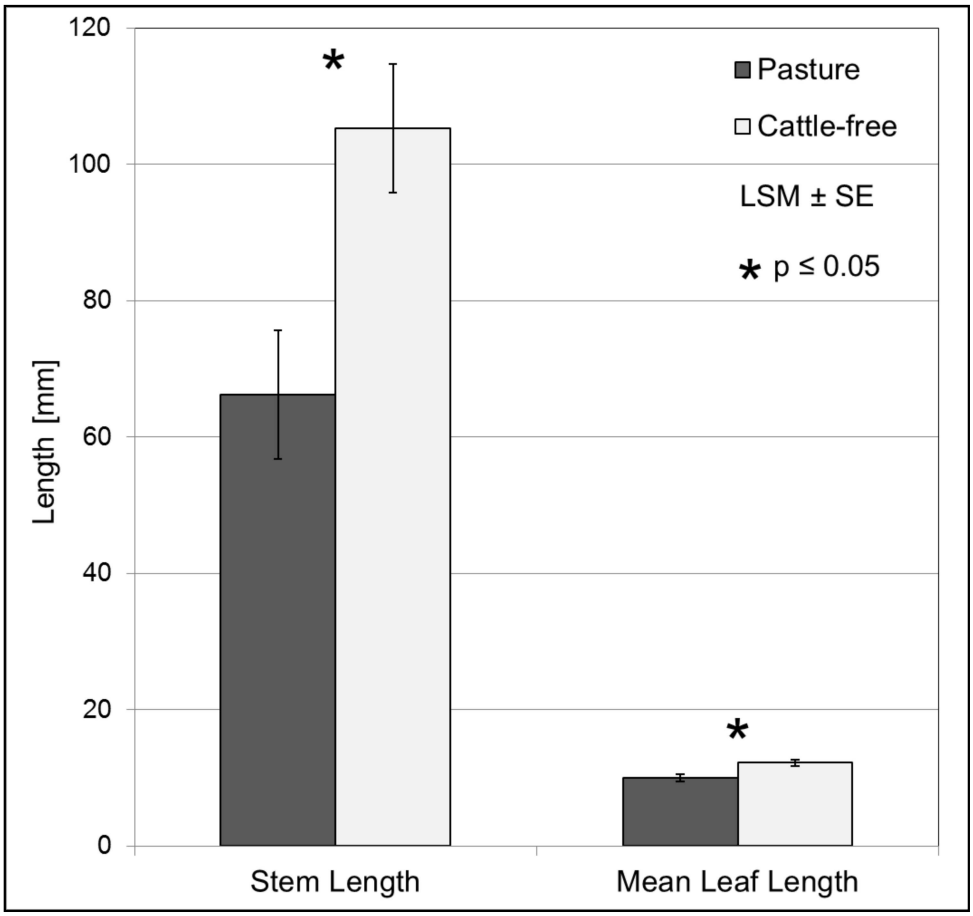

Fig. 1 Stem and mean leaf length of white clover plants sampled on pastures and on cattle-free meadows, derived from nested ANOVA analyses performed for both variables.

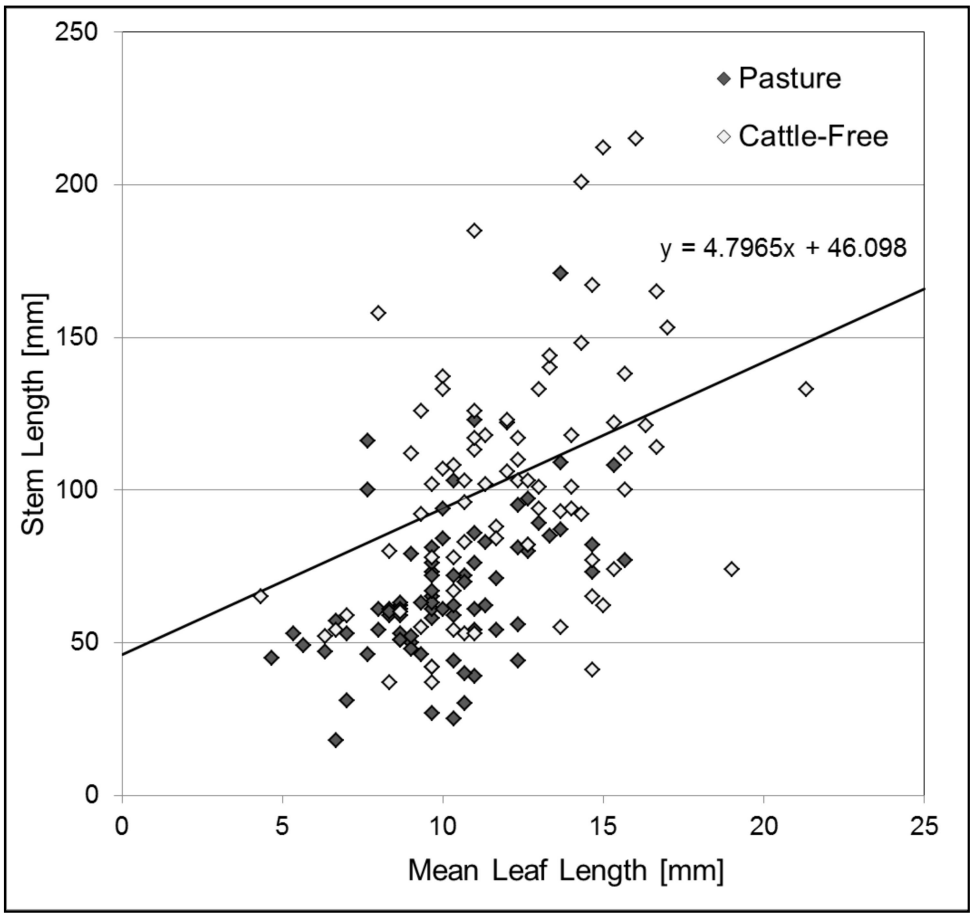

Fig. 2 Correlation between stem length and mean leaf length of white clover plants sampled on pastures and cattle-free meadows. The regression line parameters were derived from the ANCOVA analyses of mean stem length with mean leaf length as a cofactor.

