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Soil Biology and Soil Health Partnership Project 3: Molecular approaches for routine soil-borne disease and soil health assessment – establishing the scope

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1. Abstract

This review is part of a suite of integrated projects (Soil Biology and Soil Health Partnership) specifically aimed at addressing the AHDB and BBRO Soils Programme call – "Management for Soil Biology and Soil Health". This project is designated Project 3 within WP 1 (Benchmarking and Baselining; see Figure 1).

This review specifically aimed to:

- Summarise available knowledge on procedures to sample field soils to undertake DNA analysis of the presence and composition of microbial communities and their functions to provide indicators of soil health.
- Evaluate standard tools for use in routine sampling and molecular analysis of soil quality so that their value can be demonstrated to growers and agronomists during and beyond the current Soil Biology and Soil Health Research Partnership.
- Establish full lists of molecular markers that can be used to quantify:
  (a) Soil-borne pathogens for use in prediction of crop disease;
  (b) Indicators of good soil health which can influence crop yield and value.

Procedures for sampling soil and extracting DNA from the sample have been reviewed. There are no standardised sampling methods, but it is general practice to take composite samples by mixing multiple cores from the surface to 10-30 cm depth. Corers should be cleaned and flamed between collection of each separate set of composite samples. A sample size of at least 200-500g is recommended. The number and spatial arrangement of samples and sub-samples required depends on the expected distribution of the target. For unknown target distributions, it has been suggested that the area is divided into evenly-sized grids with at least 2 composite samples per grid. An internationally recognised standard (ISO 11063:2012) describes a procedure for direct isolation of DNA from soil, suitable for further analysis using qPCR and high throughput sequencing methods, but this does not include procedures for subsequent DNA purification and is only suitable for small soil samples. Procedures more suitable for direct extraction and purification of total DNA from composite soil samples of 200-500g are described in the Appendices to this report.

Various molecular techniques have been used for analysis of soil quality, including methods based on polymerase chain reaction (PCR), microarrays, DNA fingerprinting (DGGE and T-RFLP) and DNA sequencing. Two approaches are considered most suitable for routine analysis of taxonomic or functional markers; quantitative PCR (qPCR) for detection and quantification of specific markers and next generation high throughput sequencing for analysis of whole soil communities. The range of molecular markers that have been used to investigate the taxonomy and function of individual target organisms and communities of organisms in soil are described in full. These include taxon-specific markers, mainly based on selected DNA sequences from within ribosomal (rDNA) or mitochondrial (cytochrome oxidase) DNA loci. Functional markers in genes expressing key enzymes involved in

carbon, nitrogen, phosphorus and sulphur cycling are also described. Markers are also listed that have been used to assess soils for presence and activity of other key bioindicators of soil health, including mycorrhizal fungi, nitrogen-fixing microorganisms, plant growth promoting bacteria, biocontrol agents, nematode assemblages and plant pathogens.

Some technical challenges remain to be fully overcome in the application of these technologies to ensure a representative and unbiased analysis of soil microbiological communities and their function. These include further standardisation of procedures for sampling, extracting and purifying DNA from soils, improved consistency in the choice of markers to be used in the analyses and the use of appropriate internal controls that ensure accuracy of data interpretation. The high cost of molecular analysis also remains a constraint to its routine application.

