NutNet add-on to identify symbiotic N fixation

Contact:

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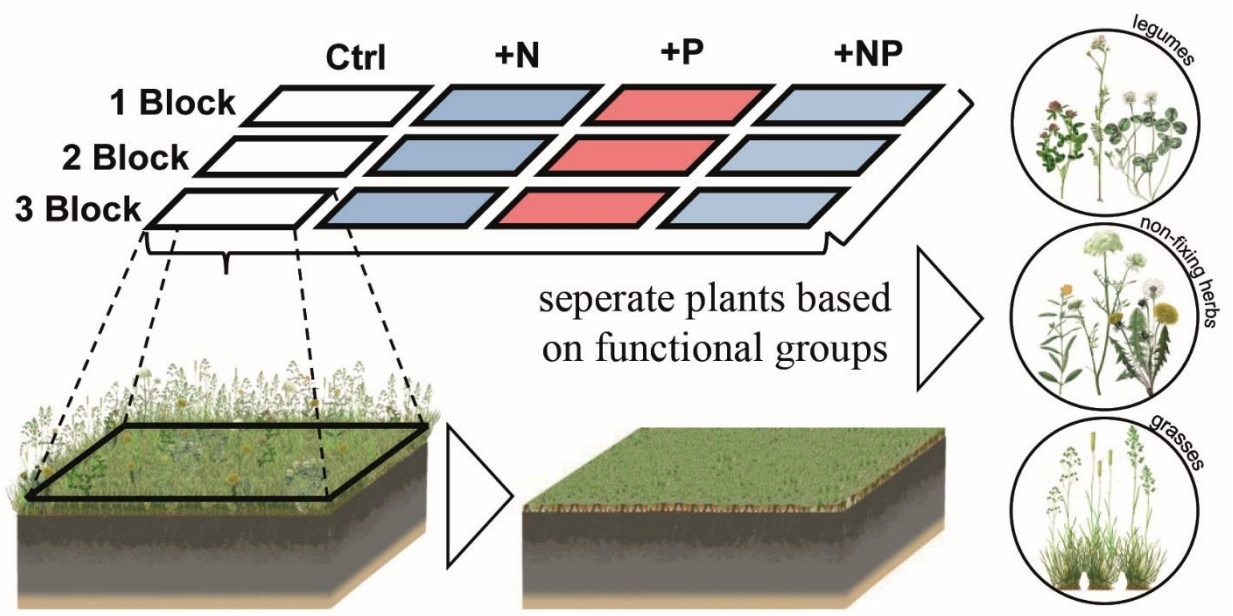
Objective: We aim at conducting an add-on project on symbiotic nitrogen fixation. The idea of the add-on project is to estimate symbiotic nitrogen fixation by legumes, and to identify how it changes following single and combined addition of N and P in grasslands. For this purpose, the ^{15}N natural abundance method will be used because most of the required data and biomass sampling is consistent with the NutNet protocol.

Methods: We will measure the isotopic signature of nitrogen ($\delta^{15}N$) in the aboveground biomass of functional groups namely legumes, forbs and grasses. Based on the isotopic signature of nitrogen in legumes in comparison to the nitrogen signature in forbs and grasses, nitrogen fixation can be calculated (differential method).

$$Ndfa\,(\%) = 100\,\frac{(\delta^{15}N_{ref} - \delta^{15}N_{fixing\ plant})}{(\delta^{15}N_{ref} - B)}$$

where N_{dfa} is N derived from the atmosphere via symbiotic N fixation, $^{15}N_{ref}$ represents the level of ^{15}N detected in a reference plant (grasses and non-fixing herbs), growing in the same soil at the same time and receiving the same fertilizer as the legume, $^{15}N_{fixing\ plant}$ is the ^{15}N abundance of the legumes and *B* is the ^{15}N abundance of legumes grown obtaining all of its N from N_2 fixation (this value will be obtained from literature and will be computed in a similar way as shown in (West *et al.* 2005) via combined boots-trapping and statistical resampling). In a next step we use the recorded legume biomass data (g m^{-2}) to extrapolate the treatment-specific N fixation (absolute data in g N_{fixed} m^{-2}). To get insights on its relevance for each site and treatment we will relate absolute N fixation to the aboveground N stock of non-fixing plants using their foliar N content (%) and the standing crop dry mass (g m^{-2}).

We encourage colleagues from all NutNet sites to send us material from the stored biomass or from this year's harvest (2017). We will consider the biomass of three functional groups (legumes, non-fixing herbs and grasses) from four treatments (ctrl, +N, +P,+NP). Additionally, we need data on aboveground biomass of the three functional groups. For most sites the workload should be relatively small, except for checking the storage rooms for subsamples, posting them to Bayreuth (Germany) and inserting required data into the excel sheet. Please see the detailed instructions below.