Discussion

In agreement with the expectations, we have found that white clover plants growing on cattle pastures were smaller in size than the ones from non-grazed meadows. However, the decrease in size involved both stem and leaf length, therefore it is not clear whether the observed relation is not due to the age difference between the specimens in specific sites. It is to be expected that the presence of grazing cattle shortens lifetime of edible meadow plants, therefore the fraction of young sprouts on a pasture could be higher than on non-grazed meadows. The data collected in this study are insufficient to make a distinction between this effect and morphological adaptation. Nevertheless we have found a trend towards shortening of stem in areas affected by cattle grazing, that would benefit the plants by making it more difficult to graze on. A manipulative study controlling for the factor of plant age would help to make a more definitive answer to that question.

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09/18/2016

The Editor:
Agriculture, Ecosystems & Environment

To whom it may concern,

Please find in attachment the manuscript entitled "The impact of cattle grazing on morphology of meadow flora- a study on white clover *Trifolium repens*".

Since our study responses to the requirements of your journal, we would like to be considered for a publication on it. Our research is based on data collected in the field and is focused on the plant community response on cattle grazing pressures in terms of size traits.

Since previous studies have largely proved how life- history traits of plants may be modified due to an intense grazing activity, we decided to introduce a new approach to the phenomenon analyzing a common pluriannual species, the white clover. This species is widely distributed in both not-grazed and grazed meadows in Poland: this has been an interesting opportunity to measure and compare general life-history traits among these two different contexts, at local level. As expected, we found that white clover plants on cattle pastures were smaller in size if compared with the ones from non-grazed meadows. However, we also found a trend of shorter stems in areas characterized by cattle grazing presence. Our results help in understanding the relevance of grazing activities on plant communities, which are modified in terms of distribution in space and time. It also suggests that grazing-resistant species (like white clover) may be strongly modified in terms of sizes.

I would like to specify that this research is presented in its original version and has not been published in other journals. Both of the authors agreed with its contents and in submitting it.

Regards,

Michela Corsini

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19.09.2016

The Editor of
Rangeland Ecology & Management

Dear Sir or Madam,

Please find attached a manuscript "The impact of cattle grazing on morphology of meadow flora – a study on white clover (*Trifolium repens*)" by Michela Corsini and myself. We kindly ask you to consider it for submission in your journal, Rangeland Ecology & Management.

We are convinced that the topic of our study is highly appropriate for your journal. To our knowledge the particular subject is novel and encourages further research in this topic. In further perspective the gathered knowledge may find use in application studies.

It is widely known that grazing cattle has a strong impact on the ecology of meadows. We have concentrated on a common meadow plant, white clover, that due to high palatability is particularly commonly grazed upon. Our hypothesis was that the plant adapts by decreasing its height, which would make it more difficult for cattle to consume. We have found that white clover plants found on pastures are generally smaller than ones sampled at cattle-free meadows, although this might be explained by age difference and cattle-mediated increase in recruitment. However, we have also found the plants growing on pastures show a trend towards developing shorter stems than the ones sampled on cattle-free meadows. In our opinion this topic is worth further investigation in wider scale.

Data and findings presented in the manuscript have not been published nor are in the process of getting published in any other journal. The project and manuscript has been prepared solely by the two authors that have been named. Both authors have approved the manuscript to be published in its current form.

Thank you in advance for considering our manuscript.

Sincerely,

Małgorzata Lipowska
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Reviews

Adam Łomnicki

Taking into account the conditions under which it was prepared, this is quite good report. However, I have the following reservations and remarks.

Line 39: the name (?) of Austrheim is given without a year of publication and it is not listed in the references. Is it a person, a product or an institution?

Lines 40-42: The comparison with *Silene dioica* is not described clearly enough.

Lines 43-45: Two sentences written there are like rewritten from other article based on several years of studies. They do not match this report.

Line 59: The description of plant collection is not precise enough to repeat this study. A three meter transect is a line and 0, 30, 60 and so on cm along this line are points without dimensions. At this point there is a clover plant or it is not. Do we select always any clover plant nearest this point or only search for such a plant well determined distance (?) but not further. According to my calculation if any plant is measured at any point in all transect one would expect that 198 plants are measured but according to line 73 only 156 plants were measured. This should be corrected so that any reader would be able to repeat precisely this study.

Line 69: More should be written on the preliminary studies, at least sample size, correlation or regression coefficients should be presented.

Lines 75-76. I do not understand what for have you applied ANCOVA with a covariate which is not independent of grazing. The only reason can be an ambition to make the statistical description unnecessarily more sophisticated.

Lines 79-80. In the description of the methods, I have not found a word on leaf damage and how it was studied and estimated. Either this sentence should be removed or more should be written on leaf damage.

Line 82, Fig.1. I know the acronym LSM does not refer to lesbian sex mafia but it has so many meanings that in should explained in the fig. description.

Anna Giermek

The paper consider a very interesting study on the impact of cattle grazing on morphology of plants. As a model species authors used clover (*Trifoluim repens*) which is commonly used plant during pasturage. The paper might enhance a general knowledge on defensive mechanisms and life history traits and could be interesting not only for the specialist from this field.

This article is well written and easy to follow, although needs some grammar corrections. Title and abstract accurately describe the contents of the article. The hypothesis is stated clearly, the methods used to test it are well designed and the results are correctly analysed. The length of article is justified, however, require more discussion of the results. Some information about performed analyses is written in the 'results' but in my opinion should be placed in 'materials and methods' (i.e. about statistical analysis in the line 76). Graphics are clear and contains all necessary elements.

Unfortunately the design of the experiment is not perfect. Although, authors found significantly shorter stems on pastured meadows, they are not able to separate effects of cattle grazing from the clover age. It would be interesting to check in the future whether the effects is heritable and lasting on the specimens which are from different treatments but at the same age (for example sampling before start of the grazing season or performance of common garden experiment).