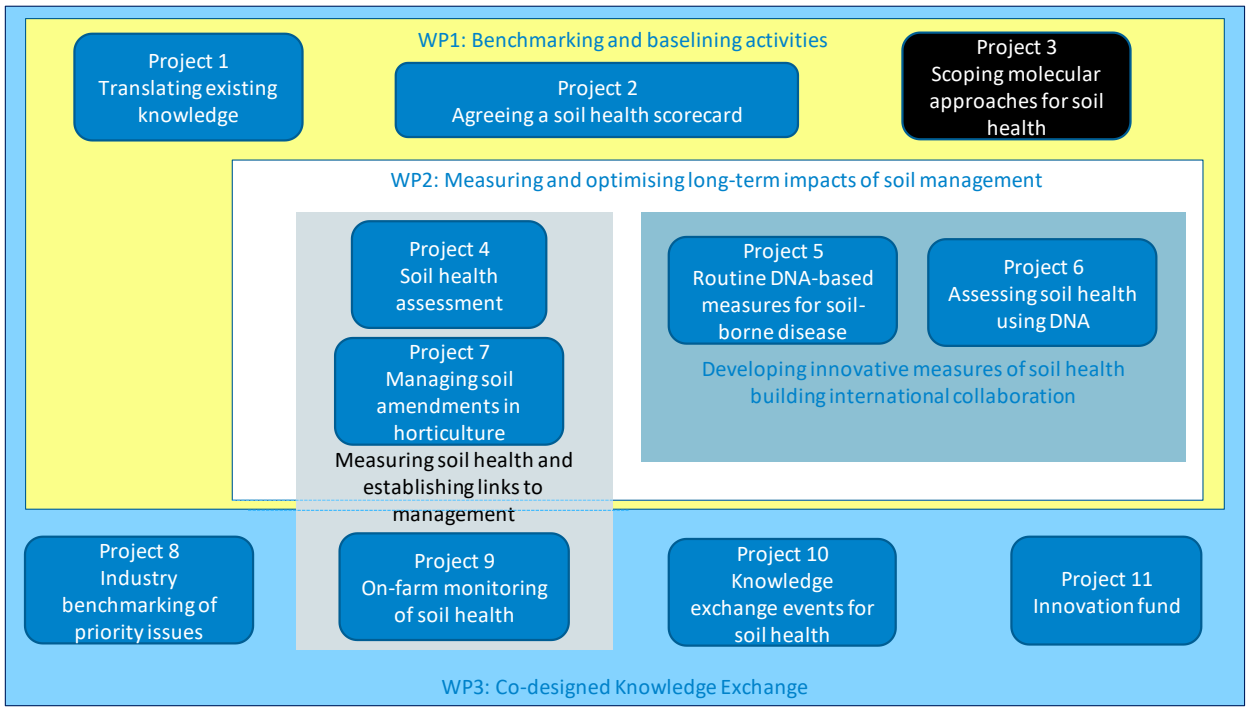

Figure 1. Diagram to show how Project 3 (in black) fits into the organisation of the Soil Biology and Soil Health Partnership.

2. Review of molecular approaches to biological soil health assessment

2.1. Introduction

In the last decade several useful biological indicators for soil health assessment have been proposed (Ritz *et al.*, 2009). There have also been advances in knowledge of the functional roles of below-ground biodiversity (Bardgett & van der Putten, 2014) and major advances in technology and a reduction in the cost of using molecular tools (Orgiazzi *et al*., 2015). The following review of a rapidly developing literature aims to describe those molecular procedures that currently offer the most potential for detection and quantification of individual target organisms or functions, or that allow analysis of the diversity of whole communities of organisms or their functional groups. The current range of DNA markers available for analysis of the biological component of soil health is also reviewed. Whilst most approaches have so far been used in a research context, their suitability for application in affordable routine soil health analyses has yet to be established.

Appropriate procedures for sampling soil and extracting DNA from the sample are also reviewed. Sampling methods capable of recognising in-field variation and methods to extract high quality DNA from statistically representative soil samples are required. Extraction methods need to be efficient across all soil types and farming practices. They also need to be geared towards the type of molecular analysis to be performed, since some methods require higher levels of purification or longer DNA fragments than others. Methods also need to be suitable for direct extraction and analysis of total soil DNA, of DNA indirectly extracted from isolated soil organisms or for extraction of e-DNA (DNA remaining in the environment after release by organisms into the soil).

2.2. Sampling

To ensure that soil samples are representative of the area being sampled, and to account for heterogeneous distribution of soil micro- and macro-fauna, the sample is usually prepared as a composite of several small cores (20-50g each). Cores are typically 100 mm long and 10 mm diameter. The standard practice is to collect sub-samples randomly across the area to be sampled and to include material from different depths, usually topsoil only and down to 10-30 cm, depending on the expected range of distribution of the target organisms. The size of the study area should also represent the expected distribution of targets. To monitor variability in nematode distribution introduced by the sampling strategy, Taberlet *et al.* (2012) recommend sampling across a regular grid pattern across the study area with at least 2 randomly collected composite samples collected per grid. Corers should be suitably cleaned to remove soil remains followed by alcohol flaming between the set of soil cores that will together form one composite sample.

