Legume Futures

Legume-supported cropping systems for Europe

Legume Futures Report 1.1

Sampling and measurement protocols for field experiments assessing the performance of legume-supported cropping systems

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FOREWORD

This report sets out the protocols for field measurements which are to be used throughout the Legume Futures research project. It fulfils Work Package1 Task 1.1 (Protocol establishment and harmonisation) which was led by the University of Helsinki and Work Package 3 Task 3.1 (Protocols for sampling and analysis of N_2O, NO_3 and biodiversity). It draws particularly on input from Work Package 3 (Environmental impact) led by Trinity College Dublin.

In some cases, more than one method is described. This is because the best method to use may be defined by the site characteristics, and also by the availability of staff, instrumentation and financial resources. This guide does not seek to define a common methodology for all variables. It provides guidance and support for those who may be new to some of these measurements. It has taken guidance from protocols followed in other European projects such as NitroEurope.

This guide is used in Legume Futures to support partners in developing standard operating procedures for all measurements carried out on sites. It was written as an internal project document and is published here as part of the project's efforts to provide full access to methods and to support other researchers in this area.

BIODIVERSITY

Susannah Cass, Trinity College, Dublin, Ireland

In Legume Futures, data are collected to assess the impacts of legume-supported cropping systems on biodiversity and ecosystem processes across the European pedo-climatic regions covered by the project. In order to generate useful data within the short timeframe of the project, these investigations are carried out in two distinct ways.

First, the diversity of non-crop vegetation and earthworms is being assessed at most partner sites. The aim of this element of the study will be to determine whether there are any discernable patterns in the biodiversity around legume crops relative to that around other crops across Europe.

Second, a smaller number of site-specific studies are being used to determine the potential impacts of legume-supported cropping on other aspects of biodiversity and the interactions between biodiversity and ecosystem processes. Ground invertebrates are surveyed at some sites to assess the potential influence of legume-supported cropping on the presence of crop pests and natural predator species. Levels of soil faunal feeding activity are also being measured and will be related to earthworm biodiversity to give an in-depth indication of soil conditions. Soil activity will also be compared to data for soil N leaching and emissions as a cross-cutting exercise linking the other environmental impact assessments being conducted as part of the Legume Futures project.

Site selection:

Most sites will carry out vegetation and earthworm surveys. For invertebrate sampling, plots must be of sufficient size to account for the mobility of the ground invertebrates under investigation. Soil activity will be investigated at a selection of sites under different legume systems.

Materials required:

Most sampling techniques require only simple and readily available materials such as 1m x 1m quadrats or small containers for pit-fall trap construction. These are listed in the materials section of each basic protocol. Each site should procure its own supplies of these items. Soil faunal feeding activity will be investigated using bait-lamina soil probes which will be supplied to selected sites from Trinity College Dublin.

Sample verification:

Vegetation is identified on site and voucher specimens of each species encountered are subsequently submitted to ensure standardisation across sites and countries, and are

held in a central data bank at Trinity College Dublin. See “Vegetation protocols” for voucher specimen preparation. Earthworm and ground invertebrate samples are also sent to Ireland for identification.

Timetable:

As the geographic spread of the Legume Futures sites is so great, seasonal sampling dates are adapted to suit local climatic conditions.

Health and safety

The appropriate risk assessments must follow local/institution guidelines. The standard hazards associated with outdoor field work must be considered as well as those related to the use of potentially hazardous chemicals such as formalin and mustard oil. See ‘Chemical Risk Assessment Guidelines’ for further information.

Vegetation: Quadrat protocol

Data on the non-crop flora of the Legume Futures field sites is collected using simple survey methods to understand the vegetation biodiversity of legume-supported cropping systems relative to other systems.

Site selection:

Most Legume Futures sites are collecting data on vegetation biodiversity to give a broad picture of patterns in legume-supported crops across the European pedo-climatic zones. Each site is required to survey vegetation in a specified selection of their available plots.

Sampling design:

Vegetation is surveyed using standard quadrat techniques. For sites with smaller plots, the required sampling density is approximately 1 quadrat per 10 m^2 to facilitate comparisons between data from the wide range of experimental sites. For sites with larger plots and field-scale experiments, samples consist of quadrats arranged along transects located within the crop.

Materials required:

1. 1 m x 1 m fixed quadrats OR 4 canes marked to 1 m.
2. Measuring tape/wheel/GPS to locate transects and sampling points.
3. Canes to mark transect start and end points in the field margin.
4. Bags to collect specimens.

5. Flower press, blotting paper, newspaper and acid-free mounting paper to prepare voucher specimens.

Sampling procedure: small plots:

Randomly distribute the required number of sampling points throughout the plot. Maintain a distance >2 m from the plot edge wherever possible.

Sampling procedure: large plots/fields:

Locate two transect origins at random distances along the field margin. Permanently mark these with canes or similar markers in the field margin. If necessary, transects may be located close to crop tramlines to minimise damage when walking through crop for sampling. To sample a transect start at the marker in the margin and walk 10 m directly into the crop, stop and place the first quadrat. *If using tramlines for access place the quadrat at least 1m into the crop to one side of the tramline.* Follow the 'quadrat methods' below to make the survey. Once that quadrat is finished, continue to follow a straight line through the crop locating sampling points at 10 m intervals until a minimum of 10 quadrats are surveyed or the far side of the field is reached (the last quadrat should be no closer than 10 m to the far field margin). Repeat this process for the second transect.

Quadrat methods:

Place a 1m x 1m quadrat around the sampling point. This may be a fixed quadrat or constructed from four 1 m canes, whichever is easier given the nature (height, density etc) of the crop being surveyed. Avoid trampling the vegetation within the quadrat. Identify all plants (including the crop(s)) present within the quadrat to species level. For each new species observed (and any which cannot be identified in the field), take a representative sample of leaves/flowers/roots and store in an individual, clearly labelled (*specimen number, field/plot, quadrat ID, date*) bag. Estimate the %cover of each species within the quadrat by eye. NOTE: where plants of different species overlap, total %cover can be greater than 100%; %cover is important for calculations of diversity.

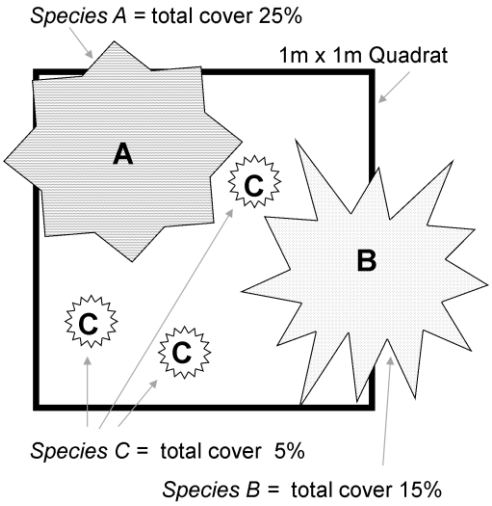

Figure 1. Example quadrat showing % cover estimates for three identified species: A, B & C.

Voucher specimens:

A voucher specimen of each identified species is pressed and dried and sent fully labelled to Trinity College Dublin.

Herbarium specimen preparation[1]

Specimens should be pressed in a plant press, which consists of a wooden frame (for rigidity), corrugated cardboard ventilators (to allow air to flow through the press), blotting paper (to absorb moisture), and folded newspaper (to contain the plant material). The plant press is tightened using straps with buckles or bolts with wing nuts. The objective is to extract moisture in the shortest period of time, while preserving the morphological integrity of the plant, and to yield material that can be readily mounted on herbarium paper (an acid-free cardstock) for long-term storage.

Each specimen should consist of a stem with attached leaves and, if at all possible, flowers and/or fruits. The roots of herbaceous plants should also be included. In the case of woody plants such as trees, shrubs, or vines, pieces should be selected to illustrate to the greatest extent possible the overall characteristics of the plant and the range of variation in flowers, leaves, and other structures. Plants should be carefully arranged as they are placed in the press to maximise preservation of diagnostic features. Leaves, flowers, and fruits should be spread out so that they do not overlap and can be observed from different perspectives.

Each specimen should be assigned a Voucher Specimen ID consisting of an abbreviation of the site name and a unique number. The collection number should be

[1] Adapted from: Frank, M.S. and Perkins, K.D. (2007) Preparation of plant specimens for deposit as herbarium vouchers. University of Florida Herbarium / Florida Museum of Natural History. http://www.flmnh.ufl.edu/herbarium/voucher.htm

clearly written on the outside of the newspaper containing each plant specimen. The plant press must be kept tight; this prevents shrinkage and wrinkling of the plant material and yields specimens that are easier to mount securely on herbarium paper. The pressed plants must be thoroughly dried prior to storage and mounting.

Specimens should be mounted on acid free paper and labelled using the following format:

Scientific name: genus, species, sub-species/variety/cultivar, authority

Site Details: Country, Site, Field/Plot, Quadrat ID

Plant description: describe characteristics of the plant which may be lost upon drying, such as flower/fruit colour and fragrance, leaf orientation and aroma

Collector name: Researcher(s) who collected and/or identified the specimen who can be contacted if further information is required.

Voucher Specimen ID: an abbreviation of the site name and a sequential straightforward numbering system (1, 2, 3,) for all plant species collected at the site, e.g., the third specimen from Solohead would be given the code Solo3

Date of collection: DD/Month/YYYY. e.g: 02 March 2011 (NOT 2/3/11 or 03/02/11)

Package the mounted specimens carefully in a box or padded envelope with plenty of padding and stiff cardboard to prevent bending

Timetable:

Data are required for spring, summer and autumn weed flora. Seasons are interpreted as appropriate for the local region and management practices.

Guideline examples:

- *Spring:* Data for first flush of weeds in a recently sown spring crop
- *Spring:* Data for established weeds in a maturing winter crop
- *Summer:* Data for mature weeds prior to crop harvest
- *Summer/autumn:* Data for weeds in fallow/follow crop
- *Autumn:* Data for first flush of weeds in a recently sown winter crop

Earthworms: Soil sampling protocol

Data on the earthworm biodiversity of the Legume Futures field sites is collected using soil sorting and chemical extraction methods. Previous studies have shown that earthworms influence the N uptake balance of intercropped legume systems [2] and increase nitrogen uptake and crop productivity[3].

Site selection:

Most Legume Futures sites are collecting data on earthworm biodiversity to give a broad picture of patterns in legume-supported crops across the European climate zones and soil types. Each site is required to sample earthworms in a specified selection of their available plots.

Sampling Design:

Earthworms are collected using soil samples and mustard oil extraction techniques. For sites with smaller plots and multiple replicates 1-5 samples are taken per plot. For field-scale experiments 5-10 samples are collected along transects previously established for vegetation surveys. The samples should be a minimum of 10 m apart and an equal number should be taken from each transect.

Materials required:

1. 30 cm ruler
2. Spade – preferably with a straight, flat blade for ease of digging regular sized holes
3. White or light coloured trays or sheets for sorting soil blocks on in the field
4. Buckets for transporting soil to the lab if necessary – due to weather conditions etc.
5. Solution of dilute mustard oil (see mustard oil protocol below)
    a. Mustard oil (allyl isothiocyanate) – 2 mL per 10 samples
    b. Isopropanol (2-propanol) – low grade/cheap
    c. Small (50 mL) containers for taking concentrate into field
    d. large water container: 20 L or more

[2] Schmidt, O. & Curry, J.P. (1999) Effects of earthworms on biomass production, nitrogen allocation and nitrogen transfer in wheat–clover intercropping model systems. *Plant and Soil* 214: 187–198.
[3] Zhang, S., Chao, Y. Zhang, C., Cheng, J., Li, J. and Ma, N. (2010) Earthworms enhanced winter oilseed rape (*Brassica napus* L.) growth and nitrogen uptake. *Agriculture Ecosystems and Environment* 139 463-468.

6. Stop-clock/timer/watch to standardise 30 min sorting period
7. Container and fresh water to rinse the extracted worms
8. Cool box or similar cool dark container with ice packs to transport live worms
9. Paper towels for drying rinsed worms
10. Watertight containers for each individual sample of worms
11. 4% formalin preservative (1:9 dilution of 40% formaldehyde in water)

Preparation of mustard oil solution (See Chemical Risk Assessment guidelines)

Complete a risk assessment according to the guidelines of your institution before working with allyl isothiocyanate. Add 2 mL mustard oil (allyl isothiocyanate) to 40 mL isopropanol (2-propanol, cheapest available is acceptable). This concentrate is enough to make 20 L of mustard oil solution, required for 10 soil block samples. Small containers of concentrate can be made, stored in a refrigerator and taken into the field for addition to water at the last minute.

Sampling procedure:

Locate sample point -> clear vegetation -> dig soil block -> mustard solution extraction -> 30 min soil sorting -> refill hole -> preserve worm samples in lab -> post to TCD

1. *Locate the sampling points*

In small plots locate the sampling points by either pacing a random number of steps along the plot edge and then a random number of steps into the crop, or by turning your back to the plot and throwing an object in over your shoulder and sampling where it lands. (On repeat sampling visits, avoid re-sampling the same place). In large fields locate the 10 samples at random intervals >10m apart along the established transects.

