1. ABOUT THE DATASET

------------

Title: Soil invertebrate and mycorrhizal activity in diverse grasslands: benefits for ecosystem service provision – PhD thesis dataset

Creator: Sarah Shepperd

Organisation(s): University of Reading.

Rights-holder(s): University of Reading.

Publication Year: 2023

Description: Data collected for PhD thesis. The thesis aim was to identify which of the commercially viable grasslands within the Diverse Forages Project had the most positive effect on the soil biota (earthworms, mesofauna and AMF) and whether changes in belowground biota affected aboveground biomass productivity. The thesis consists of three manuscript research chapters which address the soil biodiversity research gaps identified by comparing a conventionally fertilised forage pasture to three commercially available diverse grasslands.

Chapter 2 covered the changes in soil invertebrates, namely earthworms, a selection of soil mesofauna under grazed systems, and measuring arbuscular mycorrhizal (AMF) activity through trap plant root colonisation.

Chapter 3 looked at overall fungal community diversity and microbial functional diversity, with modelling effects on provisioning ecosystem service delivery of aboveground biomass.

And finally, Chapter 4 focused solely on AMF community’s contribution to the commercially available diverse grasslands in dry, stressed environments, suggestive of future climate change conditions. The experimental set up, methodologies and data used for all three research chapters are understandably interlinked, with sampling campaign breakdown identified in Table 1

*Table ‎1 Sampling campaigns for experimental Chapters 2, 3 and 4. Site column identifies the three sites used in this thesis research (Crops Research Unit (CRU): Dry site or Well-wetted site (WW), or the Centre for Dairy Research (CEDAR)). Project column identifies sample collector (DivForages: Diverse forages project team, or Sarah Shepperd (SS)).*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Sample date** | **Site** | **Project collector** | **Sample collected** | **Analysis conducted** | **Chapter** |
| September 2016 |  | DivForages | Plots sown: PRG, SG, Bio, Her | - | 2, 3, 4 (only SG, Bio, Her) |
| March-October 2019 | CRU – Dry and WW | DivForages | Aboveground biomass | - | 3, 4 |
| April 2019 | CEDAR | SS | Earthworms | Adult earthworms identified  Adult and juvenile earthworms weighed | 2 |
| April 2019 | CEDAR | SS | Soil chemistry | Soil carbon and soil nitrogen (inhouse) | 2 |
| April 2019 | CEDAR | SS | Mesofauna | Tullgren funnel. Mesofauna identified down to phylum, class or order depending on taxa | 2 |
| October 2019 | CRU – WW only | SS | Root soil cores | Root biomass – chapter 2 and 3  Root surface area – chapter 3 | 2, 3 |
| March 2020 | CRU – Dry and WW | DivForages | Soil chemistry | Sent to NRM for pH, P, N, K, Mg, OM, C | 2, 3, 4 |
| March 2020 | CRU – Dry and WW | SS and DivForages | Earthworms | Earthworm biomass | 3, 4 |
| March 2020 | CRU – Dry and WW | SS | Ryegrass AMF trap plants planted | - | 2, 3, 4 |
| September 2020 | CRU – Dry and WW | SS | Soil samples for DNA | Bulk soil fungal DNA extracted | 3 |
| October 2020 | CRU – Dry and WW | SS | Ryegrass AMF trap plants removed | AMF colonisation determined via root staining (Dry and WW)  AMF root DNA (Dry site only, Chapter 4 only)  Rhizosphere soil AMF DNA (Dry site only, Chapter 4 only) | 2, 3, 4 |
| July 2021 | CRU – Dry and WW | SS | Soil sampling for chemistry & Microresp experiment | Soil chemistry sent to NRM laboratories for total C and N (Chapters 3 and 4). Microresp data for multiple substrate induced respiration and microbial functional diversity (Chapter 3 only) | 3, 4 |

Cite as: Sarah Shepperd (2023): Soil invertebrate and mycorrhizal activity in diverse grasslands: benefits for ecosystem service provision- PhD thesis dataset. University of Reading. Dataset. https://doi.org/10.17864/1947.000514

2. TERMS OF USE

------------

Copyright 2023 University of Reading. This dataset is licensed under a Creative Commons Attribution 4.0 International Licence: https://creativecommons.org/licenses/by/4.0/.