Jowita Niedojadło

The study "The impact of cattle grazing on morphology of meadow flora – a study on white clover (*Trifolium repens*)" raised interesting topic of relations between grazing plants and cattle. Results of such study can be found interesting for many specialist working on field of ecology or botany but also many non-specialist.

Authors nicely prepared background of their study in introduction pointing several publication with mixed results, also significance of such study was clearly presented and explained. Moreover the hypothesis and predictions were formulated correctly based on literature review. Statistic prepared in study was used correctly for type of sampled data. Worth noting is clear presentence of data on graphs. In this section I personally missed F – values, which are essential part of results. Unfortunately lack of possibility to distinguish

between effect of experiment and age of plant could be a serious problem of this study. Authors are aware of this limitation and their interpretation of results is restrained. In my opinion idea standing behind project and knowledge that can be obtained from it could have serious impact on the knowledge in this field, if problem of plants' age will be solved. Authors claim on the end of discussion that laboratory manipulation will be needed to control for age effect and I also claim that this will be the most appropriate approach.

In summary I fully support concept of this study, but unfortunately method used to test the hypothesis was inadequate.

Report – final version

The impact of cattle grazing on morphology of meadow flora – a study on white clover (*Trifolium repens*)

Michela Corsini, Małgorzata Lipowska

Summary

Grazing cattle is one of the main factors affecting meadow ecosystems. Numerous studies have reported changes and adaptations of local flora to grazing activity in pastures. Our study focused on a common herbaceous meadow species, white clover (*Trifolium repens*) which is characterized by high palatability index and thus being commonly consumed by cattle. We expected to observe a reduction in plant size on pastures, decreasing the risk of being consumed. As expected, the stems of white clover plants growing on pastures were 28% shorter than those growing beyond cattle reach. The measurements of stem and leaf length of plants sampled on cattle pastures and cattle-free meadows have partially proven the thesis of size reduction on pastures. However, the observed relation may also be explained by a shift in recruitment, leading to lower average age of plants growing on cattle-grazed sites. A more in-depth study eliminating the factor of age difference between the sites should be performed. This would allow to understand the observed trend towards shortening of stem in white clover plants growing on pastures.

Introduction

The variation of plants attributes is driven by a complex combination of different, simultaneously acting factors and their interactions. Among them livestock grazing is one of the strongest, being able to produce a shift in plants' life-history traits. The identification of traits which explain and predict the response of plant communities to intense cattle grazing is one of the main tools in management of grazing systems (Coley et al, 1985). Furthermore, previous studies explain how grazing ungulates have generally shaped ecosystems around the world at local and global scale, especially because of a profound impact on plant communities. This alteration usually correlates with the nature of plants response in grazed areas (Acosta et al., 1989). All these studies document a general decrease in terms of plant height and leaf size as result of cattle grazing (Sala et al. 1986; Ellison, 1960; Diaz et al., 1992). However, all of these studies lead to different outcomes at single species level. Diaz classified species by single life-history trait (annual or

pluriannual species), plant height and grazing response (Louault, 2005). This study pointed out that grazing-resistant species were shorter in height with smaller and more tender leaves, while grazing-susceptible species disappear from pastures rather than adapt to them (Diaz et al., 2001). The experiment also shows how leaf resistance correlates with low palatability and grazing avoidance. The pressure given by cattle grazing on plant traits may have important consequences at latest stage: Austrheim observed lower recruitment in plants characterized by a higher specific leaf area on disturbed-pasture areas. The same factor did not elicit a change in recruitment capacity of *Silene dioica*, probably because of its low palatability index (Austrheim, 2003).

Understanding the impact of grazing on grassland biology is of fundamental importance for both their conservation and management. However, the knowledge of this phenomenon remains insufficient and more empirical data on plants' responses to grazing activity are needed (Pakeman, 2004). In this study we focused our attention on traits response of a herbaceous perennial plant, white clover (*Trifolium repens*), which is native to Europe and central Asia. This species is commonly found on both wild meadows and pastures, and is characterized by a high palatability index (Hovelqnd, 2014). Based on the results of previous studies, we hypothesized that the plant of interest will reduce its size as a consequence of cattle grazing. Short plants are harder to graze on, and have higher chance of survival on a pasture. We investigated the response in terms of traits variability within different sites which are characterized by the presence or absence of cattle activity.

Materials and methods

The study was performed in Ochotnica Górna (Poland), in the Jaszcze valley. For the purpose of the study three meadows being utilized as cattle pastures and three meadows beyond cattle's reach were identified in the area. On each meadow three 3-m transects were set up and white clover plants were collected at 0, 30, 60 etc. cm along the transect. A single plant has been chosen (if present) within a 3-cm radius around each designated point on the transect.

Samples were then measured by observers blind to their origin. The parameters measured were stem length and length of individual leaves. In the preliminary study the total leaf area was proven to be correlated with mean leaf length ($N=3$, $R^2=0.96$).

The data was analyzed with MIXED procedure offered by SAS 9.4 software. The influence of grazing on stem length and mean leaf length was analyzed with nested ANOVA including factors of grazing exposure, individual meadow nested within grazing exposure,

transect number within each meadow and a random factor of observer. In order to test the uniformity of plant size difference between pastures and cattle-free meadows an ANCOVA analyses was performed expanding the ANOVA model used on stem length by adding mean leaf length as a cofactor.

Results

The sampling procedure has provided measurements of 156 white clover plants sampled in the field. The nested ANOVA analyses has proven that both stems and leaves of white clover are shorter in areas subjected to grazing ($F_{1,4}$=8.58, p=0.043 and $F_{1,4}$=13.08, p=0.022 respectively, least squares means (LSM) and standard errors (SE) presented on Fig. 1). After the expansion of the model by a covariate of mean leaf length, the influence of grazing on stem length was found to be weaker. However, the trend for stems being shorter on pastures than on graze-free areas persisted ($F_{1,4}$=5.75, p=0.075). The model has also proven a strong correlation between leaf and stem length (ρ=0.21, p<0.001, Fig. 2).