Once the sampling position is selected, lay out the light coloured surface (e.g., tray, sheet, fertilizer sack etc) on which the soil will be sorted, and get a new collecting container ready to place any worms in. Whilst in the field, the worms should be kept in collecting containers that allow air circulation, and stored in a cool dark container such as an insulated cool box/picnic box containing ice packs.

2. *Clear vegetation*

Carefully clear off any surface vegetation from the 25 cm x 25 cm area. Cut away the turf layer or clip vegetation with shears if necessary, and sort through this material on the tray/sheet to extract any litter dwelling worms which should be immediately placed in the collecting pot.

3. Dig soil block

Dig out a soil block 25 cm x 25 cm x 25 cm and place onto the tray/sheet. Catch any worms immediately visible at the base of the hole. All of the worms collected at an individual collecting point should be stored in the same container clearly labelled with the site, field/plot ID, sample ID and date.

4. Mustard oil extraction method

Mix the initial mustard oil-propanol solution (42 mL) with 20 L of water. Pour approximately 1 L evenly over the base of the hole. Allow to drain into soil and repeat with a further 1 L. Observe for 5 minutes or until earthworms cease to emerge, rinse any earthworms that emerge in a container of fresh water immediately (worms will die and decompose rapidly if not rinsed free of mustard solution). Once collecting is complete, add the rinsed worms to the collecting container with the rest of those collected from the soil sample.

5. Time limited sorting of soil sample

The 25 cm x 25 cm x 25 cm soil blocks should be searched for earthworms of all stages: adults, juveniles, cocoons. To standardise sorting methods across experimental sites, only the earthworms found by eye within 30 min of sifting the sample will be counted. Start the timer and break up the soil block by hand over the light coloured tray/sheet. Extract worms to the collecting container as you encounter them. Observe the fine-sifted soil closely as some juveniles may be very small. After 30 minutes, stop sorting. Keep each sample of earthworms in a separate container within a cool dark box whilst in the field. Worms will die and begin to decompose if left exposed to heat and sunlight.

6. Refill hole

Once sampling is complete, refill the hole with the extracted soil and replace any vegetation to leave the site as tidy as possible. Move to the next sample point and repeat the previous steps.

7. Preserve earthworm samples

Earthworms may be stored in a cool (4°C) dark container for a maximum of two days before processing. When you are back in the lab, rinse the earthworms in water and blot dry with paper towels. Weigh the live mass of each sample (= all worms from 1 soil block + worms from litter layer + worms extracted with the mustard oil) as a whole. Enter data into the ‘LFs Biodiversity Data Sheet’.

Complete a risk assessment according to your institution’s guidelines before working with formalin: 40% formaldehyde (see Chemical Risk Assessment guidelines) Place each sample in a watertight container with 4x more volume of formalin preservative than

the volume of earthworms. CLEARLY LABEL with site, field/plot, sample ID and date. Leave for 2-3 weeks and then pour off the majority of the formalin so that the worms are just covered.

Prepare samples for posting by making sure containers are tightly sealed and packaged in additional sealed plastic bags. Pack containers in a sturdy box with suitable packing/cushioning material to protect containers from breaking in transit. Sample sets should be sent after each season to Trinity College Dublin. *(Please label package: FRAGILE, Biological specimens in transit, HANDLE WITH CARE)*

Timetable:

Samples are required for both Spring and Autumn when soil temperature and moisture are likely to be suitable for earthworm activity. Seasons are interpreted as appropriate to the local climate and if possible, earthworms are sampled within two weeks of vegetation surveys.

Ground invertebrates: Pit-fall trap protocol

Data on the ground invertebrate biodiversity of the Legume Futures field sites is collected using pit-fall sampling.

Site selection:

Ground invertebrate biodiversity is being investigated at a limited number of suitable Legume Futures sites. Each site is required to set pit-fall traps in a specified selection of their available plots.

Sampling design:

Ground invertebrates are sampled using pit-fall traps located along transects established for vegetation surveys. Invertebrate diversity will be compared between legume-supported crop systems and local 'conventional' crops.

Materials required:

1. A trowel or large diameter soil corer/auger to make holes in which to set traps.
2. Two plastic cups per sample – pint glasses, 500 mL glasses or large yoghurt pots are all suitable. ALL TRAPS MUST BE IDENTICAL.
3. 15 cm x 15 cm squares of plastic for rain cover – pale grey NOT transparent or bright coloured (to avoid heating effects and/or attracting flower visiting species).
4. 15 cm x 15 cm chicken-wire type mesh covers to prevent small mammals from being trapped

5. 15 cm nails to support rain cover (4 per pit-fall).
6. Killing fluid: car antifreeze (ethylene glycol 50% solution) + a couple of drops of unscented detergent as a surfactant.
7. Sealable containers/bags to collect sample and trap fluid.
8. Paper and soft pencil for labels.
9. Watertight sealed specimen bags or containers to send samples by post.
10. 80% ethanol (preferred, or isopropanol if ethanol unavailable) to preserve samples.

Sampling procedure:

Locate sample points > set pitfall traps > return in 2-3 weeks > remove sample > re-set trap > process and preserve sample > repeat every 2-3 weeks > remove traps before harvest

1. *Locate sampling points*

A minimum of 10 pitfall traps (in total) should be arranged along the two transects at random intervals, maintaining a minimum separation of 20 m from other pitfall traps and 5 m from soil disturbance related to earthworm sampling. The number of pitfalls will be based on field size.

2. *Pit-fall traps*

Dig a small hole to fit the pit-fall and set the first container into the ground so that the rim is level with the soil surface. Ensure that soil is filled back in around the edges of the container. Place a second container inside the first – it must fit closely with no gap and remain level with the soil surface. Pour in the preservative solution so it 1/3 fills the inside container. Cover the trap with the mesh mammal barrier and secure it in place. Place the rain cover over the pit-fall trap and push nails into the soil to secure it at a height of 10 cm above the ground. See Figure 3 for pit-fall trap set-up.

3. *Sample collection*

After 2-3 weeks (more frequently in hot or wet weather), return to the pit-fall traps to take the sample. Remove the roof and mesh cover and take out the internal container (leaving the external container to maintain the pitfall hole). Empty the sample (making sure all invertebrate specimens are collected) into a securely sealable container/bag. Included a LABEL (site, field/plot, trap ID, date sample collected) written in soft pencil on paper INSIDE the container with the sample.

4. *Re-set*

Re-set the trap with fresh antifreeze killing fluid and replace mesh and roof. Leave traps in place throughout the active spring-summer season, or until field is harvested/ploughed, emptying every 2-3 weeks. Remember to remove traps before harvest/ploughing!

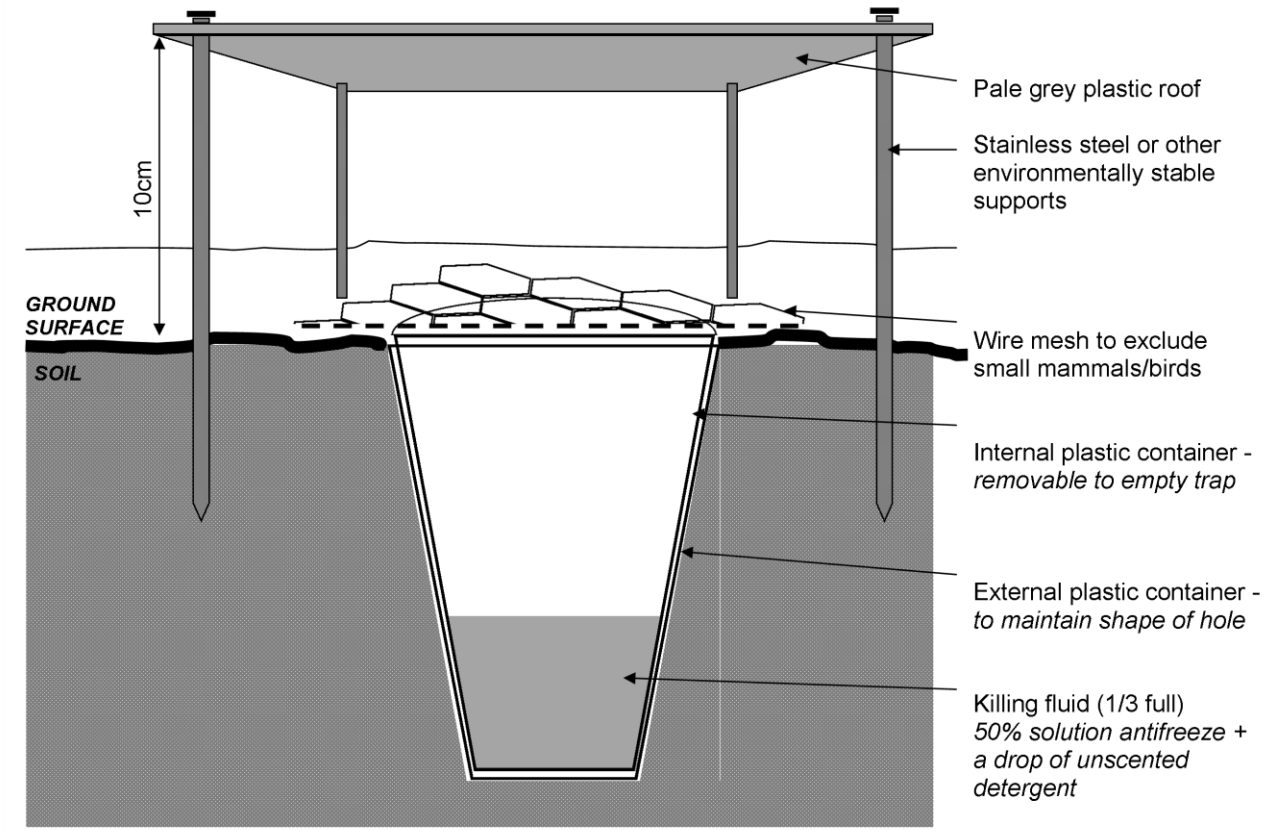

Figure 2. Pit-fall trap design and set up.

5. Sample processing

Samples should be processed as soon as possible to minimize degradation. Each sample should be emptied into the aquarium net and rinsed in fresh water for several minutes to remove the killing fluid and detergent. Large debris and pieces of vegetation should be carefully removed. The sample should then be carefully rinsed out of the net with 80% ethanol into a sealable specimen bag or container. Enough alcohol should be added to at least cover the specimens and a label included on paper INSIDE the container with site, field/plot, trap ID and date sample collected. EACH PIT-FALL SAMPLE MUST BE PACKAGED SEPARATELY. At the end of the trapping period, full sample sets should be strongly boxed and sent to Trinity College Dublin. *(Please label package: FRAGILE, Biological specimens in transit, HANDLE WITH CARE).*

Data submission:

Field and lab data are recorded in the 'LFs Biodiversity Data Sheet'. Hard copies of completed data sheets are included in the packages sent to Trinity College Dublin and copies are also emailed.

Timetable:

Sampling covers the Spring-Summer activity period for ground invertebrates with traps in place continuously throughout this time to avoid missing ephemerally active species. The spring-summer season is interpreted according to local climatic conditions although sampling should cover a minimum of 4 months.

Soil Activity: Bait-lamina protocol

Data on the soil activity of the Legume Futures field sites is collected using the bait-lamina sampling technique[4] to understand the influence of biodiversity on ecosystem processes in legume-supported crops relative to conventional crops. The aim is to produce profiles of feeding activity levels under legume-supported crops and non-legume control.

Site selection:

The bait-lamina probes are being used at selected partner sites in a selection of the available plots.

Sampling design:

Soil activity is sampled using 'bait-lamina sticks' arranged in blocks or 'base groups' which are replicated. Each base group consists of a 4 x 4 grid of bait-lamina sticks (16 in total) arranged 30cm apart. The number of base groups used depends on the size of the experimental field/plot.

[4] Törne, E. Von 1990 Assessing feeding activities of soil-living animals. I. Bait-lamina-tests. Pedobiologia 34 pp.89–101

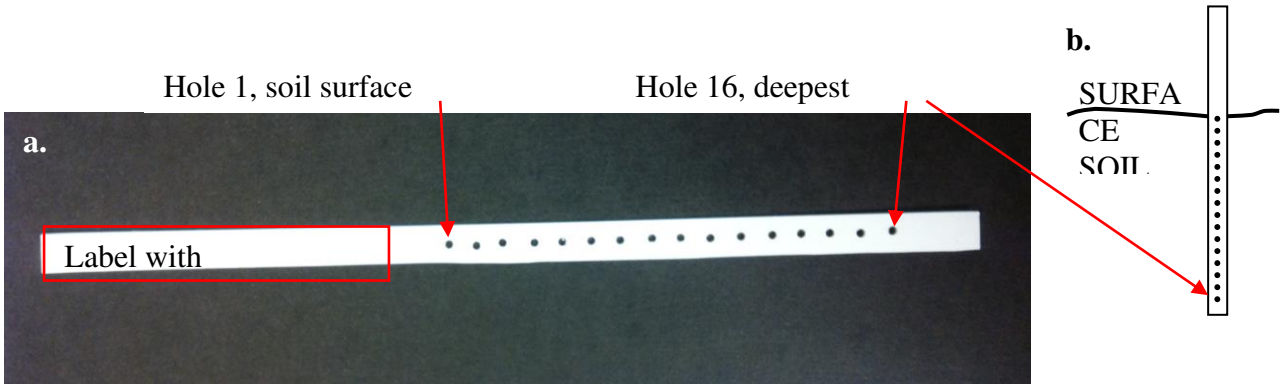

Figure 3. Photograph of a bait-lamina stick: PVC strips: 120mm x 6mm x 1mm in which 16 holes (Ø1mm) are drilled 5mm apart in the lower 85mm. Holes are baited with a standardised mixture of 65% cellulose, 15% agar, 10% bran (425µm powder) and 10% bentonite (activated carbon). Bait-lamina are placed vertically with the upper-most bait hole at surface level of the mineral soil.