3. PROJECT AND FUNDING INFORMATION

------------

Title: Soil invertebrate and mycorrhizal activity in diverse grasslands: benefits for ecosystem service provision – PhD thesis

Dates: Oct 2018 – July 2022

Funding organisation: Perry Foundation and University of Reading

4. CONTENTS

------------

File listing

SHEPPERD\_2023 EXCEL file with data per tab:

Soil 2020 – soil chemistry and physical data collected in spring 2020 over the two farms used for the research (further information in methods section). Soil cores were taken to 30cm depth and sent to NRM laboratories for analysis

Soil moisture 2020 – soil moisture taken over the two farms in spring 2020 using a soil moisture probe over 3 depths

Roots for R – Root data for the Sonning medium site only taken by soil corer over four depths down to 60cm. Root length, root surface area, average diameter, root length per volume and root volume all calculated using a winrhizo software (further information in methods below)

Microresp – percentage CO2 expelled during the microresp experiment from soil samples taken at the two Sonning sites in 2021. 7 carbon substrates and a water blank used (more information on methods below)

Root biomass – dried root biomass from the soil cores taken in 2019 at the Sonning medium site over 4 depths down to 60cm

Worm biomass 2019 – earthworm data collected at the CEDAR site which includes age (adult/juvenile), species information if the specimen was an adult and biomass. Soil chemistry of carbon and nitrogen (further methods described below)

Worm count 2019 – Earthworm count of the CEDAR data collection in 2019. Also included is soil carbon and nitrogen data (more information in methods below)

Worms 2020 – earthworm count and biomass of the earthworms sampled at the Sonning sites in spring 2020. Further information in methods below

Tullgren 2019 – Results from the Tullgren funnels from the CEDAR site with samples collected in spring 2019 (further information in methods below)

Myco col 2020 – mycorrhizal root colonisation results for the two sites at Sonning after the trap plant experiment which ran over the summer of 2020. Two trap plants per plot. Used the grid line intersect method to measure percentage root colonised (more information in methods below)

RootDNA – root mycorrhizal fungi DNA for the Sonning dry site only. Also included are other soil parameters of moisture, pH, phosphorus, potassium, organic matter, nitrogen, carbon, earthworm biomass, aboveground biomass, arbuscular mycorrhizal colonisation and results from the microresp experiment. (further information in methods below)

SoilDNA – soil fungi DNA results for the two Sonning sites. Also included are other soil parameters of moisture, pH, phosphorus, potassium, organic matter, nitrogen, carbon, root biomass and root surface area (ww site only), earthworm biomass, aboveground biomass and arbuscular mycorrhizal colonisation. (further information in methods below)

AG Biomass – aboveground biomass results for the two farms used in the study (further information in methods below)

5. METHODS

-----------

Forage mixture

Forage mixtures were sown in September 2016 at two farms belonging to the University of Reading: Centre for Dairy Research (CEDAR) and Crops Research Unit (CRU). Mixtures ranged from a single species perennial ryegrass (*Lolium perenne*) to three diverse forage mixtures of increasing species richness: SmartGrass (6), Biomix (12), and Herbal (17 species). Perennial ryegrass plots received the recommended 250kg/ha N (as ammonium nitrate), divided into four application timings annually: two at 75kg/ha N and two at 50kg/ha N. Diverse species mixture plots received no nitrogen fertiliser.

Table 2 Diverse forage mixture species selection list (PRG: Ryegrass; SG: SmartGrass; Bio: Biomix; Her: Herbal) sown September 2016 at the University of Reading Centre for Dairy Research, Berkshire and University of Reading Crops Research Unit Farm, Berkshire. Ryegrass received 250kg N/ha (as ammonium nitrate), divided into four application timings across the year: two at 75kg/ha N and two at 50kg/ha N. Diverse mixtures receive no nitrogen fertiliser

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

Centre for Dairy Research Farm (CEDAR)

Twenty 1ha paddocks representing the four forage mixtures repeated 5 times were sown at the CEDAR farm in Shinfield, Berkshire (51o24’43”N, 000o54’30”W). The paddocks were located on freely draining, slightly acid (pH 6.1) loamy soils (Cranfield University, 2019). Management of the paddocks consisted of rotational strip grazing by 40 growing dairy cattle between March and November, starting two years after paddock establishment. Surplus herbage was cut to prescribed sward heights (7cm) and removed to maintain forage quality across replicates. Land use prior to the establishment of the paddocks was Italian ryegrass (*Lolium multiflorum*) in paddocks 1-10 and perennial ryegrass (*Lolium perenne*) in paddocks 11-20.