Fig. 1. Stem and mean leaf length of white clover plants sampled on pastures and on cattle-free meadows, derived from nested ANOVA analyses performed for both variables.

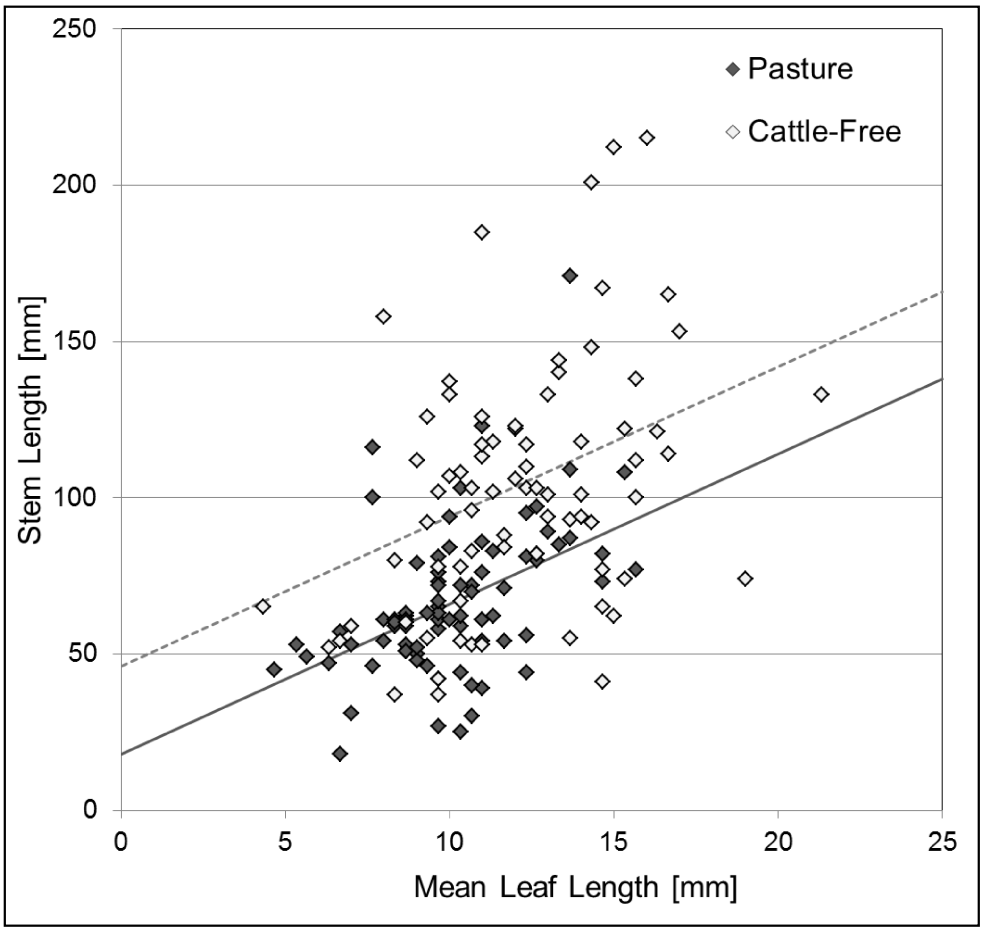

Fig. 2. Correlation between stem length and mean leaf length of white clover plants sampled on pastures and cattle-free meadows. The regression line parameters were derived from the ANCOVA analyses of mean stem length with mean leaf length as a cofactor.

Discussion

We found that white clover plants growing on cattle pastures were smaller in size than the ones from non-grazed meadows, in agreement with the results given by previous studies conducted on several species with one meadow (Ning, 2004). If compared with previous research, our project is novel in terms of the object of measurements and comparisons that was focused on single species. Earlier studies show the direct connection of leaf size and stem length as response to cattle grazing (Diaz, 2001), and our work confirms the observation. This result may be justified by a previous study (Ellison, 1960) which connected this general decrease in size with an increase of plants' density on pastures. This strategy helps the plant to avoid being picking up easily by herbivores. Our study also confirmed the finding of Louault who observed a negative correlation between leaf area and grazing activity (Louault, 2005). Although we did not perform direct measurements of leaf area, our preliminary study indicates that it is strongly correlated with leaf length.

Another idea behind the observed decrease in both stem and leaf length can be age difference between pastures and cattle-free meadows. A study conducted by Gunnar in 2003 justified this leaf area reduction with the highest recruiting probability when exposed to cattle grazing.

Even if the data collected in this study are insufficient to make a distinction between this effect and eventually a morphological adaptation, we have found a trend towards shortening of stem in areas affected by cattle grazing. Therefore, we are convinced that this topic is worthy of further studies and a manipulative experiment controlling for the factor of plant age would help to make a more definitive answer to that question.

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How cows' manure influences number of Fabaceae and number of their root nodules?

Project

Title

How different land use influences number of *Fabaceae* species and their root nodules?

Authors

Weronika Banot, Jowita Niedojadło

Summary

Agriculture modifies natural circulation of elements by supplementation of fertilizers, not only intentional by using artificial fertilizers or manure, but also by pasturage. Supplementation of nitrogen may affect *Fabaceae* presence, because of their strategy of nitrogen intake. Plants from this family cope with low nitrates presence in the soil by symbiosis with rhizobia - bacteria which inhabit roots nodules and fixate nitrogen. Therefore, we expect that increased abundance of nitrates in the soil will result in decreased number of root nodules, because plant will not need to fix nitrogen from the air. Additionally we expect elevated abundance of nitrates to increase number of *Fabaceae* species due to their big nitrogen demand which can be easier fulfilled in fertilized areas. Results of this study will add to the knowledge about human impact on the environment.

Aim/Hypothesis

The aim of this study is to check how land use influences *Fabaceae* species by testing the following hypotheses:

1. Pasturage of cows positively influence number of *Fabaceae* species.
2. Manuring influence to the greater extent number of Fabaceae than Pasturage of cows.
3. Pasturage of cows negatively influence number of root nodules in *Trifolium* sp.

