
# ANNEX 5B:

# POT TRIAL PROTOCOL TO EVALUATE SHORT-TERM EFFECTS OF RECYCLING-DERIVED PHOSPHORUS FERTILIZERS ON THE GROWTH OF LOLIUM PERENNE AND ITS RHIZOSPHERE MICROBIOTA

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# POT EXPERIMENT

# INVESTIGATING THE PHOSPHORUS-CYCLING MICROBIOTA IN SOIL

# 1. Introduction

Pot experiments are implemented in order to analyze plants, roots and soil microbiota in a controlled setting. Considering the limited amount of soil, pot trials are usually carried out as exhaustive experiments. Treatments should be applied at least in replicates of four for meaningful statistical analyses (e.g. one-way ANOVA using SPSS or R software). Results are always compared to an untreated, non-fertilized control. Here, the impact of recycling-derived fertilizers on the soil microbial community and plant growth is investigated in comparison to mineral fertilizer, focusing on the nutrient phosphorus.

# 2. Material

## 2.1. Fertilizer

- Control (no P fertilizer)
- Triple super phosphate (TSP)
- Recycling-derived fertilizers (RDFs): ashes, struvites,...

The TSP and the RDFs are added to the soil in different rates (e.g. 20 and 60 kg P/ha) based on their individual phosphorus content and considering the plants' demands. The variations of the nutrient concentration are only across the P concentration, every other nutrient is supplied in the same form and quantity across the trial.

## 2.2. Plant Host

Application of perennial ryegrass (*Lolium perenne*, diploid variety AberGreen, provided by Germinal Ireland Ltd., Tipperary, Ireland) to the pots, chosen from a list of recommended ryegrass varieties in 2018, published by the Irish Department of Agriculture, Food and the Marine in 'Grass and White Clover Recommended List Varieties for Ireland 2018' (Figure 1).

**Recommended Intermediate & Late Perennial Ryegrass Varieties 2018**

| Variety Name | Ploidy | Heading Date | Pasture Profit Index Values € / Ha / Year: Total PPI | Sub-Indices: Spring Growth | Summer Growth | Autumn Growth | Quality | Silage | Persist. | Spring (t DM/ha) | Summer (t DM/ha) | Autumn (t DM/ha) | Total Yield (t DM/ha) | Mean DMD (g/kg) | 1st Cut Silage (t DM/ha) | 2nd Cut Silage (t DM/ha) | Ground Cover Score |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | *1.17** | *7.14** | *2.35** | *10.66** | *843.6** | *4.54** | *4.07** | *6.1** |
| Aberclyde | T | 25-May | **225** | 57 | 48 | 37 | 55 | 28 | 0 | 1.35 | 7.36 | 2.25 | 10.96 | 856.2 | 5.01 | 3.73 | 5.6 |
| Abermagic | D | 29-May | **217** | 48 | 50 | 71 | 30 | 17 | 0 | 1.30 | 7.43 | 2.56 | 11.28 | 849.2 | 4.64 | 3.89 | 6.3 |
| Fintona | T | 22-May | **215** | 78 | 28 | 46 | 24 | 39 | 0 | 1.48 | 6.83 | 2.33 | 10.63 | 842.7 | 5.08 | 3.99 | 5.5 |
| Aberzeus | D | 29-May | **212** | 70 | 48 | 52 | 9 | 34 | 0 | 1.43 | 7.36 | 2.38 | 11.17 | 842.3 | 5.07 | 3.84 | 6.7 |
| Nifty | D | 27-May | **208** | 81 | 52 | 60 | -12 | 26 | 0 | 1.50 | 7.47 | 2.45 | 11.42 | 837.6 | 5.04 | 3.63 | 6.4 |
| Moira | D | 25-May | **207** | 121 | 26 | 52 | -18 | 27 | 0 | 1.74 | 6.78 | 2.39 | 10.91 | 831.4 | 4.93 | 3.80 | 6.5 |
| Abergreen | D | 30-May | **206** | 63 | 58 | 65 | 16 | 4 | 0 | 1.39 | 7.61 | 2.51 | 11.51 | 845.4 | 4.31 | 3.89 | 6.7 |
| Aberplentiful | T | 08-Jun | **203** | 60 | 49 | 45 | 29 | 20 | 0 | 1.37 | 7.39 | 2.32 | 11.07 | 847.6 | 4.42 | 4.28 | 5.6 |
| Abergain | T | 05-Jun | **198** | 32 | 43 | 45 | 60 | 30 | -11 | 1.20 | 7.23 | 2.32 | 10.74 | 857.0 | 4.77 | 4.12 | 5.8 |
| Aberchoice | D | 09-Jun | **194** | 26 | 48 | 49 | 59 | 13 | 0 | 1.16 | 7.37 | 2.36 | 10.88 | 854.4 | 4.21 | 4.33 | 6.3 |