The size of each composite soil sample has been optimised for molecular test methods (Wiesel *et al*., 2015). Soil samples for nematode extraction have traditionally been standardised at 200g (Flegg and Hooper, 1970). Although a wide range of sample sizes between 1-200g have been employed in molecular studies (e.g. Waite *et al*., 2003; Porazinska *et al*., 2010; Griffiths *et al*., 2012; Morise *et al*., 2012), it was recently concluded that a sample of at least 200g is required for accurate measure of nematode abundance and at least 100g is needed to truly represent community composition (Wiesel *et al*., 2015). Similarly, for molecular study of soil-borne fungal and bacterial populations, Ophel-Keller *et al*. (2008) collected 3 samples per field (40-300 ha) with 15 cores per sample on a 'W' transect. They suggested that DNA should be extracted from samples of at least 200-500g for 'biologically relevant analysis'. For molecular analysis of earthworm DNA in soil (Bienert *et al*., 2012), 2 layers (0-20cm and 20-40cm) have been sampled by randomly collecting subsamples to make a 500g sample per area of 10m radius.

2.3. DNA extraction and purification

Numerous procedures are described for direct extraction of total soil DNA or eDNA or indirect extraction of DNA from isolated soil organisms. The yield of extracted DNA varies with the chemical and physical properties of different soils (Feinstein *et al*., 2009), including the contents of negatively charged clay, silicates and organic matter which bind to DNA. The yield also varies according to the direct extraction process used and the relative efficiency of cell lysis of the different target organisms (Petric *et al*., 2011). The quality of extracted DNA can also vary with the method used; more intensive treatments may improve microbial cell breakdown resulting in higher DNA yields but may cause shearing of the DNA making it unsuitable for some types of molecular analyses. Different DNA extraction methods can therefore bias the results of molecular analyses in different ways. The choice of DNA extraction method therefore depends on the soil type, the target organism(s), the type and quality of DNA to be studied and the method being used for its analysis. The preferred extraction method is always a compromise between the required yield and quality of the DNA.

Significant efforts to optimize and standardize direct DNA extraction procedures have aimed to improve the reliability of quantitative and qualitative characterization of soil communities, especially bacteria, archaea, fungi and protists (Martin-Laurent *et al*., 2001; Petric *et al*., 2011; Plassart *et al*., 2012; Terrat *et al*., 2015; Santos *et al*., 2015; Dimitrov *et al*., 2017). This includes validation and improvement of a standard method for direct soil DNA extraction (ISO 11063:2012) adopted by the International Organization for Standardization (Phillipot *et al*., 2010). The method involves three main stages: (1) target cell lysis using the surfactant sodium dodecyl sulphate (SDS) followed by physical disruption by bead beating, (2) protein precipitation by sodium acetate; and (3) nucleic acid precipitation in isopropanol followed by washing in 70% ethanol. This method and its modifications (Plassart *et al*., 2012; Santos *et al*., 2015) can be

used for reproducible extraction of DNA from different soil types, which is suitable for soil community analysis using qPCR (Petric *et al*., 2011) and metabarcoding (Terrat *et al*., 2015).

Another source of bias in soil community analyses following direct DNA extraction is the effect of co-extraction of substances which inhibit DNA amplification, such as humic acids. ISO 11063 does not cover purification of the extracted DNA. There are several commercial kits available for extraction and purification of DNA from soils and their reliability has been compared (Fredericks *et al*., 2005; Dineen *et al*., 2010; Inceoğlu *et al*., 2010). Extraction kits, which have been most widely used in recent research studies, include the PowerSoil DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) and the FastDNA SPIN kit for Soil (MP Biomedicals, LLC, Solon, OH, USA). Additional DNA purification is usually also required for elimination of PCR inhibitors, usually by treatment with polyvinylpolypyrrolidone (PVPP) (Frostegard *et al*., 1999; Zhou *et al*., 1995) and/or hexadecyltrimethylammonium bromide (CTAB) (Cho *et al*., 1996; Malik *et al*., 1994; Zhou *et al*., 1995). Braid *et al*., (2003) also showed that addition of $AlNH_4(SO_4)_2$ during extraction significantly reduced the co-purification of PCR inhibitors with minimal loss of DNA yield.