Crops Research Unit Farm (CRU)

Two locations within the CRU farm in Sonning, Berkshire (51o28’22.4” N 0o54’15.3” W) were selected for their varying soil types. One site was an excessively drained shallow light sandy loam on gravel bed, while the other was a free-draining loamy coarse sand close to the river Thames (Cranfield University, 2019). The first site usually experiences severe drought in the summer (2% soil moisture as of June 2018), hereafter referred to as the dry site. The second site can typically support crop growth throughout the growing season without irrigation (7% soil moisture as of June 2018), hereafter referred to as the well-watered site. Both sites were under arable management prior to the establishment of this experiment. At both CRU sites, four replicate plots 4.2 x 5m in size of the four forage mixtures were sown in a 4x4 Latin square design. The management regime of all plots simulated animal grazing by hand cutting and removal when biomass reached 2500 kg/ha dry matter from May until September, leaving 7cm height residual forage. Cutting occurred 2 to 5 times per season (three times per year on average) over 3 growing seasons (2017 to 2019).

Soil sampling

Earthworms

Two random 20x20x20cm soil pits per paddock were dug in April 2019 at CEDAR, two and a half years after sward establishment. The CEDAR site was chosen for the destructive sampling due to the larger paddock size compared to the smaller CRU plots. Five litres of mustard flour solution (6g/l mustard flour prepared 24hr before sampling) was poured down each pit and allowed 1 hour to soak away. Destructive soil pit sampling and hand sorting ensure epigeic and endogeic earthworms are observed; chemical extraction ensures the anecic ecological earthworm group is also sampled. The mustard solution was chosen as the chemical irritant over formalin vermifuge due to its non-carcinogenic and non-phytotoxic properties (Gunn, 1992; Pelosi *et al.*, 2009). Excavated soil was placed onto a 90cm x 40cm plastic sheet in the field, where the soil cube was gently teased apart to extract earthworms. Extracted earthworms were placed in a large, loosely closed bag to ensure gas exchange and earthworm survival until identification and weighing. Earthworms that emerged as a result of the mustard solution application were collected and washed with deionized water before placing them in the same bag as the earthworms extracted from the excavated soil. Sample bags with earthworms were kept indoors in a darkened area at room temperature before identification. Earthworms were extracted from the sample bags within 48hrs of field sampling. They were placed into a container of deionized water to remove excess soil before being placed onto damp tissue paper to remove excess water and weighed. All earthworms were weighed, including half earthworms. Adult earthworms were identified using the ‘Key to Common British Earthworms’ OPAL field guide (Jones and Lowe, 2012), and diversity measured using the Shannon diversity index.

Soil total carbon: total nitrogen

Approximately 600g of soil was collected from each soil pit at CEDAR after being sifted through for earthworms. Sample bags were kept at 4oC before analysis. Samples were dried in 40cm x 20cm metal containers for one day at 105oC. Dried samples were ground using a pestle and mortar, then passed through a series of sieves (5.6mm; 1mm; 425µm). ~0.2g of dried 425µm ground sieved soil, as required by the analyser protocol, was placed into a 502-186 tin foil cup, sealed and inserted into a carbon/nitrogen analyser (LECO CHN628; LECO corporation, Saint Joseph, Michigan, USA). Each sample was analysed for 4 minutes for complete combustion at 1000oC.

Meso fauna

An additional 600g of soil was collected from each soil pit after sifting through for earthworms. Soil was placed into Tullgren funnels with 20watt bulbs within 5 hours of the collection, ethanol was used as the collection fluid. Funnels were heated continuously for 16 days; ethanol traps were checked daily. Mesofauna were identified down to phylum, class or order level depending on taxa using a dissecting stereo microscope and identification guide (Tilling, 2014).