Materials required:

- Bait-lamina sticks – provided ready baited from TCD.
- A metal skewer or similar tool to open a hole in the soil to insert the bait-lamina probe
- A light-box or light source for examining sticks after collection

Sampling procedure:

Locate sampling points > determine experimental error > arrange base group > remove first set of bait-lamina > analyse bait-lamina > remove subsequent sets and analyze > submit data

1. Locate your sampling points

Three base groups should be used in larger/un-replicated fields or plots; one or two base groups in smaller replicated plots as directly specified for each site. Avoid plot edges by >2m where plot size allows. Base groups should be separated by >5m.

2. Experimental error

At the start of sampling in each field determine experimental error by placing a "blind value bait-lamina"[5] into the soil and removing immediately. Record any loss of bait material (caused by the physical action of pushing stick into and removing from soil) on the DATA SHEETS; this will be used to determine the level of experimental error within the results. Different soil conditions may have different effects so please test each plot.

[5] Kratz, W. 1998 The Bait Lamina Test. Environmental science and pollution research 5(2):94-96

3. Base group arrangement

Use a skewer or similar tool to make a narrow slit in the soil in which to place each bait-lamina stick. Sticks should be pushed into the soil so that the uppermost bait hole is at the surface of the mineral soil (bellow litter layer if one exists). Sticks should be arranged in a 4 x 4 grid covering a 90cm x 90cm area. Bait-lamina sticks must be labelled with their position in the grid as well as the 'base-group' and plot numbers (e.g. Solo/Field1/Base1/C4) so that their location will not be confused once they have been removed from the soil for analysis; data will then be recorded in the data sheet.

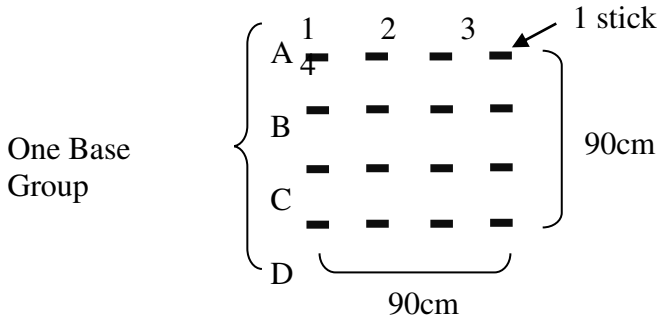

Figure 4. An over-head view of a base group of bait-lamina sticks, each column and row separated by 30cm.

4. Sampling Timing

Bait-lamina should be positioned for 'spring' sampling in approximately May, depending on regional seasons, in the early stages of growth of spring crops. All bait-lamina sticks should be put out on the same day if at all possible. As soil fauna feeding activity will depend significantly on soil conditions such as temperature and moisture level, removal dates will be flexible following the protocol described below:

- Place all bait-lamina sticks on the same 'spring' day
- Remove ROW A1-4 ONLY from each base group after 7-10 days.
- If feeding activity is evident (average of 2+ bait holes perforated) remove the next row, B1-4, in a further 7-10 days.
- If little/no (average of <2 bait holes perforated) feeding activity is evident after the first 7-10 days, wait 14-20 days before removing row B1-4.
- Continue to judge the amount of time between removal dates based on the amount of feeding activity evident in the last row analysed – remember that feeding activity is likely to accelerate after rainfall and bait-lamina will then need to be checked more regularly.

- Aim to remove the final row, row D1-4, when approximately 75-90% of the bait holes have been perforated – this may take the entire crop season. Even if this level of activity is not reached, remove the final row before crop biomass is removed/ploughed in.

- If feeding activity is high and full base groups have been removed in 28-40 days a second 'Autumn' experiment can be carried out in the later stages of the crop. All used bait-lamina must be returned to TCD promptly so that they may be re-issued with fresh bait.

5. Bait-lamina stick analysis

At each removal date pull sticks carefully from the soil and observe and rinse carefully under running water. Analysis should be done promptly to avoid accidental loss of bait giving false readings. Assess bait holes on a light-box or by holding them up in front of a suitable light source. Differentiate only between 'bait eaten' (any light penetration at all) or 'bait not eaten' (no light penetrates). Give a binary score for each bait hole: 0 = bait intact (no light gets through); 1 = evidence of feeding (some light gets through). Record data for each bait hole individually on the data sheets provided. The bait hole highest in the soil profile is number 1 and the deepest bait hole in each stick is number 16. It is important that each bait hole can be separately identified so that results can be separated into different soil layers.

Timetable:

Soil activity is measured during the spring season and may be repeated in autumn.

Site-specific requirements

Introduction

The protocols that have been chosen for use in the Legume Futures project Work Package 3 assessments have been designed and adapted with the intention of generating data that can be compared across the broad range of sites. The key to biodiversity data collection for Legume Futures is to have as many as possible simultaneous comparisons between legume-supported cropping systems and 'conventional', legume-free systems at each site.

Table 1. Work Package 3 biodiversity sampling summary.

Country	Site	Biodiversity and ecosystem processes			
		Vegetation	Earthworms	Ground Invertebrates	Soil Activity
Ireland		X	X		X
Scotland	SAC	X	X		
	SCRI	X	X	X	
Finland	Helsinki	X	X		
	MTT	X	X	X	X
Germany	VTI	X	X	X	X
Poland		X	X	X	
Greece		X	X		
Italy		X	X		
Spain		X	X		
Sweden	Stenstugu	X	X		
	Lanna	X	X		
	Saby	X	X		
Romania		X	X		X

Chemical risk assessment guidelines

Institution/national risk assessment guidelines must be followed and the appropriate safety measures taken when working with mustard oil and formalin. The details below are included for guidance only:

Substance		Associated risks	Protective measures
Mustard    Oil	*allyl isothiocyanate*	Flammability: 3 Toxicity: 3 Body Contact: 3 Reactivity: 2 Chronic: 2 Harmful if swallowed. Causes burns. Risk of serious damage to eyes. May cause SENSITIZATION by skin contact. Limited evidence of a carcinogenic effect. Toxic by inhalation and in contact with skin. Flammable. Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.	DO NOT allow clothing wet with material to stay in contact with skin Avoid all personal contact, including inhalation. Wear protective clothing when risk of exposure occurs. Use in a well-ventilated area. DO NOT enter confined spaces until atmosphere has been checked. DO NOT allow material to contact humans, exposed food or food utensils. Avoid contact with incompatible materials. When handling, DO NOT eat, drink or smoke. Keep containers securely sealed when not in use. Avoid physical damage to containers. Always wash hands with soap and water after handling. Work clothes should be laundered separately. Launder contaminated clothing before re-use. Use good occupational work practice. Observe manufacturer's storing and handling recommendations. Atmosphere should be regularly checked against established exposure standards to ensure safe working conditions are maintained. Containers, even those that have been emptied, may contain explosive vapors. Do NOT cut, drill, grind, weld or perform similar operations on or near containers.
Formalin  	*40% formaldehyde*	Flammability: 1 Toxicity: 3 Body Contact: 3 Reactivity: 1 Chronic: 4 Causes burns. Risk of serious damage to eyes. May cause SENSITIZATION by skin contact. Limited evidence of a carcinogenic effect. Toxic by inhalation, in contact with skin and if	DO NOT allow clothing wet with material to stay in contact with skin Avoid all personal contact, including inhalation. Wear protective clothing when risk of exposure occurs. Use in a well-ventilated area. Avoid contact with moisture. Avoid contact with incompatible materials. When handling, DO NOT eat, drink or smoke. Keep containers securely sealed when not in use. Avoid physical damage to containers. Always wash hands with soap and water after handling.

	swallowed. Harmful: possible risk of irreversible effects through inhalation, in contact with skin and if swallowed. Harmful to aquatic organisms.	Work clothes should be laundered separately. Launder contaminated clothing before re-use. Use good occupational work practice. Observe manufacturer's storing and handling recommendations. Atmosphere should be regularly checked against established exposure standards to ensure safe working conditions are maintained.

SOIL SAMPLING

Bob Rees, Scottish Agricultural College, UK.

One-off site measurements

At least two samples should be taken per growing season, at or just before seeding and harvest, respectively. Additional sampling, such as between harvest and sowing, follows local practice.

Soils should be sampled as a core 2-4 cm in diameter to 90 cm in 4 layers: 0-10, 10-30, 30-60, and >60 cm.

At least 10 samples should be taken per field, or 3-4 samples per plot, from locations selected randomly, which are pooled and homogenised into a single sample.

An additional 1 kg sample should be taken once, air-dried and stored for future reference purposes.

Soil description: Refer to the FAO (http://www.fao.org/ag/agl/agll/wrb/topsoil.stm) Topsoil Characterization for Sustainable Land Management with Annex A (Topsoil properties and their description) and Annex B (Examples)

Sampling procedure

Plot sampling aims to provide a representative soil sample. A topsoil sample should always be collected (0-10cm), and where possible, deeper soil layers should also be sampled (e.g. 10-30, 30-50) In order to obtain a representative soil sample, soils should be collected from across the plot in a “W” pattern, with at least 4 soil samples being bulked into one collecting bag. Samples should be returned to the laboratory and sieved though a 4 mm mesh prior to chemical analysis. Fresh soils can also be stored at 1-18^{o}C if immediate analysis is not possible.

Particle size distribution

The quickest and easiest method for determining soil texture is to use hand texturing. More accurate methods involve density separation or photoacoustic methods.

Annual site measurements

Soil pH.

Where possible pH should be measured using an electronic pH meter as follows:

Weigh (or scoop) 10 g (or 10 mL) of field moist soil in duplicate, and pass it through a 2-4 mm sieve into 40 mL disposable plastic beakers.

Dispense 20 mL of deionised water into the beaker.

Stir well with a glass rod and repeat this mixing another 3 times over the next 30 min, and just prior to the pH being measured.

Measure the pH within the next 60 min. (standard practice but not essential).

Notes: In organic soils the pH extraction ratio is 1:5 (or 1:10), as this produces enough solution in which pH can be physically measured. Alternatively, you can centrifuge the standard 1:2 extractant (5000 rpm) and measure the pH in the supernatant. Measuring pH in water provides the closest estimate of the pH in the soil solution.

Soils that have been recently fertilised (agricultural soils) may require pH measurement using 0.01 M $CaCl_2$, with 0.6 pH units added to the value obtained. This is to compensate for an increase in electrical conductivity of the soil solution due to the soluble salts from the fertiliser. Also, the $CaCl_2$ reduces the effect of the fertiliser on the soil pH measurement, thereby compensating for the influence of the history of fertiliser application.

Bulk density

Using the spade, excavate a flat area of soil, at the correct depth, on which to place the core ring, being careful not to disturb the soil being sampled.

Place the core ring on the prepared surface being careful to avoid any visible stones.

Place a flat piece of wood (a "dolly") on the ring, holding it firmly down with one hand. Strike the dolly with a hammer, in such a way that the core ring travels into the soil in a straight vertical direction. If a stone is encountered, throwing the ring off at an angle, the procedure must be abandoned and started again. If the ring goes in straight, then hammering must be stopped when the ring is just below the soil surface.

The core ring is then dug out by excavating the soil round the core with the trowel or spade, and then sliding the spade under the core, being careful not to damage the core soil. Remove any loose soil from the core, first by hand and then by using the trimming tool. Remove any soil that protrudes from either end of the core. When you are happy

that both the top and bottom surfaces of soil are flat and level with the ring, push the soil out of the ring into a numbered bag, making sure to get all the soil, then tie the bag.

Weigh and dry the sample overnight in the laboratory at 105°C

Re-weigh the sample. Bulk density (Mg m^{-3}) = Weight of dry sample (g) per volume of core ring (cm^3)

Total C and N

Total (organic) C and N may be determined by standard methods involving wet oxidation (C), Kjeldahl digestion (N) or Dumas combustion (C and N). Where carbonate is present in the soil, an acid pretreatment is required to disperse it, leaving only organic C to be analyzed.

C sequestration (where historical C content data or samples are available)

Where long term site data is available, changes in C storage at the site should be reported for defined soil layers.

Inorganic minerals: Mg, Ca, P, Mo, S.

Different research groups will use different analytical techniques for the analysis of these elements. This is acceptable providing that routine and published methods are followed. Publications such as Methods of Soil Analysis (American Society of Agronomy Series) provide useful guidance.

Multi annual measurements

Twice per year: at or just before seeding/harvest on fresh (not dried) soil.

Nitrate and ammonia determinations

Moist soil, preferably freshly sampled, is sieved through a 4 mm sieve, then 20 g of the moist soil is accurately weighed into a 350 mL plastic pot and 100 mL of 1 M KCl is added (use a 50 mL dispenser), then the mixture is shaken on an orbital shaker for 1 h.