Whilst commercial extraction and purification kits are useful for small-scale research, they are usually only suitable for small samples of soil (<10g) and their use in routine soil analyses is further limited by their slow speed of sample throughput and relatively high cost. Ophel-Keller *et al*. (2008) developed a method capable of extracting DNA from soil samples of 500g with a throughput of 160 samples per day and at a cost of less than 20% of the cost using commercial kits. However, the full details of this method remain unpublished. In the meantime, additional methods suitable for larger scale direct extractions of bacterial and fungal DNA for qPCR analysis of soil-borne fungi and bacteria have been developed (Brierley *et al*., 2009; Woodhall *et al*., 2012). Methods suitable for extraction of extracellular eDNA from larger soil volumes have also been described (Taberlet *et al*., 2012). In this case, a gentler extraction protocol is used to ensure DNA quality is suitable for metabarcoding analysis, which requires higher quality DNA (more purified and fragments longer than 500 bp) than for qPCR analysis. In this case, DNA from 4 kg composite soil samples is extracted by thorough mixing in saturated 0.12M phosphate buffer (pH 8) followed by centrifugation to remove suspended material. DNA is then extracted from aliquots of the supernatant using a commercial kit. Suggested protocols for sampling and extraction of total DNA for qPCR analysis and eDNA for metabarcoding are presented in Appendices 1 and 2.

2.4. Targeted analyses using quantitative polymerase chain reaction (qPCR)

The development of PCR has facilitated major advances in the assessment of the presence of specific target organisms or their functional genes in complex environmental samples, including water, sediments, soils, composts and manures. Real-time quantitative PCR (qPCR) is a safer

technology than conventional PCR since it does not require the use of mutagenic intercalating dyes to stain the DNA. It is also more sensitive, more reproducible, quicker and more cost-effective and is therefore now the most used molecular technique to quantify target sequences. Numerous qPCR approaches have been developed for the detection and enumeration of different organisms in various systems and the subject has been extensively reviewed (Schena *et al*. 2004; Okubara *et al*. 2005; Mumford *et al*. 2006; Cooke *et al*. 2007; Vincelli and Tisserat 2008; O'Brien *et al*. 2009; von Felten *et al*., 2010; Bilodeau 2011; Schena *et al*., 2013; Sanzani *et al*., 2014).

The main advantages of qPCR are its sensitivity, ease of use and capacity to run large sample numbers in high-throughput automated testing for quantitative estimation of DNA targets. Its limitations include the variability of the distribution of soil organisms and microorganisms, the efficiency of extraction and purification of DNA from environmental samples and the amount and variation of PCR inhibitors that can be co-extracted from environmental samples. Furthermore, the heterogeneity of PCR templates amplified from different target organisms can create artefacts and biases when attempting to compare populations of different target organisms in the same sample or of the same organism in different samples (Goyer and Dandie, 2012). The accuracy of quantification is also influenced by the number of copies of the target gene in the genome of each organism. Furthermore, since qPCR assays usually target total DNA, there is not always a relationship between DNA quantification and the viability of the detected target. Care is therefore needed when interpreting results in terms of the potential risks or benefits associated with the detection of target organisms or functional genes, which may not be viable or actively expressed. This problem can be tackled by using reverse transcriptase qPCR to detect mRNA targets that are short lived outside of the viable cell, or by using intercalating dyes such as propidium monoazide (PMA) or ethidium monoazide (EMA) which penetrate dead cells and intercalate with the DNA, preventing amplification by PCR (Fittipaldi *et al*., 2011). The extent to which these methods can be used on DNA extracted from soil and the cost implications for routine analysis have yet to be determined.

A variety of markers have been used to develop specific qPCR assays, which are described in more detail below. Whilst these are often based on unique taxonomic or functional gene markers, sequenced characterised amplified regions (SCAR) have also frequently been used (Gobbin *et al*., 2007; Hermosa *et al*., 2001; Holmberg *et al*., 2009). In these cases, unique DNA bands are generated from genomic DNA of the target organism using DNA fingerprinting techniques such as random amplified polymorphic DNA-PCR (RAPD-PCR), repetitive extragenic palindromic PCR (rep-PCR) or restriction fragment length polymorphism (RFLP). Purification and sequencing of unique DNA fragments then allows soft-ware assisted selection of primers and probes for qPCR assay development and validation. Increasingly, it is now possible to generate specific markers from whole genome comparisons using bioinformatic pipelines which terminate

in the generation of unique primer/probe combinations which are suitable for use in qPCR assays (Pritchard *et al*., 2013).