Methods

Three types of land use: control without manuring, manured meadow, meadow pastured with cow will be considered. To test first and second hypotheses, on each type of

land use two 25 m^2 areas (samples) will be randomly chosen and number of *Fabaceae* species will be counted.

To test third hypothesis, five *Trifolium* sp. specimens from each type of land use will be taken carefully with roots. Plants will be taken from randomly selected places on every meadow. On each plant number of root nodules will be counted.

Data will be analyzed using GLM. For first experiment species richness will be response variable, type of land use – explanatory variable and samples - random effect. For second experiment mean number of root nodules will be response variable and land use – explanatory variable.

Impact of results

Study will provide knowledge about impact of agriculture on biodiversity. Results will help in reasonable land management in terms of field fertilization.

Report – first version

TITLE: Effect of meadow use on root nodules in *Trifolium repens* - pilot study and methodological implications

Authors: Weronika Banot, Jowita Niedojadło

Abstract

Agriculture acts on environment by introducing fertilizing substations to the ground, intentionally, like manure, or by pasturage of cows. Changes in nitrates amount in the ground can disturb symbiotic relations between *Fabaceae* plant and rhizobia. In this pilot study, we attempted to answer the question if the meadow use influences amount of root nodules in *Trifolium repens.* We posed the following hypotheses: (1) Pasturage of cows negatively influence number of root nodules in *Trifolium repens*. (2) Manure presence negatively influences number of root nodules in *Trifolium repens*. (3) Impact of manuring in addition to cow pasturing on the number of root nodules is bigger than the impact of sole pasturing. To test the hypotheses we considered three types of meadow use: with manuring, with pasturage of cows and without these treatments. In each type we chose two meadows as repetitions and took five samples from each of them. Our results do not show differences between different meadow use. We draw from the pilot study conclusions concerning methodology. To obtain meaningful results, more repetitions of the same meadow type should be included but there is no need to take more samples from each meadow.

Keywords

Fabaceae, nitrates, manurage, pasturage, land use

Introduction

Agriculture modifies natural circulation of elements by supplementation of fertilizers, not only intentional by using artificial fertilizers or manure, but also by pasturage [1]. Supplementation of nitrogen may affect *Fabaceae* biomass, because of their strategy of nitrogen intake. Plants from this family cope with low nitrates presence in the soil by symbiosis with rhizobia - bacteria which inhabit roots nodules and fixate nitrogen

[2, 3]. Therefore, we expect that increased abundance of nitrates in the soil will result in decreased number of root nodules, because plant will not need to use symbiotic bacteria to fix nitrogen from the air for them.

We conducted pilot study to develop methodology to study the influence of land use on root nodules in *Fabaceae*. For this purpose we studied a common species from this family, *Trifolium repens*, in three types of meadows, all mowed and treated this season in one of the following ways: pasturing, manuring after pasturing and control (without any of the mentioned treatments). In the pilot study, we attempted to test the following hypotheses:

(1) Pasturage of cows negatively influence number of root nodules in *Trifolium repens*.

(2) Manure presence negatively influences number of root nodules in *Trifolium repens*.

(3) Impact of manuring in addition to cow pasturing on the number of root nodules is bigger than the impact of sole pasturing.

Methods

The study was located in Ochotnica Górna, a village set in Gorce mountains in southern Poland. Considered three types of meadows are further referred to as: 'pasturing', 'manuring' and 'control'. Each type was represented by two meadows and from each of them we took five samples of *Trifolium repens*. Samples were ca. 10x10 cm pieces of turf collected with hoe. From each sample we carefully extracted *Trifolium repens* specimens with roots and counted number of root nodules as well as weighted the mass of the overground plant parts (further referred to as 'biomass'). From these data the amount of nodules, defined as number of root nodules per gram of biomass, was calculated.

Data were analyzed using mixed model. The amount of nodules was a response variable, type of meadow – explanatory variable and repetition – random variable nested in type of meadow.

Next, to answer the question if results differed more between type of meadow than within the types and if results between repetitions in each type differed more than samples within one repetition, we calculated the between group variances and within group variances on each level using GLM.

Results

The analysis of amount of nodules showed no differences between types of land use ($F_{2.3}$=3.25, p=0.18). Results are presented on Fig. 1 as LSM ± SE.

Considering meadow types, the ratio of between group variance to within group variance was *F*=2.78 (df = 2, p=0.09). Concerning repetition in meadow type the ratio of between group variance to within group variance was F=0.31 (df = 3, p=0.82). This result means that within the meadow type repetitions do not differ from each other more than do samples within a repetition. However we did not manage to show that the meadow types differ from each other more than do repetitions within a meadow type.

Discussion

Our results did not support any of posed hypothesis about impact of meadow use on the amount of root nodules. The number of root nodules per g of biomass occurred to be not influenced by the studied types of meadow use. However, studies on soybean and legume species, show negative impact of fertilization and nitrates content in soil on many features of root nodules, e.g. the affecting the process of their formation (noduling), number of nodules or increasing their disintegration [4-8]. On the other hand, in some cases natural fertilizers such as manure were shown to have positive influence on nodules formation [9], which can be related to improvements in the soil structure and above all to avoidance of the phenomena of water stress [10]. Unfortunately our studies failed to show any impact of meadow use on the amount of root nodules in *Trifolium repens*. However, lack in differences can be explained from methodological point of view. High variance in the analyzed types of meadow use can decrease our power to detect the effect despite of the differences in the LSM (LSM±SE: in control 57.4±0.2, in pasturaged meadows 49.4±0.2, in manured meadows 22.2±0.2) (Fig. 1). Thus, we conclude that increasing the number of samples should allow to detect the difference between the effect of meadow use, at least the difference between manurage and other types.

The result considering variance between meadow types did not reach the significance level on which we could conclude that the amount of nodules differed more between meadow types than within each type. We suppose involving more repetitions within each meadow type would allow to detect this effect. In addition, comparison of

variance on the lower level of study design, i.e., the repetition within meadow type, did not show that the variance between repetitions is different than within repetition. This implicates that the number of samples per repetition is adequate.