The mixture is allowed to settle for 2-3 min before approx. 15 mL is transferred into a 16 mL disposable centrifuge tube and centrifuged at 400 rpm for 10 min. Transfer the supernatant into a 10 ml polystyrene test tube, cap then store the extracts in a fridge until analysis.

A 15 mL sample of the 1 M KCl is also centrifuged, providing a blank for analysis.

Determination of NH_4-N and NO_3-N is conducted on the auto analyser.

N.B. The top standard for NH_4-N and NO_3-N is 2 ppm. Some samples may need to be diluted - use 1 M KCl solution.

Solutions: (KCl of > 99.5 % purity must be used)

1M KCl - Dissolve 74.56 g KCl in de-ionised water and dilute to one litre.

A soil moisture determination is carried out using 30 - 35 g of fresh soil, recording the exact weight, drying in the oven at 107 ºC overnight, and recording the exact weight of dried sample after cooling.

Results are expressed as mg kg^{-1} soil (oven dry) i.e., ppm (oven dry).

Frequent or continuous measurements

Soil temperature

It is recommended that continuous records of soil temperature are maintained by using temperature probes buried at 10 cm in the soil.

Soil moisture

Weigh accurately a metal can with lid (W1). Place about 50 g of soil in the can and weigh accurately along with the lid (W2). Place the can with the lid under it in a drying oven at 105°C for 24 – 48 h, or until constant weight is reached. Remove the can from the oven, cover it tightly with the lid, and place in a desiccator to cool.

After cooling, weigh the can accurately with the oven-dry soil in it. Record the weight (W3). Compute percent moisture content on oven-dry basis.

Calculation:

Weight of water = W2 - W3

Weight of oven dry soil = W3 - W1

$$\text{Moisture \% (oven dry basis)} = \frac{W2 - W3 * 100}{W3 - W1}$$

Leaching

Drainage plots, ceramic suction cups, wells, soil percolate. This is an optional measure, but may involve the installation and measurement from lysimeters, or calculations based on the change in soil nitrate concentrations multiplied by estimated net drainage during the winter period.

MOLECULAR CHARACTERISATION OF THE SOIL BACTERIAL COMMUNITY

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Introduction

The application of molecular biology techniques based on the analysis of soil-extracted DNA and/or RNA represents an emerging strategy in microbial ecology to describe microbial diversity, and achieve a deeper understanding of diversity-function interactions in the soil system. Community fingerprinting methods of PCR-amplified 16S ribosomal RNA (rRNA) gene-coding fragments from soil-extracted DNA is nowadays one of the most popular tools in soil microbial ecology, and are widely used to profile complex microbial communities in many diverse habitats. In recent years, a multitude of different extraction methods and community profiling procedures have been published (see for instance Akkermans et al., 1995[6]; Kowalchuk et al., 2004[7]). This proliferation of protocols is often explained by the large variations between soils in chemical characteristics, which require the application of different procedures to almost every new soil.

In this section, we describe the use of two nucleic acid extraction methods developed and used routinely in our laboratories: method I, which is a direct extraction method modified from van Elsas et al., 1997[8], and an alternative extraction method (method II) developed by Qbiogene Inc., Montreal, Canada. Furthermore, two techniques are presented for the molecular characterization of soil bacterial community, which are both based on the analysis of 16S rRNA gene-coding fragments: polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), and length heterogeneity-polymerase chain reaction (LH-PCR).

[6] Akkermans, A.D.L., van Elsas, J.D., de Bruijn, F.J., 1995. Molecular Microbial Ecology Manual. Kluwer Academic Publishers, Dordrecht, The Netherlands.
[7] Kowalchuk, G.A., de Bruijn, F.J., Head, I.M., Akkermans, A.D.L., van Elsas, J.D., 2004. Molecular Microbial Ecology Manual, second ed. Kluwer Academic Publishers, Dordrecht, The Netherlands.
[8] van Elsas, J.D., Mäntynen, V., Wolters, A.C., 1997. Soil DNA extraction and assessment of the fate of Mycobacterium chlorophenolicum strain PCP-1 in different soils by 16S ribosomal RNA gene sequence based most-probable-number PCR and immunofluorescence. Biol. Fertil. Soils 24, 188-195.

Soil nucleic acid extraction method I

This method will primarily extract total community DNA from soil, although RNA is sometimes coextracted. The extraction method is based on an efficient lysis of bacterial cells in the soil matrix (lysis *in situ*) followed by removal of soil particles and interfering coextractives (i.e. humic compounds, polysaccharides, glycoproteins) using sequential purification steps. It is indicated bead beating of soil slurries in a Braun's cell homogenizer (B. Braun Diessel Biotech, Melsungen, Germany) as the method of choice for cell lysis, since it was shown to provide larger yield of high molecular weight DNA (van Elsas et al., 2006[9]). After cell lysis, the DNA is purified by CsCl precipitation followed by glassmilk spin column filtration.

Soil extraction

1. Re-suspend 2 g freshly sampled soil in 3 ml 120 mM sodium phosphate buffer at pH 8.0. Add 3 g of sterile acid-washed glass beads (0.10 - 0.11 mm diameter) in a 10 ml polypropylene tube.

2. Homogenize the soil suspension three times for 90 s in the bead beater (MSK, cell homogenizer, B. Braun) operating at 4000 oscillations/min with intervals of 20 s.

3. Add 180 µl of 20% of sodium dodecyl sulfate (SDS), mix well and leave on ice bath for 30 min.

4. Add 3 ml of Tris-buffered phenol at pH 8.0 to the lysed cell slurry.

5. Mix well and store on ice (30 min ca).

6. Centrifuge for 5 min at 6000 *g* (not less).

7. Recover the aqueous (upper) phase in a new polypropylene tube. If necessary (little aqueous phase) add sterile water (1 ml ca) and centrifuge again.

8. Extract the aqueous phase (ca 2 ml) with an equal volume of chloroform/isoamyl alcohol (24:1).

9. Centrifuge for 5 min at 6000 *g* and recover the aqueous (upper) phase in a new polypropylene tube.

10. Add 0.1 volume of 5 M NaCl and two volumes of ice-cold 96% ethanol. Keep at –80°C for 20 min or at –20°C for at least 1 h (often overnight).

11. Centrifuge for 15 min at 6,000 *g*.

[9] van Elsas, J.D., Top, E., M., Smalla, K., 2006. Soil microbial community fingerprinting based on total community DNA or RNA, in: Bloem, J., Hopkins, D.W., Benedetti, A. (Eds.), Microbiological Methods for Assessing Soil Quality. CABI Publishing, Wallingford, UK, pp. 187-203.

12. Discard the supernatant and wash the pellet with 3 ml ice-cold 70% ethanol.

13. Centrifuge for 5 min at 6000 *g.*

14. Discard the supernatant and air-dry the pellet (approximately 10 min).

15. Resuspend the pellet in 200 µl (sterile) TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). Store the crude extract at -20°C. Quantity and quality of extracted DNA by may be checked by electrophoresis in 0.8% (w/v) agarose gel.

Purification step I: CsCl precipitation

1. Add 50 µl sterile water and 30 mg CsCl to 50 µl crude extract in an Eppendorf centrifuge tube.

2. Incubate for 1.5 hours at 24°C.

3. Centrifuge at 1.000 for 30 s.

4. Recover the supernatant in a new Eppendorf centrifuge tube.

5. Add 400 µl sterile water and 300 µl iso-propanol.

6. Mix and incubate for a minimum of 5 min at room temperature.

7. Centrifuge at 15,600 g for 15 min. Check degree of pelleting.

8. Discard the supernatant and resuspend the pellet in 50 µl TE buffer. Store the partially purified extract at -20°C.

Purification step II: CsCl precipitation

Use GeneClean® Spin Kit (QBiogene/MP Biomedicals, LLC, OH; cat no. 1101-400) according to manufacturer's instructions. You can either process 50 or 100 µl of extract from purification step I. The purified DNA extract is suitable for PCR amplification and can be stored at -20°C. Yield of purified DNA by may be checked by electrophoresis in 0.8% (w/v) agarose gel.

Soil nucleic acid extraction method II

This nucleic acid extraction protocol is based on the use of a commercially available kit (FastDNA® SPIN Kit for Soil, QBiogene).

PCR-DGGE

Since DNA amplification via PCR is extremely sensitive, care should be taken not to contaminate the sample material or new PCR reaction mixes with target DNA or PCR

products resulting from previous reactions, since this might lead to false positives. Sample preparation and processing, setting up of the PCR reaction mixtures and handling of PCR products all have to be performed with extreme care. Use of high-purity PCR reagents is strongly recommended. To avoid the occurrence of false-positive results, we suggest having separate sample preparation, PCR and product analysis rooms, with separate equipments including pipettes. PCR reagents should be handled under a laminar flow cupboard.

The PCR protocol described in the following will address the amplification of bacterial 16S rRNA gene sequences by using the primer pair F984GC/R1378, which is suitable for total community fingerprinting. At a finer level of resolution, a picture of the diversity of less dominant soil bacterial groups (such as □-Proteobacteria, □-Proteobacteria, Actinobacteria, Pseudomonads) can obtained by using group specific primer systems (see for instance Kowalchuk et al., 2006[10]).

PCR reaction mixture preparation

Prepare a 50-µl reaction mixture containing: Stoffel buffer (10 mM Tris-HCl, pH 8.3, 10 mM KCl) (Applied Biosystems, Foster City, CA), 200 µM each deoxynucleotide triphosphate, 3.75 mM $MgCl_2$, 0.2 µM primer F984GC, 0.2 µM primer R1378, 2% (v/v) formamide, 0.25 µg/50 µl T4 gene 32 protein (Roche Diagnostics, Mannheim, D), 5 Units/50 µl of AmpliTaq® DNA Polymerase, Stoffel Fragment (Applied Biosystems), 1 µl of template DNA (ca 10 ng), and sterile dd-H_2O to a final volume of 50 µl.

Primer sequences used for PCR amplification of 16S rRNA gene-coding fragments

Primer[a]	16S rDNA target (positions) [b]	Sequence (5´-3´)
R1492	Bacteria (1492-1513)	TACGG(C/T)TACCTTGTTACGACTT
F984GC	Bacteria (968-984)	gc.-AACGCGAAGAACCTTAC
R1378	Bacteria (1378-1401)	CGGTGTGTACAAGGCCCGGGAACG
gc.		CGCCCGGGGCGCGCCCCGGGCGGGGCGG GGGCACGGGGGG

[a] F, forward primer; R, reverse primer; (gc.), G+C-rich sequence (clamp) attached at 5´ end.
[b] Positions according to *Escherichia coli* 16S rDNA sequence.

A GC-rich sequence is added to the 5' end of primer F984GC in order to prevent complete melting during the separation in the denaturant gradient.

PCR running conditions

[10] Kowalchuk, G.A., Drigo, B., Yergeau, E., van Veen, J.A., 2006. Assessing bacterial and fungal community structure in soil using ribosomal RNA and other structural gene markers, in: Nannipieri, P., Smalla, K. (Eds.), Nucleic Acids and Proteins in Soil. Soil Biology, volume 8. Springer-Verlag, Berlin.

DNA amplification is performed by using a "touchdown" PCR in order to reduce the formation of spurious by-products. During the touchdown PCR the annealing temperature, which is initially set 10°C above the expected annealing temperature (i.e. at 65°C), is then decreased by 2ºC every second cycle until a touchdown of 55°C, at which temperature 25 additional cycles are carried out. PCR running conditions are: an initial denaturing step at 94°C for 5 min followed by 35 thermal cycles consisting of 1 min of denaturation at 94ºC, 1 min for primer annealing at the appropriate temperature, and 2 min at 72ºC for primer extension. Cycling is followed by a final extension step at 72ºC for 10 min and cooling to 4°C. Check quantity and quality of the PCR products by electrophoresis in 2% (w/v) agarose gel.

DGGE

PCR-amplified 16S rDNA fragments can be community fingerprinted using a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Load PCR products (15 µl) onto 6% (w/v) acrylamide gels (acrylamide:N,N'-methylenebisacrylamide, w/w, 37.5:1) containing a linear chemical gradient ranging from 40 to 58% denaturant (100% denaturant corresponds to 7 M urea plus 40%, v/v, deionized formamide). The polyacrylamide gels can be prepared by using a Model 475 Gradient Delivery System (Bio-Rad Laboratories) according to the manufacturer's instructions. Run the electrophoresis in a 1x TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA, pH 8.3) at 60ºC at a constant voltage of 100 V for 7 h. After the run, stain the gels with SYBR® Gold nucleic acid gel stain (Molecular Probes, Eugene, OR) and rinse with water. Gel images can be digitally captured under UV light (λ = 312 nm) by a digital camera equipped with a SYBR® Gold gel stain photographic filter.

Denaturing gradient gel electrophoresis (DGGE) allows for DNA fragments of the same length but with different nucleotide sequences, such as those generated by PCR with 16S ribosomal RNA gene-based primers, to be separated on the basis of differential melting behaviour in a linearly increasing gradient of denaturants (urea and formamide). This results in a banding pattern, in which each band may represent different molecular ribotypes. The fingerprints obtained via DGGE separation provide a representative picture of the numerically dominant ribotypes, from which a representation of the microbial community structure in environmental sample may be inferred. DGGE community fingerprints can be processed by statistical methods (cluster analysis, multivariate analysis) to facilitate comparison of resulting banding patterns from differing soil treatments.