2.5. Methods suitable for soil community analyses

Molecular approaches for the analysis of the functions and diversity of whole soil communities have evolved rapidly in the last 10 years and have been widely reviewed and discussed (e.g. Sørensen *et al*., 2009; Orgiazzi *et al*., 2015; Drummond *et al*., 2015; Creer *et al*., 2016). In addition to qPCR targeting taxon-specific barcode sequences (see above), various other molecular approaches have been used in soil community research, including the following:

2.5.1. Microarrays

The attachment of labelled DNA probes of known sequences to a solid slide or "DNA chip" enables probing with fragments obtained from soil communities. Detection of hybridization between the immobilised DNA probes and the soil DNA, then indicates the array of genes associated with the soil community. For example, development of the microarray GeoChip, with up to 82000 probes covering 141 995 coding sequences from 410 functional gene families, has allowed investigation of the ecological functions of soil microbial communities involved in nitrogen, carbon, sulphur, and phosphorus cycles as well as energy metabolism, antibiotic resistance, metal resistance/reduction, organic remediation, stress responses, bacteriophage, and virulence (He *et al*., 2010; Tu *et al*., 2014).

2.5.2. DNA fingerprinting methods

These methods combine PCR amplification of conserved marker genes across a soil community and differentiation between the resulting PCR amplicons according to variation in their DNA sequences. For example, when applied to 16S rRNA gene markers, the method allows the dissection of microbial communities at the level of the phylogeny of their constituents (Smalla *et al*., 2007;). Similar approaches have been used to estimate nematode diversity using the 18s rDNA gene (Waite *et al*., 2003; Foucher *et al*., 2004). Two fingerprinting approaches have been most widely used:

- ***Density gradient gel electrophoresis (DGGE)*** separates the double stranded DNA PCR amplicons during electrophoresis in a polyacrylamide gel with an increasing density gradient of DNA denaturing agents (usually urea and formamide). Fingerprint patterns are formed in the gel when amplicons varying in sequence are denatured at different gradients and their migration is slowed down or stops at different distances during their migration along the gel.

- ***Terminal restriction fragment length polymorphism (T-RFLP)*** assesses variation in the sequence of PCR amplified DNA by treating with restriction enzymes that cleave the

amplicons into several fragments at specific points in the sequence. The use of fluorescently labelled PCR primers enables the terminal fragments to be separated according to their size in a capillary sequencer.

Chen *et al*. (2010) compared these methods to other molecular methods, including qPCR and DNA barcoding, for monitoring soil nematodes as biological indicators. They concluded that T-RFLP was well suited for routine monitoring purposes based on the advantages of high-throughput, ease of comparison between samples and rapid data analysis. T-RFLP has also been used to survey the bacterial biogeography of British soils (Griffiths *et al*., 2011) However, it has been shown that such fingerprinting techniques sometimes only consider the most abundant phylotypes and so diversity estimates can be poorly correlated with true community diversity (Lalande *et al*., 2013).

2.5.3. Next generation high throughput sequencing

While PCR-based methods have been widely used to study community interactions at high taxonomic levels, the specificity of available primers and the labour and expense involved in sequencing or otherwise differentiating PCR amplicons has limited both sample throughput and the resolution to which individuals can be identified within the soil community. With the development of the first true high throughput sequencing (HTS) 454 Life Sciences platform (Margulies *et al*. 2005), it became possible to pyrosequence millions of individual amplified molecules in parallel. Subsequent developments in sequencing technology have further increased the depth of sequencing and opportunities for high sample throughput (Loman *et al*. 2012). Three new next generation sequencing (NGS) platforms were released in 2011 alone: Ion Torrent's PGM, Pacific Bioscience's RS and the Illumina MiSeq (Quail *et al*., 2012). Alongside these advances in technology are developments of bio-informatics tools that manage large data flows, compare data with specialised databases and extract relevant information, creating new perspectives for investigating the soil microbiome (Uroz *et al*., 2013). Illumina sequencing-bysynthesis has particularly enabled greater sequencing depth and higher sample throughput alongside reduced costs. More recently, single molecule sequencing technologies, such as Pacific Biosystems and Oxford Nanopore, have allowed the generation of much longer reads from samples where DNA is only present at low concentrations. However, higher costs, reduced throughput and increased error rates of the latest platforms mean that Illumina currently remains the platform of choice for community ecology research (Schmidt *et al*., 2013; Creer *et al*., 2016).