**Figure 1 Overview of the recommended intermediate and late perennial ryegrass varieties in 2018, published in 'Grass and White Clover Recommended List Varieties for Ireland 2018' by the Department of Agriculture, Food and the Marine, Ireland. The variety highlighted in the red box was chosen.**

## 2.3. Soil Type

The soil used should have a low P availability with a P index of 1 or 2 out of 4 determined with Morgan's P test (classification of soils according to the plant growth response to the available P present in soils commonly used in Irish soils, see https://www.teagasc.ie/crops/soil--soil-fertility/soil-analysis/soil-index-system/ ). Within this range, a response to P fertilization is expected.

## 2.4. Equipment

- Round plant pots (height = 10 cm, $diameter_{top}$ = 12 cm, $diameter_{bottom}$ = 9 cm)
- Pot saucers with drainage (diameter = 11 cm, height of elevated part = 1 cm)
- Soil sieves (5.60 mm and 3.35 mm mesh size)
- Fan oven
- Pestle and mortar
- Top pan balance
- Analytical balance
- 25 mL glass pipette

- Nylon mesh (20 µm; PlastOk, Birkenhead, UK)

## 2.5. Fertilizer Preparation

If the granule size of the TSP and the struvites varies greatly the granules are broken down into smaller pieces using a pestle and mortar. The finer texture of the fertilizers allows for more accurate weighing and ensures a better distribution, when mixed with soil.

If the materials appear to be moist, they can be dried in a fan oven, until a weight equilibrium is reached. For struvites, temperatures above 40°C should be avoided.

## 2.6. Soil Analyses

The soil samples taken from a field are mixed and air-dried to some extent (residual moisture content of 10 – 20 %) to allow for sieving. First, the soil is sieved through a sieve with a mesh size of 5.60 mm, in order to exclude bigger stones. Then the soil is subsequently passed through a sieve with a mesh size of 3.35 mm to remove smaller gravel and roots and to break down bigger soil aggregates.

### 2.6.1. Moisture Content

- Take the empty weight of an aluminium tray ($m_0$)
- Weigh 50.00 g of sieved soil (3.35 mm) into the aluminium tray ($m_1$)
- Place the tray with the soil in a fan oven at 105 °C for 24 h
- Remove the tray from the oven after 24 h, let it cool down for 30 min at room temperature (RT) and take the weight again ($m_2$)
- Calculate the dry mass of the soil: $m_{dry} = m_2 - (m_0 + m_1)$
- Calculate the moisture content of the soil: Soil Moisture (%) = $(m_1 - m_{dry}) / m_1 * 100$

### 2.6.2. Field Capacity

- Take the empty weight of a plant pot with a nylon mesh (20 µm) covering the bottom ($m_0$)
- Add 500.0 g of air-dried and sieved (3.35 mm) soil ($m_1$)