LH-PCR protocol for bacterial community fingerprinting

The protocol is modified from Tiirola et al. (2003) [11] by Anu Mikkonen

1. PCR is done using general bacterial primers fD1 (AGAGTTTGATCCTGGCTCAG) and 5' FAM-labelled PRUN 518r (ATTACCGCGGCTGCTGG) are used for DNA amplification using one microlitre of 1/10 water-dilution of the DNA extract (1-5 ng of DNA) as template. PCR is done in a final of 50 µl-reaction volume containing 0.2 mM of each dNTP (Finnzymes, Finland), 0.3 mM of both primers (Oligomer, Finland), 0.05% of BSA (Promega), 1x Biotools reaction buffer with 2 mM $MgCl_2$, and 1 Unit of DNA polymerase (Biotools, Spain).

2. Amplification programme. Initial denaturation at 95°C for 5 min, followed by 30 cycles of: 94°C for 45 s, 55°C for 1 min, and 72°C for 2 min. Check quantity and quality of the PCR products by electrophoresis in 2% (w/v) agarose gel.

3. Fragment separation. Separation based length with polyacrylamide capillary electrophoresis using ABI PRISM 310 Genetic Analyser with a 47 cm capillary and POP-6 Polymer (Applied Biosystems). Between 0.5 and 3 µl of the PCR product or its water-dilution is mixed with Hi-Di Formamide (Applied Biosystems) and 0.5 µl of self-made molecular size standard comprising similarly amplified HEX-labelled PCR products of known length to give the final sample volume of 15 µl. The amount of loaded PCR product was optimised to increase sensitivity by high signal/noise ratio (highest sample peak > ~1000 relative fluorescence units) but avoiding signal saturation by overloading (highest sample peak < ~8000 relative fluorescence units). The mixture is denaturated at 98°C for 2 min and ran by the Genetic Analyser with the following conditions: injection seconds 10 s, injection and run voltage 15.0 kV, run temperature 60°C, run time 70 min.

4. Fingerprint analysis. The fingerprint electropherograms for HEX- and FAM-traces of each sample run are imported from GeneScan 3.7 (Applied Biosystems) to BioNumerics 5.0 (Applied Maths, Sint-Martens-Latem, Belgium) for analysis.

Microbial biomass measurements by the chloroform fumigation-extraction method

The microbial biomass is defined as the living component of soil organic matter, and responds more quickly than most other soil fractions to changing environmental conditions. Thus, the biomass can serve as an early warning of such changes, long before they are detectable in other ways.

[11] Tiirola et al. 2003. Microbial diversity in a thermophilic aerobic biofilm process: analysis by length heterogeneity PCR (LH-PCR) Water Research. 37, 2259–2268.

The most widely used procedure for determining microbial biomass involves soil fumigation with chloroform, which results in lysis of the microbial cells. The C and N released from microbial cells after fumigation are extracted with a dilute salt solution (0.5 M K_2SO_4) and measured directly.

Soil sampling and analytical procedure

Soil sampling should provide about 10 samples / field or 3-4 samples / plot, taken from randomized points from the upper 20-cm layer and then pooled together into a composite soil sample. Time of sampling: before sowing (I), at the flowering stage (II), and at or just after the harvest (III).

After sampling, the soil should be kept moist and cool, and when possible, immediately processed. Alternatively, it can be stored at 4°C for up to 7 days. Freezing and complete drying is not recommended because of their potential biocidal effects. The soil sample is normally sieved through a 2- to 6-mm sieve to improve sample uniformity.

Weigh out duplicate moist soil sample, generally 20 to 50 g (dry weight equivalent) each. A third sample should be weighed for gravimetric soil moisture determination (105°C for 24 h). Because soil water content can affect the determination of soil microbial biomass, sample moisture should be adjusted to approximately 60% of water-holding capacity (WHC).

The non-fumigated control samples are placed in 250 ml conical flasks and then immediately extracted with 100 ml 0.5 M K_2SO_4 (1:4, w/v, soil:extractant ratio) for 30 min at room temperature. After shaking, the suspensions are filtered through a Whatman n. 42 filter paper, and the filtrate is stored at -20°C.

For the fumigated treatment, 50 ml glass vials containing the moist soils and labelled with a pencil (chloroform will dissolve ink) are placed in a desiccator containing at the bottom a wet tissue paper. A beaker containing 25 ml ethanol-free chloroform (chloroform for synthesis stabilised with 2-methyl-2-butene can be used) and antibumping granules are added. Then the desiccator is evacuated until the chloroform boils vigorously for approx. 2 min. Because of the carcinogenic and volatile properties of chloroform all work must be done with care using an adequate fume hood. The desiccator valve is closed and the samples are incubated in the dark at 25°C for 24 h. After the fumigation period, the paper towel and the beaker containing the chloroform are removed, and the residual chloroform occurring in soil pores is removed by repeated (eightfold) evacuation for 2 min, letting air pass into the desiccator after each evacuation. Then soil samples are transferred to 250 ml conical flasks for extraction with 0.5 M K_2SO_4 as seen above. Filtered extracts are stored at -20 °C.

Filtrates are analysed for their concentration of total C and N by wet chemical procedures (i.e. dichromate oxidation or Kjeldahl method, respectively).

Microbial biomass C (B_C) is calculated from the equation:

B_C (µg C g^{-1} dry soil) = (F_C-UF_C) / K_{EC}

where: F_C is the organic C extracted from fumigated soils; UF_C is the organic C extracted from non-fumigated soils; K_{EC} = 0.38 and represents a conversion factor that expresses the fraction of biomass C extracted from the soil[12].

Microbial biomass N (B_N) is calculated from the equation:

B_N (µg N g^{-1} dry soil) = (F_N-UF_N) / K_{EN}

where: F_N is the total N extracted from fumigated soils; UF_N is the total N extracted from non-fumigated soils; K_{EN} = 0.54 and represents a conversion factor that expresses the fraction of biomass N extracted from the soil [13].

Additional remarks

Soil microbial biomass typically comprises 1 to 5% (w/w) of the total soil organic C, and 1 to 6% (w/w) of total soil organic N. Expressing the size of the microbial biomass in relation to other soil variables, such as total soil organic C and N, mineralizable C and N or respiration, may provide a measure of soil organic matters dynamics and thus soil quality.

Detailed description of the procedure for estimating soil microbial biomass C and N is available in the following literature:

1. Horwath, W.R., Paul, E.A., 1994. Microbial biomass, in: Weaver, R.W. (Ed.), Methods of Soil Analysis. Part 2 – Microbiological and Biochemical Properties. No. 5 in the SSSA Book Series, SSSA, Madison, WI, pp. 753-773.

2. Brookes, P.C., Joergensen, R.G., 2006. Microbial biomass measurements by fumigation-extraction, in: Bloem, J., Hopkins, D.W., Benedetti, A. (Eds.), Microbiological Methods for Assessing Soil Quality. CABI Publishing, Wallingford (UK), pp. 77-83.

[12] Joergensen, R.G., 1996. The fumigation-extraction method to estimate soil microbial biomass: calibration of the K_{EC} value. Soil Biol. Biochem. 28, 25-31.
[13] Joergensen, R.G., Mueller, T., 1996. The fumigation-extraction method to estimate soil microbial biomass: calibration of the K_{EN} value. Soil Biol. Biochem. 28, 33-37.

EVALUATION OF CROP ESTABLISHMENT

Fred Stoddard, University of Helsinki, Finland.

The following guidelines are intended to be used to support the recording of management and measurement data relating to crop establishment.

Record the presence of legume, non-legume co-crops, or the next non-legume crop in a rotation. Results should specify the species and cultivar used.

Land preparation (e.g., tillage, preceding crop). Include a description of the nature and timing of tillage operations prior to the establishment of a crop.

Record all dates of preparation and sowing of crops.

Record seeding depth (mm).

Record the nature of seeding machinery used.

Record sowing density (kg seed ha^{-1}) and row spacing (mm).

Perform an emergence count (established crop density as plants m^{-2} or plants in row-meter, for grain legumes - not needed for pasture/forage legumes).

Record the use of rhizobial (or other) inoculants, which one, and when it was applied. (yes/no: which type).

Record the use of cover, catch or nurse crops, and record dates of planting (yes/no: which type).

BIOLOGICAL NITROGEN FIXATION

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There is a range of methods available that can be used to measure biological nitrogen fixation (BNF). These include:

- The acetylene reduction assay (ARA).
- The N difference method.
- ^{15}N natural abundance.
- ^{15}N enrichment or dilution method.

Nitrogen fixation may also be estimated by calculation from known constants.

All methods have their advantages and limitations, but for the purposes of peer-review the natural abundance is the most accurate and recommended method.

ARA was used widely in the past, but it provides only an instantaneous and indirect measure of nitrogenase activity. At best this method indicates whether a legume nodule has active nitrogenase and serves to indicate only if there is an active nitrogen-fixing symbiosis.

With the N-difference method, the total amounts of N are compared in legume and non-legume (grass, cereal) plots growing close to each other. This assumes that N fixed from air accounts for the difference. This method is cheap, but it can either underestimate or overestimate BNF because of the assumption that legumes take up as much soil N as grasses or cereals do.

The ^{15}N isotopic techniques (natural abundance and enrichment) are regarded as the most reliable techniques for BNF estimation under field conditions. They rely on the differences in ^{15}N abundance between soil N and N_2 in the atmosphere. A non-fixing reference plant is used to measure the $^{15}N/^{14}N$ ratio in the soil. A non-fixing “reference” plant (*e.g.* weeds and/or a non-legume crop) that obtains its N needs from the soil will have a $\delta^{15}N$ value close to that of the plant-available N in the soil. An N_2-fixing plant that derives its N primarily from the atmosphere will have a $\delta^{15}N$ value closer to atmospheric. Natural abundance can be used, if the abundance of ^{15}N in the soil is higher than in atmospheric N_2 (>0.3663 atom % or $\delta^{15}N$ >2 ‰), and it requires an atom mass

spectrometer. When necessary, the difference between soil N and N_2 is expanded by incorporation of ^{15}N enriched compounds into the soil, but this has become less usual since the mid-1990s as the precision of mass spectrometers has improved.

There are also formulas for BNF calculation in perennial mixed swards based on legume (red clover, white clover, lucerne) yield. They are simple to use, but not as reliable as measurements, because they do not take into account either the N content of the legumes, or the nitrogen proportion derived from the air. They are recommended only for farm- or field-level N balance estimations.

A useful reference is:

Unkovich M., Herridge D., Peoples M., Cadisch G., Boddey R., Giller K., Alves B. and Chalk P. 2008. Measuring plant-associated nitrogen fixation in agricultural systems. ACIAR, Canberra, Australia. <http://aciar.gov.au/publication/MN136>. ISBN 978 1921531262 (print), 978 1921531279 (online).

Principles of the $\delta^{15}N$ (^{15}N natural abundance) technique

The following are to a few points to consider (after Unkovich *et al.* 2008) in determining biological N_2 fixation (BNF) in legume crops –

1. More conventional (and practical) to use delta units ($\delta^{15}N$ ‰) than atom % to express natural variations in ^{15}N abundance relative to the international standard of atmospheric N_2. The atom% of ^{15}N in the atmosphere is 0.3663, and so $\delta^{15}N$ (‰) can be calculated thus:

$$\delta^{15}N\ (‰) = \frac{(\text{sample atom\%}^{15}N - 0.3663)}{0.3663} \times 1000 \qquad \text{(Equation 1)}$$

2. Soil is usually slightly enriched in ^{15}N compared to atmospheric N_2 (which logically must have a $\delta^{15}N$ value of 0 ‰).

3. A non-fixing “reference” plant (e.g. weeds and/or a non-leguminous crop) that obtains its N needs from the soil will have a $\delta^{15}N$ value close to that of the plant-available N in the soil.

4. An N_2-fixing plant that derives its N primarily from the atmosphere will have a $\delta^{15}N$ value closer to atmospheric.

5. To calculate the proportion of N derived from air (%Ndfa) you need to know the $\delta^{15}N$ of the putative fixer and the $\delta^{15}N$ of the plant-available N in the soil, which is represented for practical purposes by the $\delta^{15}N$ of a non-fixing reference plant grown in the same soil as the “fixer”:

$$\%Ndfa = \frac{\delta^{15}N \text{ of reference plant} - \delta^{15}N \text{ of } N_2\text{-fixing legume}}{} \times 100 \qquad \text{(Equation 2)}$$

$\delta^{15}N$ of reference plant - $\delta^{15}N$ of N_2 1

6. It is useful to obtain a $\delta^{15}N$ "B-value" for the legume shoot when it is grown only on fixed N. This B-value (which will typically be in the range 0 to -3 ‰) can then be used to calculate more accurate %Ndfa values when only shoot samples are taken for analysis (which is likely), as it takes into account the isotopic fractionation that occurs within the plant for the particular legume-rhizobial symbiosis that is being measured.

$$\%Ndfa = \frac{\delta^{15}N \text{ of reference plant} - \delta^{15}N \text{ of } N_2\text{-fixing legume}}{\delta^{15}N \text{ of reference plant} - B} \times 100 \quad \text{(Equation 3)}$$

7. The higher the reference $\delta^{15}N$, the more precise the estimate of %Ndfa. The $\delta^{15}N$ of a reference plant should thus ideally be >4‰, but even better if it is >6‰.