Detailed Protocol:

1. Check if there are dried aboveground biomass samples from both biomass stripes for each functional group (grasses, leguminous forbs and non-leguminous forbs) in your storage. The biomass should be separated according to the experimental NutNet protocol for all replicates of the Ctrl, +N, +P, and +NP treatment. In case your site is older than 2 years, we would also be interested in receiving samples from several consecutive years to study how N fixation has changed over time. If samples from previous years are still available, it would be excellent to get these samples and data sets, too.

   For some sites this might be not given i.e.:

   a) *when samples were not stored:* in this case send us material and data from this year`s harvest. Please follow the ordinary sampling procedure using both biomass harvest stripes.
   b) *when biomass is sorted on species level:* in this case plants have to be pooled by functional group. It is crucial that this is done relative to the standing aboveground biomass for each plot (Ctrl, +N, +P, +NP).
   c) *when biomass is not separated:* in this case dried bulk biomass has to be separated according to the three functional groups. If this is not possible, biomass should be sampled for this year (see point 1a).
   d) *when legume biomass is scarce:* as long enough biomass can be delivered for ^{15}N analysis (see point 2a, below) and data sets on cover and standing biomass are available there should be no problem.
   e) *when legumes are not present at all:* in this case symbiotic N fixation cannot be estimated. It means there is no use to send us material from the reference groups.
   f) *when not all treatments contain legumes:* with regard to our hypotheses, we are particularly interested in the combinations of (Ctrl vs. +N) or (Ctrl vs. +P). If for one

of these combinations biomass material and corresponding data can be provided, we would be also interested in receiving these samples.

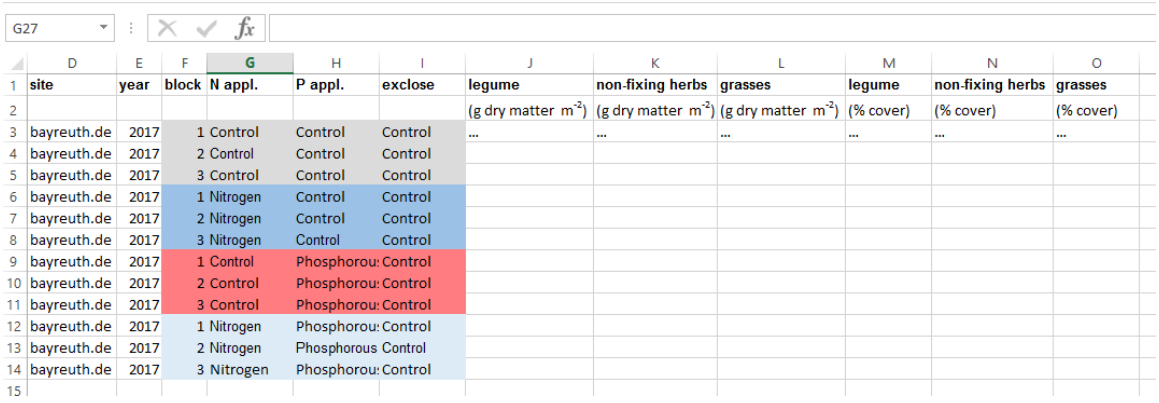
g) *when legumes are not present in all three replicates of a single treatment:* Please check if at least one of the combinations (see 1f) is complete. A complete sampling is required to ensure appropriate statistics. If this is not given, please contact us then we can discuss what to do.

h) *when there other problems that are not included:* Please also contact us! We presumably will find a solution.

2. After harvesting and separating plants to functional groups for both biomass stripes, prepare a representative subsample of each functional group. Take randomly about 5 g for each functional group and cut the biomass in small pieces (ca. 3-4 cm). Put each subsample into a small paper bag (or plastic bag when paper bags are not available) and close them with tape to prevent losses during transport. Add a label indicating site, year, treatment and functional group on each bag.

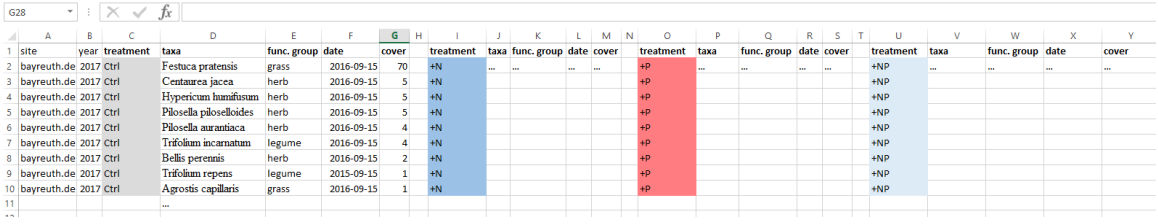
   a) *when you have less than 5 g dry mass material for a functional group:* then please send as much as possible! The stable isotope measurements requires ca. 50 µg N in the sample. However, the required biomass weight depends on the element content of the plant tissue and further it is likely that material losses will occur during sample preparation (i.e. milling). This makes it difficult to state an accurate minimum of the required biomass. We assume that below a plant biomass of 0.5 g dry matter the measurements will be challenging and probably will fail.

3. Please insert the corresponding standing biomass (in g m^{-2} dry weight) and vegetation cover (in %) for each functional group into the excel sheet.