- Add 500.0 g of water to the pot ($m_{H2O}$)
- Place the pot on a pot saucer with drainage and allow the water to leach by gravity (make sure there is enough space for the leachate)
- Weigh the pot after 24 h again ($m_{24h}$)
- Calculate the water addition: $m_{H2O, Add} = m_{24h} - (m_0 + m_1)$
- Calculate the total water content: $m_{H2O, total} = m_{24h} - (m_0 + m_1 * (100 - \text{Soil Moisture [\%]})$
- Calculate 100 % field capacity: Field capacity = $m_{H2O, total} / m_1$
- Calculate the amount of water needed to reach X % of field capacity:
- $m_{soil}$ * Field capacity * X % - $m_{soil}$ * Soil Moisture [%]

### 2.6.3. Soil pH

- Weigh 5.0 g of air-dried and sieved (2 mm) bulk soil into a 50 mL assay tube
- Add 20 mL of 0.01 M $CaCl_2$ solution
- Rotate solution (Elmi Intellimixer RM-S2) for 5 min at 70 rpm at room temperature (RT)
- Allow mixture to settle for 2 h
- Determine the pH in the supernatant

## 2.7. Potting Procedure

In general, the pots are set up in the following way:

1) Determine the soil capacity of the pots (e.g. 875 g soil)
2) Place a nylon mesh (20 µm mesh size) at the bottom to minimise roots growing past the pots and loss of bulk soil
3) Add soil mixed with RDF/TSP (e.g. 825 g soil)
4) Add seeds with residual soil without fertilizer addition on the top (e.g. 50 g soil)
5) Water the soil to around 70 % of field capacity (soil moisture is dependent on crop used in the experiment)

Below the steps are described in more detail:

### 2.7.1. RDF Application to Soil

The different RDF and the TSP are applied mixed throughout the soil, considering that the pot experiment is a rather short-term trial and therefore differences in micro-environmental conditions should be avoided. In addition, given the small amount of fertilizer added to the soil, the mixing of the soil with the fertilizer is carried out for each individual pot to ensure that every pot will receive the same amount of fertilizer. Only a small layer of soil on the top is applied without fertilizer addition to allow for undisturbed seed germination.

### 2.7.2. Seed Application

The amount of seeds is determined in accordance to sowing recommendations. The seed mixture applied in Teagasc (40 % AberGreen (D), 30 % AberChoice (D) and 30 % AberGain (T)) had a recommended sowing rate of 14 lb/ac (34.6 kg/ha). However, in case a single variety (AberGreen (D)) is used in the pot trial, the weight difference between the mixture of di- and tetraploid seeds of the mixed varieties and the diploid seed variety is corrected by weighing 100 seeds of both the seed mixture and the single variety in triplicates. Furthermore, the amount of seeds is adjusted for the low germination rate in soil tested beforehand. The calculation of the seed amount per pot with all the considerations mentioned above is carried out as follows:

$$Initial\ sowing\ rate\ \left[\frac{kg}{ha}\right] \cdot \frac{m_{mixed\ varieties}[kg]}{m_{single\ variety}\ [kg]} \cdot Germination\ rate\ [\%] \cdot Surface\ area\ \left[\frac{ha}{pot}\right] = Amount\ of\ seeds\ to\ apply\ \left[\frac{kg}{pot}\right]$$

The plants are incubated in a greenhouse. The temperature within the greenhouse is set to not fall below 18 °C. The soil temperature is monitored with a temperature sensor (Mätman G2-USB, Eltex of Sweden) in a separate pot, which is set up like a control and is irrigated the same way as the other pots. The pot positions are rotated weekly in order to randomise small differences in environmental conditions.

## 2.8. Pot Irrigation

Apply a sufficient amount of water to the pot that covers the plants' needs, but does not cause leaching. The same amount is applied to each pot, using a glass pipette. Irrigation takes place every second day. The pots are placed on pot saucers with drains to prevent stagnant moisture. The pots are weighed back once a week to monitor the moisture status.