8. Please note that non-fixing reference plants should exploit the same N-pool, and have a similar duration of growth and pattern of N-uptake as the legume. Much thought should, therefore, be taken into selecting appropriate reference plants.

9. Take care that the $\delta^{15}N$ of the putatively N_2-fixing legume falls between the "B-value" and the $\delta^{15}N$ of the reference plant, as Equation 3 will give %Ndfa values <0% or >100%. Although there are ways to overcome this problem, if this is the case you might consider abandoning the use of the $\delta^{15}N$ method and consider using the ^{15}N isotope dilution method instead.

10. Do not use the $\delta^{15}N$ method when soil N-content is very high (e.g. because of previous N-fertiliser applications), as this will inhibit both nodulation and BNF by the legume.

When should you consider using the ^{15}N isotope dilution method, and how does it work?

^{15}N enrichment or dilution method

The ^{15}N isotope dilution method has fallen somewhat into disuse since the '90s. This is because of great improvements in the practicality and accuracy of the $\delta^{15}N$ technique made possible by the development of high precision mass spectrometers with a precision of $\delta^{15}N$ ± 0.3‰ and ± 0.0001 atom% ^{15}N. However, if there is access to such equipment, or if the $\delta^{15}N$ of plant-available N in the experimental fields is <2‰, then you might consider artificially enriching the soil with ^{15}N-labelled fertilisers.

In this technique:

$$\%Ndfa = \frac{\text{atom\% } ^{15}N \text{ excess } N_2\text{-fixing plant}}{\text{atom\% } ^{15}N \text{ excess reference plant}} \times 100 \quad \text{(Equation 4)}$$

The principle of the method is essentially the same as for the $\delta^{15}N$ technique, except that the soil is artificially greatly enriched in ^{15}N above background ^{15}N abundance.

The main assumption underlying the technique is that the ^{15}N enrichment of the non N_2-fixing reference plant accurately reflects the ^{15}N enrichment of soil N taken up by the legume. For this to be valid, the ^{15}N enrichment of the soil N needs to be relatively constant over time, space and depth, or the time course and depth of soil N uptake by the reference and N2-fixing plants the same.

However, the successful application of the technique depends on fulfilling point (3), but obtaining a spatially and temporally stable ^{15}N-enriched soil is neither easy nor quick. Theoretically, it should simply be a matter of incorporating an appropriately labelled fertiliser (at a ^{15}N level of approx. 4 atom% excess) into the soil via thorough mixing down to at least the maximum rooting depths of the legume and the non-fixing reference plants, but owing to the various physical and chemical processes that will act upon the added ^{15}N, mixing alone will not be enough to ensure stability of the ^{15}N signal with respect to time and depth. Time will be required, and it may take a whole season for the ^{15}N signal to stabilise sufficiently before the labelled soil can be used with any confidence. It is for this reason, and because of the additional expense of buying the ^{15}N-labelled fertiliser, that I strongly advocate that the feasibility of using $\delta^{15}N$ (natural abundance) technique be investigated in the first instance.

Choosing the most appropriate method for BNF measurements

The following key is designed to help select the best method for quantifying nitrogen fixation (note the Natural Abundance method is preferred wherever possible). It includes the options of using the ^{15}N natural Abundance Method, Dilution- or Enrichment-Method and N-Difference Method. There is also guidance on the use of appropriate BNF calculations.

A key to selecting the best method for quantifying nitrogen fixation

Q1	Is the abundance of ^{15}N in the soil higher than in atmospheric N_2?		
Q2	2.1	Yes.....	^{15}N natural abundance method
	2.2	No.....	go to next question
Q3	Do you have resources for ^{15}N fertilizer?		
	3.1	Yes.....	^{15}N enrichment method
	3.2	No.....	go to next question
Q4	Are you growing a mixture or a pure crop?		
	4.1	Mixture.....	Estimate by calculation
	4.2	Sole crops.....	N Difference method

If you do not have access to a mass spectrometer to determine the ^{15}N abundance in your (soil) samples, it may be determined in a Legume Future partner laboratory, or via a commercially operated analytical chemistry service-provider, of which there are many.

Plant Sampling

There should be a reference plant growing in a plot beside the legume plot, or in the mixture with legume. The reference plant is typically a grass for fodder legumes and a cereal for grain legumes.

It is recommended that the scissors that are used for cutting the plants as well as the sieves in mills are disinfected with pure (95 %) ethanol after each sample, to avoid cross-contamination

The above-ground dry matter yield (DM) of the legume, excluding roots, has to be determined. For fodder legumes, record the total harvested yield. For grain legumes, record the seed and straw yield.

Samples comprise 100 - 200 g of fresh material, dried at 60°C, milled through a 1 mm sieve and finally ground to a fine powder with a 0.2 mm sieve in a ball mill.

The ^{15}N natural abundance method[14]

This method is the best and recommended method where possible. It is useful and easy when the abundance of ^{15}N in the soil is higher than in atmospheric N_2 (>0.3663 atom % or $\delta^{15}N$ >2 ‰). This method often requires that the atom mass spectrometer has not been used for ^{15}N-enriched material, i.e., there is no danger of contamination from previous samples.

$$\delta^{15}N\ (‰) = ((\text{sample atom\%}^{15}N - 0.3663) / 0.3663) \times 1000 \qquad (1)$$

[14] An example of calculating using the Natural Abundance method for a rotation on Balruddery Farm (The James Hutton Institute, Scotland – is shown in Annex IV)

Every time the plants are harvested, a sample of legume and non-legume is taken from each plot, as in 1.2 above.

Samples are analysed for atom % ^{15}N and total N with atom mass spectrometer.

The proportion of N derived from the atmosphere (Ndfa, in the legume) is calculated by equation (2) using the non-legume as a reference plant representing the soil-derived N:

$$Ndfa = (\delta^{15}N_{non\text{-}legume} - \delta^{15}N_{legume}) / (\delta^{15}N_{non\text{-}legume} - B) \quad (2)$$

The B value describes the ^{15}N abundance of the legume grown either in hydroponics or in sterilized soil with no N source other than atmospheric N_2. For grain legumes, the B-value should be determined separately for seeds and straw and is usually between 0 and -3‰. When the $\delta^{15}N$ of the legume falls between the $\delta^{15}N$ of the non-legume and B, this equation gives Ndfa outside of the 0-100% range, and it is probably necessary to use the ^{15}N enrichment method (step 3, below).

Apply the BNF calculation as shown above.

^{15}N enrichment (dilution)

This method should be used when the abundance of ^{15}N in the soil is low.

The success of this technique requires that the ^{15}N enrichment of the non-legume accurately reflects the ^{15}N enrichment of soil N taken up by the legume. *For this to be valid, either the ^{15}N enrichment of the soil N needs to be relatively constant over time, space and depth, or the time course and depth of soil N uptake by the non-legume and legume needs to be the same. Obtaining a spatially and temporally stable ^{15}N-enriched soil is neither easy nor quick*. Although it should be an easy matter of incorporating an appropriately labelled fertilizer (about 4 atom% excess ^{15}N) into the soil by thorough mixing, down to at least the maximum rooting depths of the legume and the non-legume, this is not enough because of the physical and chemical processes that act upon the added ^{15}N. Time is required, and a whole growing season is often required until the ^{15}N signal has stabilized sufficiently and the labelled soil can be used with confidence. Hence, and also because of the high cost of the ^{15}N-labelled fertilizer, the $\delta^{15}N$ (natural abundance) technique is preferable.

At the beginning of the growing season (perennial crops) or at seeding time (annual crops), the enriched N is added to the soil. The aim is to expand the difference between soil N and atmospheric N_2.

A 1 m^2 micro-plot is placed in all experimental plots and divided into 400 squares (5 cm × 5 cm each). A solution of ^{15}N (5 – 20 atom%)-double labelled NH_4NO_3 is prepared in deionized water and 2 mL of fertilizer solution is pipetted over each cell of the grid, followed immediately by 5 mL of deionised water, giving a net rate of 5 g N m^{-2}. 'Normal' fertilization practices can be followed thereafter.

Every time the plants are harvested, samples of legume and non-legume are taken from an area of 0.25 m x 0.25 m in the middle of each micro-plot, as above.

Samples are analysed for atom % ^{15}N and total N with atom mass spectrometer.

The proportion of N derived from the atmosphere (Ndfa, in the legume) is calculated as in equation (3) using the non-legume as a reference plant representing the soil-derived N:

$$Ndfa = (1- (atom\ \%_{legume} – 0.3663 / atom\ \%_{non\text{-}legume} – 0.3663)) \times 100 \quad (3)$$

Here the atom % ^{15}N of the atmosphere is 0.3663 and it is subtracted from the atom % ^{15}N of non-legumes and legumes to determine the excess of atom% ^{15}N.

Apply the BNF calculation as shown above.

The N-difference method

This method should be used when (1) you grow sole crops, (2) the abundance of ^{15}N in the soil is lower than in atmospheric N_2 (0.3663 atom %), and (3) you have no funds for ^{15}N fertilizer.

Every time the plants are harvested, a sample of legume and non-legume is taken from each plot, as described above.

Dry matter yield is determined (step 1.4) and total N content is determined using Dumas or Kjeldahl.

Apply the BNF calculation as shown above.

BNF calculation

The amount of BNF in the harvested biomass of legume is calculated by equation 4:

$$BNF\ (kg\ N\ ha^{-1}) = (DM\ yield_{legume}\ (kg\ ha^{-1}) \times N\%_{legume} \times Ndfa) / 100 \quad (4)$$

Ndfa is determined by ^{15}N abundance or ^{15}N enrichment method, and if this is not possible then an estimated may be made. If the mineral N content of the soil is low and no extra N fertilization has been used, the Ndfa is probably 80-90 %. In high soil and/or mineral N concentrations, however, the Ndfa value can be 50-60 %.

The amount of BNF in the harvested biomass of the legume in N difference method is calculated by equation 5:

$$\text{BNF (kg N ha}^{-1}) = (\text{DM yield}_{\text{legume}}\ (\text{kg ha}^{-1}) \times \text{N\%}_{\text{legume}}) - (\text{DM yield}_{\text{non-legume}}\ (\text{kg ha}^{-1}) \times \text{N\%}_{\text{non-legume}}) \quad (5)$$

For forage legumes grown in a mixture with grasses, equations 6-8 developed by Carlsson & Huss-Danell (2003)[15] can be used:

$$\text{Red clover: BNF (kg ha}^{-1}\ \text{y}^{-1}) = \text{clover DM yield (kg ha}^{-1}\ \text{y}^{-1}) \times 0.026 + 7 \quad (6)$$

$$\text{White clover: BNF (kg ha}^{-1}\ \text{y}^{-1}) = \text{clover DM yield (kg ha}^{-1}\ \text{y}^{-1}) \times 0.031 + 24 \quad (7)$$

$$\text{Lucerne: BNF (kg ha}^{-1}\ \text{y}^{-1}) = \text{lucerne DM yield (kg ha}^{-1}\ \text{y}^{-1}) \times 0.021 + 17 \quad (8)$$

The harvested biomass includes only 60 - 70% (as a mean from results varying from 50% - 90%) of the BNF of the total biomass of the legumes, since much remains in the roots and stubble. Therefore the BNF in the harvested yield should be multiplied by 1.6.

[15] Carlsson G, Huss-Danell K. (2003). Nitrogen fixation in perennial forage legumes in the field. *Plant and Soil* **253**, 353-372.

MEASURING N_2O USING STATIC CHAMBERS

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The measurement of emissions of N_2O from soil should be carried out using static chambers. The majority of measurements of N_2O emissions reported in the literature arise from the use of static chambers inserted into the soil, so-called non-flow-through, non-steady state chambers (NFT-NSS chambers). There is no single accepted chamber design or measurement protocol although in recent reviews on experimental procedure a number of quality control criteria have been established (Rochette and Hutchinson, 2005; Rochette and Bertrand, 2007; Rochette and Eriksen-Hamel, 2008). A summary table adapted from Rochette and Eriksen-Hamel (2008) is illustrated below highlighting accepted good practice.

Table 2. Grading of chamber design and N_2O sampling protocol according to good practice.

ACCEPTED PRACTICE WEIGHTING				
CHAMBER DESIGN	VERY POOR	POOR	GOOD	VERY GOOD
Chamber type	push-in to soil types		Dedicated base + chamber	
Insulation	No		yes	
Vent	No		yes	
Height (cm)	<10	10 to <20	20 to <40	≥40
Base insertion (cm)	<5	5 to <8	8 to <12	≥12
Area/perimeter ratio	<2.5	2.6 to <6.25	6.26 to <10	≥10
Duration (min.)	>60	>40 to 60	>20 to 40	≤20
SAMPLING PROTOCOL				
Type of sample vial	Plastic syringe	Glass Syringe	All other vials	Exetainers vacutainers, Al tubes
Pressurized sample (fixed volume container only)		No		Yes
Quality control sample	No			Yes
Time zero sample		No		Yes
Number of samples	1	2	3	>3
Duration of sample storage				
plastic syringe	>2	1-2	<1	
glass syringe	>4	>2-4	1-2	<1
other	>90	>45 - 90	>15 - 45	≤15
CALCULATION OF FLUX				
Non-linear model considered	No			Yes
Temperature corrections		No		Yes

Chamber design

There are many types of NFT-NSS chamber described in the literature, the dimensions and design being dictated by the crop. The minimum height above ground (including

collar or sleeve) should be 25 to 50 cm for grass swards/young arable crops. Taller crops may require stackable types where an extension layer can be added when necessary. Such chambers normally include a water filled channel onto which the extension can be fitted maintaining an air-tight seal. FOR THESE CHAMBERS IT IS ESSENTIAL THAT THE WATER CHANNEL IS LEVEL DURING MEASUREMENT TO MAINTAIN THE SEAL. Covering chambers with reflective tape or simply using white paint will help in mitigating any temperature changes within the chamber during measurement.