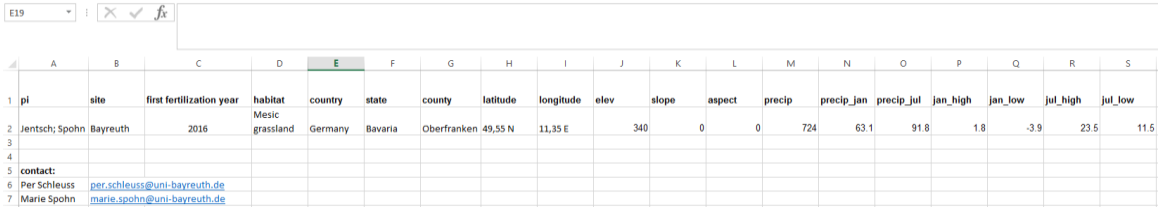
	D	E	F	G	H	I	J	K	L	M	N	O
1	site	year	block	N appl.	P appl.	exclose	legume	non-fixing herbs	grasses	legume	non-fixing herbs	grasses
2							(g dry matter m^{-2})	(g dry matter m^{-2})	(g dry matter m^{-2})	(% cover)	(% cover)	(% cover)
3	bayreuth.de	2017	1	Control	Control	Control	...	...	...	...	...	...
4	bayreuth.de	2017	2	Control	Control	Control						
5	bayreuth.de	2017	3	Control	Control	Control						
6	bayreuth.de	2017	1	Nitrogen	Control	Control						
7	bayreuth.de	2017	2	Nitrogen	Control	Control						
8	bayreuth.de	2017	3	Nitrogen	Control	Control						
9	bayreuth.de	2017	1	Control	Phosphorou	Control						
10	bayreuth.de	2017	2	Control	Phosphorou	Control						
11	bayreuth.de	2017	3	Control	Phosphorou	Control						
12	bayreuth.de	2017	1	Nitrogen	Phosphorou	Control						
13	bayreuth.de	2017	2	Nitrogen	Phosphorous	Control						
14	bayreuth.de	2017	3	Nitrogen	Phosphorou	Control						
15												

4. Please insert the species composition for each site according to the vegetation cover of the corresponding year (at least for the dominant species).



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y
1	site	year	treatment	taxa	func. group	date	cover		treatment	taxa	func. group	date	cover		treatment	taxa	func. group	date	cover		treatment	taxa	func. group	date	cover
2	bayreuth.de	2017	Ctrl	Festuca pratensis	grass	2016-09-15	70		+N	...	...	...	...		+P	...	...	...	...		+NP	...	...	...	...
3	bayreuth.de	2017	Ctrl	Centaurea jacea	herb	2016-09-15	5		+N						+P						+NP				
4	bayreuth.de	2017	Ctrl	Hypericum humifusum	herb	2016-09-15	5		+N						+P						+NP				
5	bayreuth.de	2017	Ctrl	Pilosella piloselloides	herb	2016-09-15	5		+N						+P						+NP				
6	bayreuth.de	2017	Ctrl	Pilosella aurantiaca	herb	2016-09-15	4		+N						+P						+NP				
7	bayreuth.de	2017	Ctrl	Trifolium incarnatum	legume	2016-09-15	4		+N						+P						+NP				
8	bayreuth.de	2017	Ctrl	Bellis perennis	herb	2016-09-15	2		+N						+P						+NP				
9	bayreuth.de	2017	Ctrl	Trifolium repens	legume	2015-09-15	1		+N						+P						+NP				
10	bayreuth.de	2017	Ctrl	Agrostis capillaris	grass	2016-09-15	1		+N						+P						+NP				
11				...																					

5. Add the site specific information into the provided excel sheet. Please also include the year you started the nutrient manipulation.



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1	**pi**	**site**	**first fertilization year**	**habitat**	**country**	**state**	**county**	**latitude**	**longitude**	**elev**	**slope**	**aspect**	**precip**	**precip_jan**	**precip_jul**	**jan_high**	**jan_low**	**jul_high**	**jul_low**
2	Jentsch; Spohn	Bayreuth	2016	Mesic grassland	Germany	Bavaria	Oberfranken	49,55 N	11,35 E	340	0	0	724	63.1	91.8	1.8	-3.9	23.5	11.5
3																			
4																			
5	**contact:**																		
6	Per Schleuss	per.schleuss@uni-bayreuth.de																	
7	Marie Spohn	marie.spohn@uni-bayreuth.de																	

6. Please contact us before sending the samples that we can send you the required documents for customs. We will cover the costs for customs.

7. **(optional)** I would be nice to have some seeds of the dominant legumes from your site (if possible: ca. 20 seeds should be enough). If time allows, we will grow them in N free soil to extend the data set for the B value calculation.

Reference:

West JB, HilleRisLambers J, Lee TD, Hobbie SE, Reich PB (2005) Legume species identity and soil nitrogen supply determine symbiotic nitrogen-fixation responses to elevated atmospheric CO2. *The New phytologist*, **167**, 523–530.