## 2.9. Nutrient Solution Application

Dependent on the soil type and main goal of fertilizer application (nutrient build-up in soil, pasture establishment, reseeding, hay, silage production, grazing) the amount of nutrients may differ. Here, fertilizer recommendations from Teagasc – The Agriculture and Food Development Authority for Lolium perenne are used. In order to prevent precipitation of nutrients, every ingredient is dissolved and applied individually. The nutrients nitrogen, potassium, sulphur, magnesium, calcium,

copper, zinc and chloride (exclusion of phosphorus) are applied to all treatments. Before application, the pH of the solutions is measured. The nutrient solutions are added in 5 – 10 mL aliquots per pot on 2 subsequent irrigation days instead of watering the pots. The soil is preconditioned with 5 mL of water before the nutrient application. The nutrient solutions are applied once during the pot experiment, two weeks after the start of the experiment and shortly after the seed germination. An additional application of N and K after a grass cut can be considered.

**Table 1 Nutrient Application Rates recommended by Teagasc and the nutrient concentrations finally applied to the pots.**

| | *Recommended Concentrations [kg/ha]* | *Concentration Applied [kg/ha]* | *Concentration [mg/cm²]* | *Mass /Pot [mg]* |
|---|---|---|---|---|
| *N* | 220 | 220 | 2.20 | 248.8 |
| *K* | 182 | 185 | 1.85 | 209.2 |
| *Ca* | 2 kg Ca / 1 kg N | 440 | 4.40 | 497.6 |
| *S* | 20 | 20 | 0.20 | 22.6 |
| *Mg* | 30 | 30 | 0.12 | 13.7 |
| $ZnSO_4$ | 5 | 5 | 0.05 | 5.7 |
| $CuSO_4$ | 20 | 15 | 0.15 | 17.0 |

**Table 2 Amount of chemical compounds needed to meet nutritional requirements for 50 pots, prepared either in 0.25 or in 0.5 L of deionized water.**

| | *Mass [g]* | *Volume [L]* | *Concentration [g/L]* | *Concentration [mol/L]* |
|---|---|---|---|---|
| $KNO_3$ | 27.05 | 0.25 | 108.21 | 1.07 |
| $Ca(NO_3)_2 \cdot 4H_2O$ | 73.30 | 0.25 | 293.21 | 1.24 |
| $CaCl_2$ | 34.45 | 0.50 | 68.90 | 0.62 |
| $MgSO_4 \cdot 7H_2O$ | 6.94 | 0.25 | 27.76 | 0.11 |
| $ZnSO_4 \cdot 7H_2O$ | 0.44 | 0.25 | 1.77 | 0.01 |
| $CuSO_4 \cdot 5H_2O$ | 1.33 | 0.25 | 5.31 | 0.02 |
| $MgCl_2 \cdot 6H_2O$ | 8.47 | 0.25 | 33.87 | 0.17 |

# 3. Possible Further Analyses

After harvesting the bulk and rhizosphere soil (soil closely associated with the plant roots), the following experiments can be conducted:

- Gravimetric assessment of the fresh and dry weight of the plants
- Elemental analysis of the plant nutrient uptake in the dry biomass
- Colorimetric determination of the acid and alkaline phosphomonoesterase activity in soil
- Cultivation-dependent analysis of P mobilisation capabilities of the soil microbiome
- Most Probable Number (MPN) approach (minimal media with single phosphorus source, either phytate or phosphonoacetic acid, and R2A medium)
- Tri-Calcium Phosphate (TCP) Agar colony count
- Microscopic investigation of arbuscular mycorrhizal root colonisation
- Extraction of DNA from rhizosphere soil
- PCR and subsequent denaturing gradient gel electrophoresis (DGGE) for 16S rRNA and ITS (internal transcribed spacer)
- Quantification of P-mobilising gene copy numbers via qPCR (e.g. phoD; NGS and/or clone libraries of P cycling genes)