Root biomass

Soil cores (8cm diameter) were taken at the CRU well-watered site only over 4 depths (0-15cm, 15-30cm, 30-45cm, 45-60cm) in the autumn of 2019, three years after plot establishment. Soil cores were emersed in water and soaked for 24 hours to ease the separation of roots from the soil. Roots were washed clean of soil and then dried at 50oC to constant weight for dry biomass measurements.

AMF colonisation

Sterile sand (Sibelco UK Ltd) absorbent clay substrate was created as 4 parts sand to 1 part Terra Green (Oil Dri UK), with 0.025 g/kg calcium hydrogen orthophosphate and 10% deionised water. The mixture was autoclaved at 105oC for 1 hour, rested for 24 hours then autoclaved again at 105oC for 1 hour. Five-centimetre diameter hydroponic pots were placed within the same size closed bottom cups and each was filled with 140g of the sterile substrate. Afterwards, 14ml deionised water and 20mg ryegrass seeds (Cotswold Seeds Ltd, UK) were added at 1cm depth into the substrate.

Five pots per sunbag (Sigma-Aldrich Inc., Germany) were placed in a growth cabinet set to 22oC 16-hour days and 15oC 8-hour nights at 75% humidity for 1 month. Sunbags are transparent plastic bags with 0.02µm pores to enable plant growth and prevent contamination from the external environment, in this instance, the sunbags were used to prevent fungal contamination. Pots were fertilised with 1ml Long Ashton solution, a nutrient solution which contains no phosphorus, and 1ml deionised water twice over the 4 weeks the plants were in the growth cabinets. One ryegrass pot per sun bag was checked for mycorrhizal colonisation to confirm ryegrass plants free from mycorrhiza were planted in the field. All plants were removed from the growth cabinet and given one week to acclimate to ambient conditions in a sheltered outdoor space while remaining in the sun bags to ensure no mycorrhiza colonisation before planting in the field. In late March 2020, three and a half years after establishing the plots, two trap-plants per plot were planted at the two CRU sites only (*N*=64). Ryegrass trap-plants were removed from the closed-bottom pots but retained in the hydroponic pots and sterile substrate. Plants were given 5ml deionised water upon planting and monitored to ensure field establishment. Monitoring of the trap-plants continued through the spring and summer 2020.

Trap-plants, retained within their hydroponic pots, and an extra 5x5x5cm of soil from below the hydroponic pots to cover new root growth were extracted from the CRU plots in early October 2020 using a trowel sterilised in 1% virucidal disinfectant Virkon S (Lanxess, Cologne, Germany). Trap-plant and associated soil were kept at 4oC before processing.

Roots were washed free of soil, placed in 10% KOH, and left at room temperature for 3 days. Roots were then rinsed with deionised water and stained with Ink/Vinegar solution (1-part Shaeffer black ink to 19 parts white vinegar) for 1 hour at room temperature. Roots were rinsed again using deionised water and stored in lactoglycerol (1-part lactic acid, 2 parts glycerol, 1 part water; Walker & Vestberg, 1994). Percentage root AMF colonisation was measured using the grid line intersect method (Giovannetti and Mosse, 1980) with a compound microscope.

Soil chemistry

Three 10cm deep soil cores were taken in late spring 2020 from each plot at CRU only and mixed to create a composite sample. For soil moisture, a subsample of fresh soil was weighed, heated at 105oC for 24 hours and reweighed. Soils were dried, passed through a 2mm sieve, and sent to NRM Laboratories (Cawood Scientific Limited, Bracknell, UK) for full soil chemical analysis. The analysis included pH measured in water [1:2.5], and available phosphorus determined through sodium bicarbonate extractable Olsens and calculated colour-metrically. Ammonium nitrate extractable method was used to determine soil available potassium and magnesium, and determined by ICP-OES. Loss on ignition at 430oC was used for OM, and total nitrogen and total carbon were determined through the Dumas method.