Pilot study showed that to allow detection of differences in meadow use on the amount of root nodules the number of samples should be increased. Comparison of variances leads to the suggestion that increasing number of samples could be reached by including more repetitions of the same meadow type but does not require taking more samples from each meadow.

Competing interests

We have no competing interests.

Authors' contribution

WB and JN designed and contucted the study. JN did the statistical analysis. WB and JN interpreted the results and prepared the manuscript.

Acknowledgements

We would like to thank local people of Ochotnica Górna for the access to their grounds. We thank Joanna Rutkowska for advise in study design and statistics. We thank other course participants for conclusive discussions.

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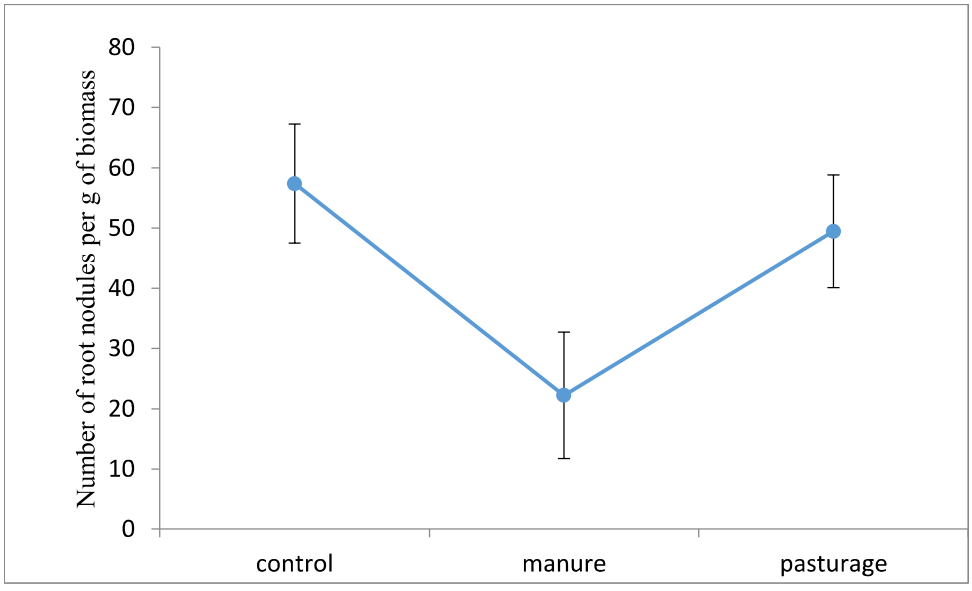

Fig. 1. Number of root nodules per g of *Trifolium repens* above ground biomass. Results are presented as LSM ± SE.

18. September 2016

The Editor
Crop Science

Dear Sir or Madame,

We would like to ask you to consider our manuscript entitled 'Effect of meadow use on root nodules in *Trifolium repens* - pilot study and methodological implications'.

The aim of our research was to find if meadow management type differing in natural fertilization influences the amount of root nodules in *Trifolium repens.* Plants belonging to the *Fabaceae* family comprise insufficient nitrates availability by symbiosis with rhizobia which fixate atmospheric nitrogen. Thus we found it interesting if different nitrates supply affects the strategy of nitrogen acquisition reflected by the amount of nodules. Our approach was to conduct pilot study and draw conclusions for broader study design. The pilot study did not detect significant influence of the type of meadow use, however, data analysis led us to the methodological conclusion. Increase of the number of samples could allow to detect the effect and should be performed by concerning more repetitions of each meadow type but within a repetition sampling used by us is sufficient.

We think that our conclusions can be meaningful for researchers who want to conduct study in this field and thus are worth publishing in your journal which covers topics concerning agricultural studies and plant physiology.

Our manuscript is not previously published in or simultaneously submitted to any other scientific or technical journal.

Yours faithfuly,
Weronika Banot
Institute of Environmental Sciences
Jagiellonian University in Kraków

Ochotnica, 19.09.2016r.

Dear David Richardson,

I am writing to submit our manuscript entitled “Effect of meadow use on root nodules in *Trifolium repens* - pilot study and methodological implications” for consideration for publication in Symbiosis. Since the main interest of this journal is to show ecological implications on symbiosis on organismic level, I would like to introduce you studies prepared by me and my colleague, in which we raised a question of the impact of agriculture on root nodules in *Fabaceae*. We tested effect of different meadow use, manured, with pasturage or without treatment, on number of root nodules in *Trifolium repens*. We unfortunately did not show effect of treatment, but our investigation lead to important methodological implications about sampling of data, which can be in interest of many researchers.

This manuscript describes original work and is not under consideration by any other journal. All authors approved the manuscript and this submission.

Thank you for receiving our manuscript and considering it for review. We appreciate your time and look forward to your response

Kind regards,

Jowita Niedojadło

Reviews

Adam Łomnicki

It is quite good report, taking into account the conditions under which it was prepared, but I have the some serious reservation concerning statistical applications.

If we apply the hierarchical analysis of variance, then the significance of variance between the highest levels (manuring, pasturing, control) should be tested against the variance next in the hierarchy in our case between the meadows. It is not allowed to test against the variance of lower levels which can be quite small. I have tried to explain it in the last paragraph on the page 188 of *Wprowadzenie do statystyki dla przyrodników* fifth edition 2014. The authors seem to understand it at the end of Discussion suggesting the we need more meadows in each meadow type, not more samples and more plants. For the reason given above, testing of the variation of meadow types against variation of lower hierarchies should not presented in the report. I would also like the table of the hierarchical analysis of variance to be included in the manuscript, since now the presentation in lines 62-66 is not quite clear.

My other small remarks are:

Line 39: I would not call this study a pilot one, with such a short time all the studies made in Ochotnica are the pilot studies.

Line 63: I know that the acronym LSM is not Linux Server Management but it has so many meaning the it should be explained.

Line 98-99: I know that Dr J. Rutkowska wants every report to look as prepared for publication in "Nature" but I am afraid that the statement on competing interests in this report is an exaggeration.