High throughput sequencing technology is revolutionising the way in which the functions and diversities of soil communities are investigated through analysis of directly or indirectly extracted total DNA or of the eDNA. Three main approaches are currently being followed:

- ***Metabarcoding*** involves PCR amplification of pre-selected barcode sequences in phylogenetically relevant marker genes followed by simultaneous high throughput sequencing of all amplicons generated and bioinformatic sequence comparisons using databases of verified specimen sequences. Several sequence databases have been employed, including RDP (Cole *et al*., 2014), Greengenes (DeSantis *et al*., 2006), SILVA (Pruesse *et al*., 2007), UNITE (Abarenkov *et al*., 2010), BOLD (Ratnasingham & Hebert, 2007), & Genbank (Benson *et al*., 2012). First used to describe meiofauna community structures by analysing eDNA in sediments (Creer *et al.*, 2010), metabarcoding methods were also developed to analyse soil for DNA from fungi, bryophytes, enchytraeids, beetles and even birds (Epp *et al*., 2012). High-throughput metabarcoding studies on fungi and other eukaryotic microorganisms are rapidly becoming more frequent and more complex, and several new bioinformatic pipelines have been described for metabarcoding bacteria (Gellie *et al*., 2017), fungi (Bálint *et al*., 2014) and fauna (Yang *et al*., 2013; de Groot *et al*., 2016) including protists (Geisen *et al*., 2015), nematodes (Posazinska *et al*., 2010 and 2010b; Sapkota and Nicolaisen, 2015) and earthworms (Bienert *et al*., 2012).

- ***Metagenomics*** entails random (shotgun) sequencing of long strands of soil DNA to elucidate the taxonomic structure and potential functional genomic capability of a community. In contrast to metabarcoding, metagenomics does not require an initial PCR step, thus avoiding potential biases associated with the use of different primer sets with varying amplification efficiencies (Logares *et al*. 2013). Shotgun sequencing provides an amplification independent method for assessing community diversity, additionally allowing for the capture of information from groups that are otherwise difficult to survey (Narasingarao *et al*. 2012). For example, Delmont *et al*. (2012) described the metagenome of a Rothamsted grassland soil using 454 pyrosequencing and showed that only 1% of the annotated sequences found corresponded to known sequenced genomes. Unexpectedly low seasonal and vertical soil metagenomic functional class variations were also observed. Metagenomic analysis of soils across global ecosystems is indicating major differences in soil microbiomes in terms of both taxonomic and functional representation (Noronha *et al*., 2017). Differences in functional and/or taxonomic diversity are also shown to vary with agricultural land use (Manoharan *et al*., 2017), including tillage and crop management practices (Souza *et al*., 2015), pH and fertilizer adjustment (Fierer *et al*., 2012; Zhalnina *et al*., 2015) and the use of organic amendments (Jenkins *et al*., 2017). Significant investment is being targeted towards sequence-based approaches to understand the soil microbiome. For example, The Earth Microbiome Project (www.earthmicrobiome.org) alone is characterizing 200 000 samples from researchers all over the world. Results of metagenomic analysis are known to vary with the DNA extraction method, database choice and the annotation procedure (Jacquiod *et*

al., 2016). In support of this, improved curated genomic databases, such as RefSoil (Choi *et al*., 2016) and FuSiON (Zhu *et al*., 2015), are being developed to provide a soil-specific framework with which to annotate and understand soil sequencing projects.

- ***Metatranscriptomics*** allows study of the actual functional activity of the entire soil microbiome through shotgun sequencing of the messenger RNA (mRNA) (Carvalhais and Schenk, 2013; Myrold *et al*., 2014; Thies, 2015). Presence of mRNA reflects the portion of the soil microbial community that is active at the time of sampling (Blagodatskaya and Kuzyakov, 2013) and thus provides a robust means to examine biological responses to soil management. The process is challenging as mRNA typically represents less than 5% of the RNA extracted (Carvalhais *et al*., 2013) and is often extremely short-lived. The mRNA in the extract must be enriched and the rRNA removed. Subtractive hybridization, treatment with endonucleases that preferentially degrade rRNA or duplex specific nuclease treatment are used to remove rRNA (Yi *et al*., 2011). Eukaryotic mRNA can be separated by binding the 3'-poly-A tails of the transcripts to surfaces coated with poly(dt)probes, thus enriching mRNA from bacteria and archaea in the extract. The mRNA is then reverse-transcribed (RT) into cDNA for high-throughput sequencing.