Soil sampling

Five 10cm deep soil cores were taken in a W pattern per plot and mixed to create a composite sample; this was repeated three times for the three different analyses required. The soil corer was sprayed with a 1% virucidal disinfectant Virkon (Lanxess, Cologne, Germany) and rinsed with deionised water between plots for aseptic sampling. Soil samples destined for DNA analysis were taken in September 2020, four years after plot establishment, and frozen at -20oC before further analysis. Samples for the MicroRespTM experiment to measure soil microbial functional diversity and chemical analysis were taken in July 2021. Samples destined for the MicroRespTM were 2mm sieved for homogenisation and stored at 4oC for 72 hours. Subsamples were taken for water holding capacity (WHC) and soil moisture measurements. Briefly, a subsample of fresh soil was saturated in water overnight and allowed to drain for 8 hours the next day. A weighted subsample of soil was placed in an oven set to 105oC until no further water loss occurred (24 hours). Dried soil was reweighed to work out WHC. For soil moisture, a subsample of fresh soil was weighed, heated at 105oC for 24 hours and reweighed. Samples destined for chemical analysis had roots and stones removed, and soil was dried and ground before being sent to NRM Laboratories (Cawood Scientific Limited, Bracknell, UK).

Earthworms

One random 20x20x20cm soil pit per plot was excavated in March 2020 at the dry and well-watered site three and a half years after sward establishment.Excavated soil was placed onto a 90cm x 40cm plastic sheet in the field, where the soil cube was gently teased apart for 15 minutes per pit to extract earthworms. Extracted earthworms were weighed, including half earthworms.

Roots

Soil cores (8cm diameter) were taken at the well-watered site at 4 depths (0-15cm, 15-30cm, 30-45cm, 45-60cm) in the autumn of 2019, three years after plot establishment. Soil cores were submerged in water and soaked for 24 hours to ease the separation of roots from the soil. Roots were washed clean of soil, suspended and spread in clean water in a clear plastic tray and scanned. Roots were dried at 50oC to constant weight for dry biomass measurements.

WINRHIZO (Regent Instruments Canada Inc) software was used to process scanned images. Calibration using the object of known dimensions method was used to obtain accurate readings for the root surface area data outcomes. Batch image analyses was used with settings detailed in Table ‎3.

Table ‎3 criteria settings for the WINRHIZO software (Regent Instruments Canada Inc) for root samples taken in the autumn of 2019 at the well-watered site

|  |  |
| --- | --- |
| **Criteria** | **Setting** |
| Greyscale | Yes |
| Root and background | Automatic |
| Rough edges | Off |
| Debris | 1.5 |
| L:W | 7.5 |
| Lagarde | 64 px |

Aboveground biomass

Three 50x50cm quadrat cuts per plot were taken three times during the growing season, each time leaving a residual height of 7cm. The material was dried at 60oC for 72 hours. Aboveground biomass results are shown as the total of all three cuts and converted into tonnes per dry matter weight per hectare (t DM/ha).

Soil chemical analysis

Soil chemical analyses were conducted by NRM Laboratories (Cawood Scientific Limited, Bracknell, UK). Chemical analysis on soil samples from July 2021 included total carbon and total nitrogen determined through combustion in pure oxygen at 1200oC. The resultant gas mixture led through a splitter with the carbon dioxide gas measured by Infra-Red (IR) detector. Nitrogen oxide gas was reduced to nitrogen by passing through a copper reduction oven, and nitrogen was determined by a thermal conductivity detector, the Dumas method (AOAC Official Method, 1997).

DNA extraction and amplification

DNeasy powersoil pro kit (QIAGEN LLC, Hilden, Germany) was used to extract DNA from the dry and well-watered soil samples at the University of Reading: 0.25g of soil was weighed, and the DNeasy powersoil pro kit extraction protocol followed, using the Tissuelyser II (QIAGEN, Hilden, Germany). DNeasy plant mini kit (QIAGEN LLC, Hilden, Germany) was used to extract DNA from the ryegrass trap-plant roots. 0.12g of root sample was ground using a pestle and mortar with liquid nitrogen. Samples were checked for DNA yields using 1.5µl of the sample on the NanoDrop 2000/2000c spectrophotometer (ThermoFisher Scientific, Waltham, Massachusetts, US). Extracted DNA samples were adjusted to 10ng/µl using elution buffer and stored at -18oC before amplification.