Stanisław Bury

The manuscript concerns the problem of habitat management on plant-microbial interaction, namely the effect of pasturing or fertilizing on root nodules in *Trifolium repens*. Although the manuscript contains all expected parts and explores interesting and important problem I have several comments:

a) Introduction provides almost no information about current state of knowledge and no justification on the importance of presented research. Reader who is not a specialist in plant ecology may not get what are the bases of the hypotheses presented in the end of the introduction. Moreover in the introduction authors name the research as a pilot study. Even if it is a pilot study providing such information in the very beginning diminish the value of the research in the eyes of readers.
b) In the methods as well as in the results section there is information about comparison of variation coefficient within and between groups. This is something that confuses the reader because there is nothing about such aim in the introduction. Moreover in both sections the information how variation coefficient was obtained and compared is described unclearly. Author also claim that were not able to show that variation between groups is larger than within groups, yet they do not provide any information what is the reason – was the result not significant or did they not know how to perform the analysis?
c) The discussion is focused on methodological aspects, because authors assume that with larger sample size would enable to get significant results. Yet one should keep in mind that maybe there is just no effect of pasturage or manure on measured traits? It needs to be mentioned since it corresponds to primary results.

Michela Corsini

This study underlines the consequences of fertilizers input in agricultural substrates and takes a closer look at how different sources of nitrogen may affect *Fabaceae* biomass. The topic itself is important in terms of promoting sustainable agricultural techniques but, in the meantime, suggests the relevant role of these nutrients at soil level. Since the nutrient circle shows how nutrients move from the physical environment into living organisms, the topic assumes a decisive role in terms of agricultural ecosystems conservation. This altered composition of soils' nitrogen, in fact, may influence the natural balances of soil elements with a marked reflection in terms of rhizobia structure. The researchers here tried to explain this phenomenon and introduce a fascinating example given by the pluriannual herbaceous species white clover "Trifolium repens", commonly distributed in Europe and central Asia. Even if this particular section of the report is clear in terms of hypothesis and predictions and valid in all of its arguments, a general main reason concerning the importance of this study has not been clearly reported. In other words: why do the authors

consider important to study the effects of different nitrogen sources at the *Fabaceae* biomass level? which would be the main application of this results into a wider perspective?

The introduction illustrates clearly which are the effects of natural circulation of nutrients given by the addition of fertilizer and points out both the aims and the 3 main hypothesis of the study. However, I would suggest the authors to search for earlier studies concerning the effect of nitrogen in soils and its reflection in terms of rhizobia structure responses. In case this type of method would be considered as something new, I suggest to describe it as novel in terms of "nitrogen input index" measurements. It would probably be another good reason for its consideration.

Generally I have found the topic really interesting and its structure well-organized: the methodic part such as the statistics are clear and the text in its complex is fluent. Even if the results are not that strong in terms of meaning as the authors expected, this is due to a reduced number of samples and will be enough for a pilot study. Since the main hypothesis have not been fully proved or explained in terms of results, I would not suggest the authors to publish in an important journal. However, the results showed a trend in this pilot study and I still believe it may play an important role as a start for something more relevant in the future.

Dorota Lutyk

The study entitled „Effect of meadow use on root nodules in *Trifolium repens* - pilot study and methodological implications" concern important topic of impact of agriculture on plants. In the introduction the authors clearly explain the aim of the study and present their hypothesis. They are also confident to the importance of their research.

Even though the researchers did not confirmed any of their hypothesis, their results were close to significance. Furthermore, they performed additional statistical analysis to get closer insight in what could be done to improve the study. Thanks to that, they are aware of methodological mistakes they made and clearly explain it in the manuscript. Their conclusions about the methodology can be very helpful for other researchers interested in this topic. The discussion is really comprehensive, relate the study with other findings and emphasize the advantages of the research.

I had some problems while reading the results because of complicated description of differences between within- and between-group variances. Maybe a simple scheme could help the readers with better understanding this section. It would also be easier to follow if the figure concerning main results was located within the text, not below. Moreover the figure could has bigger font.

Despite this comments I find the manuscript interesting and worth publishing.

Effect of meadow use on root nodules in *Trifolium repens* - methodological implications

Weronika Banot, Jowita Niedojadło

Abstract

Changes in nitrates amount in the ground can disturb symbiotic relations between *Fabaceae* plant and rhizobia. In our study, we attempted to answer the question if the meadow use influences amount of root nodules in *Trifolium repens.* We posed the following hypotheses: (1) Pasturage of cows negatively influences number of root nodules in *Trifolium repens*. (2) Manure presence negatively influences number of root nodules in *Trifolium repens*. (3) Impact of manuring in addition to cow pasturing on the number of root nodules is bigger than the impact of sole pasturing. To test the hypotheses we considered three types of meadow use: with manuring, with pasturage of cows and without these treatments. In each type we chose two meadows as repetitions and took five samples from each of them. The amount of root nodules was in manured meadows twice lower than in other meadow types; however, the difference between meadow types was not significant. We draw from the study conclusions concerning methodology. To obtain meaningful results, more repetitions of the same meadow type should be included but there is no need to take more samples from each meadow.

Keywords

Fabaceae, nitrates, manurage, pasturage, land use

Introduction

Agriculture modifies natural circulation of elements on meadows by supplementation of fertilizers, not only intentional by using artificial fertilizers or manure, but also by pasturage [1]. Insufficient availability of nitrogen from the soil is by plants from *Fabaceae* family compensated by cooperation with rhizobia, nitrogen fixing soil bacteria which are present in root nodules [2, 3]. However, the development and nitrogen fixation activity of root nodules are known to be suppressed when nodulated roots are exposed to a high concentration of combined nitrogen. Nitrates, a major form of inorganic

nitrogen in soil, strongly inhibit nodulation and N_2 fixation activity [3–6]. Nitrate inhibition has been shown to have many effects, including a decrease in nodule number or nodule mass [4]. Therefore, we found it interesting to study the effect of meadows use on physiology of one of the most common plants from *Fabaceae* family present on meadows, white clover – *Trifolium repens*.