Figure 5. Example of chamber and soil collar with pressure vent deployed in the field. Soil collar is constructed from Waven tubing with main body of the chamber being a white PVP bucket that fits tightly over the collar. The pressure vent is a simple loop of soft plastic tubing.

The use of a short sleeve or 'collar' already inserted into the soil and onto which the chamber is temporarily attached during deployment is considered best practice. This reduces the influence of soil disturbance on gas fluxes. The collar is inserted into a 5cm deep slot cut into the soil preferably at least a week before measurements begin, this collar having an above-ground extension of 4-5 cm. A diagram of one type of simple chamber and collar design is given in Figure 6.1. Note that a venting tube is attached. This is important to overcome the effect of chamber volume and pressure changes that arise when sliding the chamber onto the collar or removing air samples. A sampling port should also be included incorporating replaceable butyl rubber seals

With regard to the ground area the chamber covers then one factor to consider is the area /perimeter ratio. Here any influence of a poor chamber seal on the estimated flux will decrease with increasing chamber diameter. Rochette and Eriksen-Hamel (2008)

calculate an ideal ratio of the order of 10 cm or above, i.e., in the case of a cylindrical chamber one with a diameter of 40 cm.

Air sampling and storage

Air is sampled from the chamber by using a 50 mL plastic syringe with needle attached. Best practice dictates the use of air tight glass vials for the collection and storage of the air samples (i.e. crimp top glass vials with butyl rubber seal and tightly fitting crimp cap). These should be standard vials for gas analysis using an automated headspace sampler. The size of these vials will depend on each individual GC system. These vials may either be pre-evacuated or not depending on sampling procedure. We have found no difference in flux measurements using either pre-evacuated vials or vials flushed through with sampled air (Fig. 6.2).

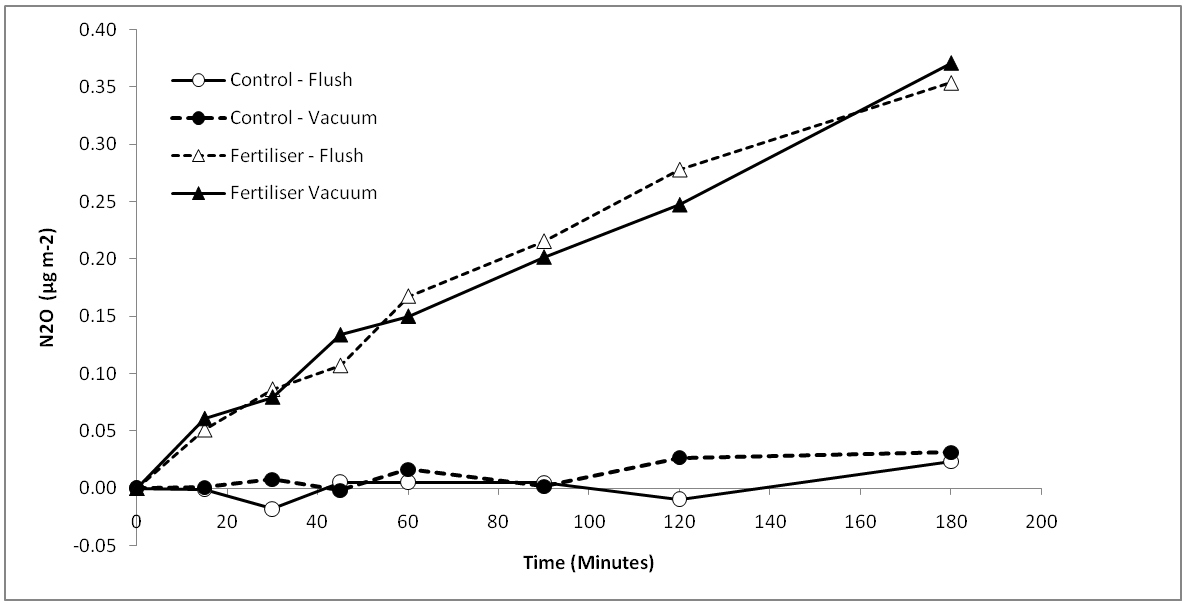

Figure 6. Increase in concentration of N_2O in air samples removed from soil chamber on either fertilized or control plots. Air sample vials were either pre- evacuated or flushed through with excess air sample on site (Brendan Roth, TCD, unpublished data).

The accuracy of flux estimation depends upon the number of air samples taken during deployment of the chamber. It is recommended that a minimum of three samples are taken with one of these representing the concentration of N_2O in the chamber headspace at time t=0. It is important not to assume ambient air conditions but sample from the chamber immediately after deployment.

The duration of deployment is another factor to consider in reducing errors associated with air sampling. In most situations the increase in N_2O concentration is linear over a 40 to 60 min time period but this may vary with chamber design and site conditions. However, long deployment times (≥40 min) may increase the possibility of changes in

temperature and humidity within the chamber and also the possibility of a negative feedback on N_2O flux. Optimum deployment times with respect to linearity of flux should therefore be determined for each site, as should the temperature of the chamber headspace at the time of air sampling.

The number of chambers to deploy at any one time is dependent on the ground area of the chamber and the area of the plot to be measured. There is no one solution given the spatial variability that may be present at any one site. For chambers with a ground area of 0.16m^2, SAC personnel have recommended 5 chambers *per* plot.

Unfortunately all gas containers used for air sampling leak, whether they be plastic syringes or glass vials. It is therefore important to reduce the time between collection of the samples for storage and measurement using gas chromatography. It is recommended for good practice that the duration of sample storage be no longer than 45 d from collection to analysis, and ideally less than 15 d.

Chamber deployment and sampling

Gas sampling should be carried out between 10:00 and 14:00 h due to diurnal variation in N_2O emissions, and ideally between 10:00 and 12:00 hrs as indicated by IPCC good practice. Linearity of fluxes should be tested on a number of chambers beforehand to determine the best three sampling times once the chamber is installed, and one of these three readings must be at t=0 min). Plastic septa on each chamber must be changed regularly as should syringes and needles.

Attach chamber to soil collar ensuring gas-tight seal and that chamber is upright. Record the height of the top of the chamber to the ground.

Take a plastic 50 mL or 100 mL syringe fitted with a needle and pierce the septum in the lid. Slowly remove 50 or 100ml of headspace gas without withdrawing the needle or disconnecting the syringe. Depress the plunger to force this sample back into the chamber and to ensure that a representative gas sample is taken.

SLOWLY withdraw another 50 or 100 mL sample, hold at 50 or 100 mL until the plunger stays fast then remove the syringe from the chamber.

For evacuated gas vials

Pierce the septum of the appropriate labelled gas vial. As the vial is pierced, the gas will automatically be withdrawn from the syringe to equalise the pressures in the vial and syringe. Push the plunger in as far as it will go and hold the syringe in position. Pierce the septum with another single needle (narrow bore) while pushing down the plunger of the syringe until it reaches 0 mL (the expelled gas should make a hissing noise). Hold both the syringe and needle in place for 3 s so that the pressure equalises (the hissing

noise should cease) then withdraw the venting needle first, immediately followed by the syringe needle.

SAMPLE VIALS SHOULD BE EVACUATED AS CLOSE TO N_2O SAMPLING AS POSSIBLE. DO NOT USE VIALS THAT HAVE BEEN EVACUATED FOR MORE THAN ONE WEEK.

For non-evacuated (flushed) gas vials

First pierce the septum of the appropriate labelled gas vial with a narrow bore syringe needle to act as a vent then pierce the septum with the sampling syringe needle and SLOWLY press the plunger of the syringe so that the gas vial is flushed with approximately 3 times the volume of sampled air (for 20 mL vials or larger, the 100 mL syringe is best). Withdraw the venting needle first immediately followed by the syringe needle.

Record the gas vial identifier, such that the vial and N_2O concentration in the particular chamber headspace can be matched following subsequent analysis by GC. Also record the time the gas samples are taken on the proforma.

Glass vials can easily be broken, so ensure they are well packaged to avoid damage in transit. A photocopy of the proforma sheets should be kept in the working file used by the gas ampler for ready reference.

For sites with no GC facility and where samples are to be sent to either SAC or Trinity College, the sample vials (with a photocopy of the signed proforma sheets) should be posted as soon as possible to the designated lab after collection. Best practice dictates that standards are added to each batch of vials for analysis to correct for gas loss if there is a delay in analysis by GC.

Ancillary measurements

Ambient N_2O

- Although the estimation of N_2O flux includes a measurement of the concentration of the gas in the chamber head space at time t = 0, it is also useful to determine the ambient concentration of N_2O in the atmosphere on the site on the day of measurement. To avoid contamination from car exhaust or soil efflux these samples are taken 1 m from the ground, away from any roads. Take 5 vial samples at the beginning of the chamber measurements and 5 vial samples at the end.

Soil temperature

- Soil temperature should be measured for each chamber position after each of the three air samples has been taken.

Soil moisture and dry bulk density

- Soil samples for gravimetric soil moisture content should be taken at a depth of 0-10 cm on each day of sampling. This is so % water filled pore space (WFPS) can be determined. In order to convert the gravimetric soil moisture values to WFPS, the soil dry bulk density must also be known. It is suggested that the core cutter method is used, with small cylinders that are hammered into the soil. As this parameter changes with management of the soil, one annual measurement is not sufficient.

Soil nitrate

- These measurements are already being taken in WP1. Monthly measurements would be useful.

Meteorological measurements

- At each experimental site, daily rainfall and air temperature (minimum and maximum) should be measured.

Frequency of measurements

The frequency of sampling throughout the year is an important factor in reducing errors in the estimation of annual flux. Ideally, measurements are taken each week with a greater frequency following N additions or ploughing. For many of the sites, this will not be possible, so a programme of monthly measurements should be designed with a greater frequency associated with times of fertilizer addition and ploughing. A typical strategy is given in Table 2.

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Summary of recommended methodology for using static chambers[16]

Please note the minimum set of criteria for reliable soil N_2O flux measurements using static chambers that is given in the six-point recommendation list below.

1. Chambers should be insulated, and have pre-inserted collars.
2. The height of the chamber should be greater than 10 cm.
3. The collar should have a minimum insertion depth of 5 cm.
4. For air sample storage pressurized fixed-volume containers of known efficiency should be used (i.e. avoid plastic syringes).
5. A minimum of three discrete air samples should be taken during deployment of the chamber, including one at time zero.
6. The non-linearity of changes in headspace concentration with time should be tested when determining dC/dt.

Chamber deployment, air sampling and sample storage

Materials and equipment

1. Chambers and Collars (collars pre-installed in the experimental plots).
2. Numbered pegs to identify chambers, timer and needles and 50 or 100 mL plastic syringe.
3. Crimp- top glass vials with butyl rubber seal and tightly fitting crimp cap (either evacuated or not) exact details depend on GC used.
4. Sampling proformas to be completed for each sample day.

Chamber deployment and sampling

1. Gas sampling should be carried out between 10:00 and 14:00 hrs.
2. Attach chamber to soil collar ensuring that seals are gas-tight and that chamber is upright. Record the height of the top of the chamber from the ground.
3. Prepare glass evacuated or non-evacuated glass sample vials.

[16] Accepting these recommendations, the methodology presented is adapted from the joint ADAS, AFBI, NW-Res and SAC N_2O flux sampling and calculation protocol and with comments abridged from Rochette and Eriksen-Hamel (2008).

4. Take a plastic 50 mL or 100 mL syringe fitted with a needle and pierce the septum in the lid. Slowly remove the syringe's maximum volume of headspace gas without withdrawing the needle or disconnecting the syringe. Depress the plunger to force this sample back into the chamber and to ensure that a representative gas sample is taken. **SLOWLY withdraw** another maximum-volume sample, hold at the maximum volume until the plunger stays fast, then remove the syringe from the chamber.

5. Record the gas sample vial identifier, such that the vial and N_2O concentration in the particular chamber headspace can be matched following subsequent analysis by GC. Also record the time the gas samples are taken on the proforma shown below.

6. Samples are to be sent immediately for processing Air is sampled from the chamber by using a 50 mL plastic syringe with needle attached.

7. Take a minimum of three samples: one at time zero, the other two later.

8. Accumulation time should not exceed ≥40 min (record the incubation time).

9. The number of chambers to deploy: for chambers with a ground area of 0.16m^2, SAC have recommended 5 chambers per experimental plot.