Fungal community analysis was performed at LGC Group (Teddington, UK) for ITS data generated from amplicon sequencing on 32 DNA samples using a two-step PCR approach with primer pair ITS1FKyo2 (TAGAGGAAGTAAAAGTCGTAA) and ITS86R (TTCAAAGATTCGATGATTCA) run on illumina Inc (San Diego, CA, US) MiSeq v3 pair-end sequencing of 2x300bp. The second amplification used i7- and i5- sequencing adaptors. First amplification settings were 1-minute 96oC pre-denaturation followed with 30 15s cycles at 96oC denaturation, 58oC 30s annealing, 68oC 90s extension, 70oC 2-minute final extension and 8oC final hold. The second amplification followed the first amplification process with annealing changed to 3 cycles at 50oC followed by 7 cycles at 58oC. The process involved demultiplexing, clipping, primer detection and forward and reverse reads using BBMerge. Amplicon pre-processing included chimera removal, resulting in high-quality reads clustered into operational taxonomic units (OTU) picked using Mothur at the 97% identity level. The total number of reads came to 638,574, an average of 19955.438 ± 22.404 sd per sample (Table ‎4). Taxonomic classification of the fungi was processed against the UNITE database, totalling 626,983 fungal sequences obtained from the 32 samples.

Table 4 OTU counts per sample for the two CRU sites per forage mixture (PRG: perennial ryegrass; SG: SmartGrass; Bio: Biomix; Her:Herbal)

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Dry site** | | | | **Well-watered site** | | | |
|  | **Sample number** | | | | | | | |
|  | **1** | **2** | **3** | **4** | **1** | **2** | **3** | **4** |
| **PRG** | 19947 | 19961 | 19965 | 19968 | 19941 | 19953 | 19954 | 19956 |
| **SG** | 19953 | 19954 | 19970 | 19967 | 19894 | 19962 | 19968 | 19967 |
| **Bio** | 19931 | 19952 | 19958 | 19965 | 19954 | 19960 | 19964 | 19971 |
| **Her** | 19863 | 19961 | 19975 | 19966 | 19956 | 19976 | 19973 | 19969 |

MicroRespTM

Soil samples were wetted to the same moisture content (40% WHC as per Campbell *et al.*, 2003). Each soil sample was used to fill 24 deepwells per plate, with the plate weighed after each filling. Fully filled and parafilmed deepwell plates were placed into a container with soda lime and wet paper towel for 3 days at 25oC to allow the soil to settle after the initial disturbance.

Indicator solution was made with 18.75mg cresol red, 16.77g KCl and 0.315g NaHCO3 dissolved by heating in 900ml water. Agar gel was made with 3g agar dissolved in 100ml water. Indicator gel solution was created in a 1:2 agar gel to indicator solution ratio. 150µl of the indicator gel was pipetted into each well of a microplate. Microplates were covered with parafilm and placed in a desiccator with soda lime and a beaker of water in the dark until use.

Carbon substrates chosen were malic acid, citric acid, D-(+)-glucose, D-(+)-galactose, α-ketoglutaric acid, ϒ-amino butyric acid, L-arginine and a water blank (Campbell *et al.*, 2003; Creamer *et al.*, 2009, 2016; Andersen *et al.*, 2013; Moscatelli *et al.*, 2018). Substrates were prepared as 30mg g-1 of soil water calculated from the prior weighing of the deepwell plates during soil addition.

25µl of each substrate was pipetted into each of the soil containing deepwells (3 replicates per substrate). Initial measurement for time zero was taken with the microplate filled with indicator gel on the SpectraMax i3x (Molecular Devices Limited, San Jose, CA, US) with absorbance set to 570nm. The read microplate was clamped together and divided with a rubber seal with the substrate/soil filled deepwell plate for 6 hours at 25oC. The microplate was reread after the 6 hour incubation to measure final absorbance, again set to 570nm.

Multiple substrate-induced respiration (MSIR) was calculated by subtracting the water blank basal rate from the substrate-induced respiration values recorded as µg C-CO2 g-1 hr-1 . Microbial functional diversity values were calculated from the Shannon-Weiner biodiversity (Hmic) derived from substrate use (Klimek *et al.*, 2016).