The study was designed to investigate the effect of land use on root nodules in *Fabaceae*. Another aim was to establish methodology for study in this field. We sampled plants with roots on meadows which were all mowed and treated this season in one of the following ways: pasturing, manuring after pasturing and control, without any of the mentioned treatments. In the study, we attempted to test the following hypotheses:

(1) Pasturage of cows negatively influences number of root nodules in *Trifolium repens*.

(2) Manure presence negatively influences number of root nodules in *Trifolium repens*.

(3) Impact of manuring in addition to cow pasturing on the number of root nodules is bigger than the impact of sole pasturing. We expected that increased abundance of nitrates in the soil would result in decreased number of root nodules both in pasturage and manured meadows, because plant would not need to use symbiotic bacteria to fix nitrogen from the air for them.

We also expected to draw conclusions for further study design.

Methods

The study was located in Ochotnica Górna, a village set in Gorce mountains in southern Poland. Considered three types of meadows are further referred to as: ‘pasturing’, ‘manuring’ and ‘control’. Each type was represented by two meadows and from each of them we took five samples of *Trifolium repens*. Samples were ca. 10x10 cm pieces of turf collected with hoe. From each sample we carefully extracted *Trifolium repens* specimens with roots and counted number of root nodules as well as weighted the overground plant parts (further referred to as ‘biomass’). From these data the amount of nodules, defined as number of root nodules per gram of biomass, was calculated.

Data were analyzed using mixed model. The amount of nodules was a response variable, type of meadow – fixed explanatory variable and repetition – random variable nested in type of meadow. To show the effect of type of meadow, variance between

meadow types was tested against variance of repetitions. This result was used to conclude about the study design in terms of sampling.

Results

The amount of nodules (number of root nodules per gram of biomass; shown as least square mean ± standard error, LSM ± SE) on 'manure' meadows (21.8 ± 6.4) was two times smaller than both in 'control' (56.3 ± 5.9) and 'pasture' meadows (49.4 ± 5.5, Fig. 1). However, the difference was below the significance level ($F_{2,3} = 8.71$, $p = 0.0526$) . Therefore, we did not prove that the meadow types differed from each other more than did repetitions within a meadow type. The results of analysis are presented in Table 1.

The repetition within meadow type did not occur to influence the amount of nodules ($F_{3,21} = 0.31$, $p = 0.8150$). This means that within the meadow type repetitions did not differ from each other more than did samples within a repetition.

Discussion

Although we obtained in manured meadows twice lower amount of root nodules than in other meadow types, the difference between meadow types was not significant. Therefore, our results did not support any of posed hypothesis about impact of meadow use on the amount of root nodules. Lack of differences can be explained from methodological point of view. The result considering variance between meadow types did not reach the significance level on which we could conclude that the amount of nodules differed more between meadow types than within each type. We suppose involving more repetitions within each meadow type would allow to detect the effect. In addition, differences at the lower level of study design, *i.e.,* between repetitions within meadow type, were not significant. This implicates that the number of samples per repetition is adequate.

Although our study did not find the effect of meadow use on root nodules, studies on soybean and legume species, show negative impact of fertilization and nitrates content in soil on many features of root nodules, *e.g.* affecting the process of their formation (noduling), number of nodules or increasing their disintegration [4-8]. On the other hand, in some cases natural fertilizers such as manure were shown to have positive influence on nodules formation [9], which can be related to improvements in the soil structure and above all to avoidance of the phenomena of water stress [10].

In conclusion, our study demonstrated that to allow detection of differences in meadow use on the amount of root nodules the number of samples should be increased. Comparison of variances leads to the suggestion that increasing number of samples could be reached by including more repetitions of the same meadow type but does not require taking more samples from each meadow.

Authors' contribution

WB and JN designed and conducted the study. JN did the statistical analysis. WB and JN interpreted the results and prepared the manuscript.

Acknowledgements

We would like to thank local people of Ochotnica Górna for the access to their grounds. We thank Joanna Rutkowska for advise in study design and statistics. We thank other course participants for conclusive discussions.

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Table 1. Results of mixed model concerning the effect of meadow type and repetition on the amount of root nodules. SS – sum of squares, MS – mean squares, Df – degrees of freedom

factor	effect type	SS	MS	Df	*F*	p
meadow type	fixed	5325.11	2662.56	2	8.7110	0.0526
repetition(meadow type)	random	903.629	301.21	3	0.3141	0.8150
error	-	20137.890	958.95	21	-	-

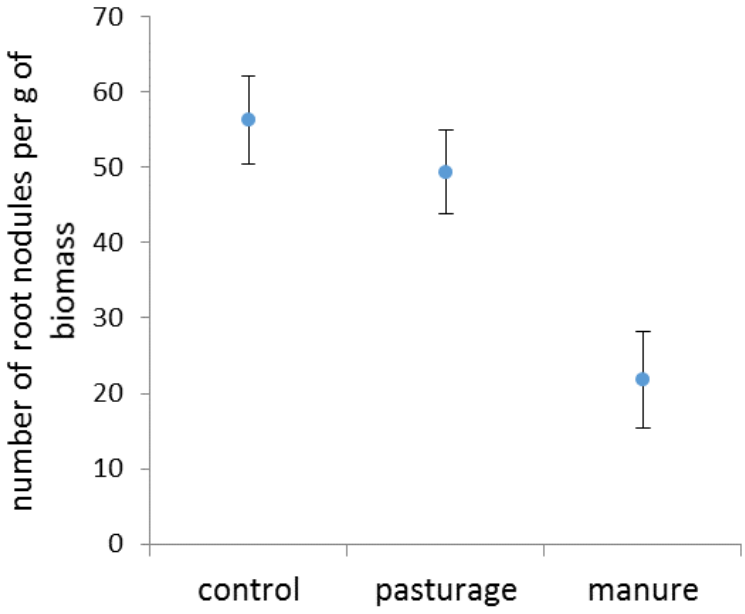

Fig. 1. Number of root nodules per g of *Trifolium repens* above ground biomass. Results are presented as LSM ± SE.