10. Minimise the time between sample collection and measurement using gas chromatography to less than 15 d.

11. Calculation of N_2O flux.

The following formula is used to calculate the flux of N_2O from any NFT-NSS chamber, the important variables being the total chamber volume, the surface area covered by the chamber, and the volume of 1 mole of N_2O at the air temperature within the chamber headspace.

$$N_2O \text{ flux } (gN_2O\text{-}N\ ha^{-1}\ d^{-1}) = (\delta C/\delta t) \times (VM_{mol}/AV_{mol}) \times (1.44 \times 10^7) \quad (9)$$

Here $\delta C/\delta t$ is the rate of increase in concentration of N_2O within the chamber (ppm), V is the total chamber volume (m^3), M_{mol} is the molar mass of N_2O (28 g mol^{-1}), A is the surface area covered by the chamber (m^2) and V_{mol} is the volume occupied by 1 mole of N_2O at the temperature recorded for the chamber headspace at the time of sample collection.

Proforma for gas sampling for analysis by GC

Experiment Code:	
Experiment Title:	
Site Name:	
Field Name:	
Sampling Date:	
Number of Ambients and Time Taken:	
Number of stored samples and concentration (ppm): (To be entered by lab staff)	
Weather during sampling:	**Raining / Dry** **Hot / Warm / Cool / Cold** **Calm / Light breeze / Windy**
Soil conditions:	**Water on surface / Soil wet / Soil moist / Soil dry**
General Comments:	

Sampled by:..... Certified by:.....

Sample Identifier	Plot Number	Chamber Number	Chamber and lid equipment numbers	Chamber Height (cm)	Time Chamber On	Time Gas Sampling	Notes

Sampled by:..... Certified by:.....

ANNEX 1 - MEASURING BNF USING THE 15N NATURAL ABUNDANCE METHOD

Euan James, James Hutton Institute, UK.

1. Assess the N content of the soil in the prospective experimental sites to ensure that is not so high that legumes will be inhibited from nodulating and fixing N_2.

2. Assess the $\delta^{15}N$ signal of the plant-available N in the soil by sampling non-nodulated weeds growing within the prospective sites, and then select the site(s) with the highest $\delta^{15}N$ value to conduct the experiment.

3. Plot size will depend upon the objective of the experiment and the number of harvests planned. If the objective is to estimate %Ndfa alone, then plots do not have to be too large, but if estimates of kg N fixed ha^{-1} are required then determinations of plant biomass will be also be needed and plot sizes will consequently be larger. Edge effects also have to be taken into account regardless of the experimental objective.

4. Before starting the field experiment, grow the same cultivars of the legumes in sterile sand-filled pots in the greenhouse, and inoculate them with an effective strain of their Rhizobium. Harvest the shoots of the plants at 2 or 3 growth stages from early vegetative stage up to and including the end of pod fill, and evaluate the $\delta^{15}N$ signature and total N content at each harvest. This should allow for the calculation of a B-value for each growth stage, which can then be used to calculate the %Ndfa of the field-grown plants at the same growth stages.

5. Once B-values and soil/non-legume $\delta^{15}N$ values are obtained, it should be possible to assess the feasibility of using the technique in the field, e.g., the soil/non-legume $\delta^{15}N$ signature is suitably high (>2‰) and the B-value(s) are not too low (see Equation 3).

6. Sow the grain legumes together with the non-legumes in an appropriately randomized manner that also reduces errors due in estimating N_2 fixation due to soil heterogeneity (see Unkovich et al. 2008 for examples of plot layouts).

7. There should be more than one species of reference non-legume, and they should have a similar duration of growth and pattern of N-uptake as the legume. A brassica (e.g., oilseed rape) and barley would be appropriate for Balruddery. Dicot weeds of a similar age to the legume can also be harvested as additional references during the experiment.

8. Do not add N-containing fertilizer to either the legumes or to the references, as they must exploit the same soil N pool for the technique to be valid.

9. At each harvest randomly select 10 subplots (0.5 – 1 m^2) per plot, harvest whole shoots from 5 legume plants per subplot, bulk the 5 shoots, and oven dry them as soon as possible. Do likewise for the non-legume reference plants in the same plots.

10. The samples are weighed, milled, and analysed for total N concentration and atom% ^{15}N values.

ANNEX 2 - MEASURING BNF USING N DIFFERENCE AND THE ^{15}N DILUTON TECHNIQUE

William Burchill, James Humphries, Gary Lanigan and Dejun Li, Teagasc, Ireland

Mike Williams, Trinity College Dublin, Ireland

Un-grazed plots

Materials

- Etesia mower
- Oven
- Milling machine
- Plastic sample bags
- Weighing scales
- Fencing materials (to set up plots)
- ^{15}N (99 atom%) $(NH_4)_2SO_4$
- Wash bottle with narrow nozzle (application of ^{15}N $(NH_4)_2SO_4$)
- Measuring tape
- Deionised water
- Gardena grass shears
- Timber pegs

Method: (N difference technique)

Here the difference in protein N content of herbage between clover mix and non-clover plots will be compared with a more specific determination of biological N fixation using ^{15}N.

Clover and non-clover control plots (1.5 m x 10 m) have been set up adjacent to one another and receive no grazing or N fertilizer. Herbage is cut on a regular basis throughout the year to mimic grazing frequency on the farm and the dry weight and protein N content determined.

Field: A central strip is cut through the each plot using the Etesia mower and the herbage fresh weight determined. A 300 g sub-sample is taken for analysis and the remaining herbage removed. Any remaining grass on the plots is cut to 4cm and the herbage removed.

Laboratory: A fresh 100 g sample is taken from each 300 g sample of herbage from the clover and non-clover plots. These samples are dried at 40°C for 48 h, milled and the N content (crude protein) determined in the laboratory. Another 100 g fresh sample is weighed out and dried at 100°C for at least 16 h. This dried herbage sample is weighed and the kg dry matter ha^{-1} calculated. Biological nitrogen fixation as calculated by N difference is determined according to Carranca et al. (1999).

Method: (^{15}N dilution technique)

Here 1 m^2 micro plots adjacent to the clover mix and non-clover plots have been set up that receive no grazing or N-fertilizer. The vegetation in each case is representative of the clover mix plots.

The ^{15}N (99 atom%) $(NH_4)_2SO_4$ will be applied to these micro plots at a rate of 0.1 g N m^{-2} in a solution of 700 mL at four-monthly intervals. The micro-plots will be harvested on the same day as the larger plots used for the N difference technique (once a month for two years). On each harvesting day an area of 0.5 m x 0.5 m in the centre of the plots will be cut to 4 cm and the harvested plant material separated into white clover and ryegrass and both fractions weighed.

Laboratory: A portion of the white clover and ryegrass material (100 g) is dried at 40°C for 48 h to determine DM%, milled to a fine powder and sent for analysis of total N and ^{15}N. The fraction of N derived from N fixation (%Ndfa) and biologically fixed N are calculated according to methods adopted by Hansen and Vinther (2001) and Carranca et al. (1999).

References

Hansen, J.P. and Vinther, F.P. (2001) Spatial variability of symbiotic N_2 fixation in grass-white clover pastures estimated by the N^{15} isotope dilution method and the natural N^{15} abundance method. Plant and Soil 230: 257 – 266.

Carranca, C., de Varennes, A and Rolsten, D.E. (1999) Biological nitrogen fixation estimated by N^{15} dilution, natural N^{15} abundance, and N difference techniques in a subterranean clover-grass sward under Mediterranean conditions. European Journal of Agronomy 10: 81-89.

ANNEX 3 - ROUTINE MEASUREMENTS OF N_2O FLUXES AT SOLOHEAD, IRELAND

William Burchill, James Humphries, Gary Lanigan and Dejun Li, Teagasc, Ireland.

Mike Williams, Trinity College Dublin, Ireland.

Materials

- N_2O gas flux chambers and collars
- 12 mL evacuated vials
- Thermometer
- Soil moisture meter
- Gas chamber fans
- Syringe and needles
- GC

Method

N_2O flux will be measured from grazed clover paddocks on a low stocking management system of Jersey and Holstein Friesian cows in order to provide data for calculation of N emissions. Six paddocks will be used with five gas flux chambers randomly placed in each.

Field Procedure

N_2O fluxes will be recorded weekly. At each sampling time the chambers are placed on the inserted collars for 30 minutes with the chamber fans turned on. Gas samples are taken at time T_0 and T_{30}. The change in the N_2O concentration in the headspace over the 30min cover period is used to calculate N_2O fluxes.

Sampling will be increased from every week to every two days after application of synthetic fertilizer and slurry for a period of two weeks. This is required to capture the increase (peak) in N_2O emissions after N application. Air temperature inside each chamber is also recorded at times T_0 and T_{30}. Soil temperature and soil moisture are recorded on each sampling day. Chamber volume sampling and analysis by GC/ECD is as described in the main Legume Futures protocol (Legume Futures Report 1.1).

ANNEX 4 - MEASURING OTHER N FLUXES AT SOLOHEAD, IRELAND

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Mike Williams, Trinity College Dublin, Ireland

This is about measuring N_2 emissions to air and N emissions to water

Materials

$^{14}NH_4{}^{15}NO_3$, $^{15}NH_4{}^{15}NO_3$, typically 98 atom% ^{15}N

Gas collection chambers and collars for $^{15}N_2$

12 mL and 7 mL evacuated vials

Syringes and needles

Isotope ratio mass spectrometer (UC Davis USA)

Thermometers

Delta T Probe

Soil sampler

KCL extraction solution

Water sample containers

Well depth dipper

Wash bottle with narrow nozzle (for application of $^{15}NH_4{}^{15}NO_3$ and $^{14}NH_4{}^{15}NO_3$)

Pick axe and sledge hammer

Whatman no.2 filter paper

Hand water pump

Experiment design

Two collars will be placed in each of six paddocks as well as in three control plots receiving no fertilizer or grazing. At each paddock $^{15}NH_4{}^{15}NO_3$ or $^{14}NH_4{}^{15}NO_3$ will be

applied to the two adjacent collars four times per year over a two year period. The position of the collars at each site will be changed each time the labelled fertiliser is applied. Water and soil samples will also be collected from these paddocks over the two year sampling period.

Method:

The $^{15}NH_4{}^{15}NO_3$ and $^{14}NH_4{}^{15}NO_3$ will be applied to the two collars at each site to derive two ratios ($\text{Ratio A}=\frac{N_2O_{denit}}{N_2O_{nit}+N_2O_{denit}}$; $\text{Ratio B}=\frac{N_2}{N_2O_{denit}}$).

Given the two ratios determined eight times in the year can represent annual N_2 emission and seasonal variation can be derived in combination with the data of routine N_2O fluxes (F_{N2O}), which was collected in 2009/2010, as the following:

$F_{N2O\text{-}denit} = F_{N2O} \times$ Ratio A;

$F_{N2} = F_{N2O\text{-}denit} \times$ Ratio B

Where $F_{N2O\text{-}denit}$ means the fraction of N_2O evolved from denitrification in the routine samples, and F_{N2} means N_2 fluxes.

^{15}N labelling Procedure

Before labelling the grass in each soil collar will be cut to 2 cm and the cuttings removed.

Soil within these collars will be treated with solutions of either $^{14}NH_4{}^{15}NO_3$ or $^{15}NH_4{}^{15}NO_3$. The fertilizers are applied at a rate of 10 kg N ha^{-1}. This is a rate of 0.2g $^{15}NH_4{}^{15}NO_3$ and $^{14}NH_4{}^{15}NO_3$ at 98 atom % excess ^{15}N per collar.

The fertiliser is mixed to form a solution (100 mL) which is then applied evenly across the soil surface within the collars. The wash bottle used to apply the solution is then washed with deionised water and the washings also applied to the collar.

Gas Sampling procedure

Two, four, seven and ten days after treatment two samples are collected (a) for $^{15}N_2O$ and $^{15}N_2$ analysis (12 mL vials) and (b) for N_2O, CO_2 and CH_4 analysis (7 mL vials). This sampling procedure will be reviewed once the first set of results has been analysed. At time 0, one sample is taken and placed in a 7 mL evacuated vial for concentration analysis.

At each sampling time the chambers are placed onto the collars. Gas samples are then withdrawn from the headspace after 2 hours. Three ambient air samples are also collected on each sampling day to determine the natural abundance of both N_2O and N_2. If the natural abundance concentration of N_2O and N_2 remains constant over the first

number of sampling days, then further collection of ambient air will not be necessary. In addition, temperature within and outside the headspace is recorded on each day of measurement.

The following soil characteristics associated with each sampling site will also be recorded on each sampling day: soil moisture at 5cm depth to determine WFPS, soil temperature at 5 cm, and topsoil samples for determination of soluble organic carbon and soluble mineral nitrogen (nitrate N and ammonium N). Soil pH will also be measured once a year.

Groundwater sampling procedure

Groundwater levels from adjacent wells will be recorded to determine the depth of the water table during the period of gas sampling. Ground water samples will be taken to determine N loss to ground water (nitrate-N and ammonium-N).

Groundwater (GW) samples will be taken fortnightly during the winter drainage period and after periods of high rainfall. All the resident water will be removed from the wells using a hand pump and wells will be allowed to recharge for at least three hours prior to sampling. After refill, a 50 mL sample will be taken from each well. Water samples from each paddock will be bulked and composite samples filtered (Whatman no.2 filter paper) within an hour after sampling. The collected samples will be frozen at -15°C until analysis.