2.6. Molecular markers for bio-indicators of soil health

The size and diversity of the biological communities sustained within different soils can be determined according to the prevalence of molecular markers within their DNA, which have been selected to identify different taxonomic groups, functional groups or individuals within the community. Ritz *et al*. (2009) listed several bio-indicators that can be targeted in this way when assessing the overall health status of soils in relation to crop productivity and long-term sustainable land management:

2.6.1. Biological communities contributing to soil biodiversity

For taxonomic barcode markers within bacteria and archaea, highly conserved target sequences within the 16S ribosomal RNA gene have been most widely used (Fierer *et al*., 2005; Sogin *et al*., 2006), although hypervariable regions within the gene and the 16-23S intergenic spacer have also been used for more taxa-specific analyses (e.g. Becker *et al*., 2000; Suzuki *et al*., 2000; Takai & Horikoshi, 2000 and Blackwood *et al*., 2005). Various housekeeping genes have also proven useful as taxonomic barcodes since they are essential and are therefore not lost from genome, but evolve more quickly than 16S rDNA. The most commonly used taxonomic markers used to identify soil-borne fungi and oomycetes also often include rDNA markers, including the intergenic transcribed regions ITS1 and ITS2, located between the small sub unit (SSU) 18S and the large sub unit (LSU) 28S genes and separated by the 5.8S gene (Schoch *et al*., 2012; Schena *et al*., 2013). For other taxonomic

groups, a diverse set of loci from the analogous eukaryotic rRNA gene array (e.g. ITS, 18S or 28S rRNA) (Bik *et al*., 2012a; McGuire *et al*., 2010; Epp *et al*. 2012) have been employed. Barcoding of nematodes has mainly relied on rDNA targets, including ITS1, ITS2, 18S, D2-D3 expansion segments of the 28S rRNA gene, 5S and intergenic spacer (IGS) targets, although other targets also include the mitochondrial cytochrome oxidase gene (CO1) (Blok, 2010). Some 51 taxon-specific markers based mainly on the rDNA locus (Floyd *et al*., 2002; Griffiths *et al*., 2006; Holterman *et al*., 2006; Rybarczyk-Mydłowska *et al*., 2012; Vervoort *et al*., 2012) have been used for characterisation of nematode assemblages (Quist *et al*. 2016 and 2017). Similarly, for protists, conserved sequences within the 18S, ITS and CO1 genes are often used (Pawlowski *et al*. 2012). The 18S and CO1 genes are also widely used for barcoding meiofauna and macrofauna (Hebert *et al*., 2003; Deagle *et al*., 2014) together with additional sequence information from the 12S and 16S genes for macrofauna (Epp *et al*., 2011). Capra *et al*. (2016) also recently described a new set of markers based on 18S rRNA for metabarcoding of soil metazoa. In general, markers based on rDNA often provide useful sequence barcodes with good resolution at genus or higher, although resolution to species level is not always possible. Furthermore, quantification based on total gene copies detected can be variable due to the different numbers of rRNA copies associated with different species (Klappenbach *et al*., 2000).

2.6.2. Microbial activity affecting soil nutrient availability/retention

Organisms involved with biogeochemical processes such as carbon, nitrogen, phosphate and sulphur cycling are important indicators of soil health. Genes controlling these processes have been used as markers to quantify functional groups of these organisms in soils. Recent examples describing the use of qPCR to monitor presence and quantify various functional microbial groups according to their expected activities in soils and sediments are shown in Table 1.

For carbon cycling organisms, these include genes expressing enzymes involved in oxidation of organic matter to CO_2 (soil respiration), which include ß-glucosidases, cellulases and phenol oxidases, as well as the consumption of methane by methanotropic bacteria (methane oxidases). Functional groups of bacteria can also be quantified using marker genes for enzymes involved in nitrogen cycling, including:

- ***Nitrogenases*** in nitrogen-fixing *Rhizobium* species and cyanobacteria (blue green algae), which assimilate atmospheric nitrogen into ammonia.
- ***Ureases and amydases*** in nitrogen mineralising bacteria, which convert organic nitrogen to ammonium ions.
- ***Ammonium oxidases*** in nitrifying bacteria, which produce nitrite and nitrate from ammonium.

- ***Nitrate reductases***, nitrite reductases and nitrous oxide reductases in denitrifying bacteria, which release gaseous nitrous oxide and nitrogen from nitrates and nitrites.

Similarly, functional groups of bacteria and fungi contributing to phosphate and sulphur cycles have been quantified according to marker genes expressing the key enzymes involved. Acid and alkaline phosphatase enzymes produced by bacteria, fungi and plant roots serve to transform complex and sometime unavailable forms of organic P into assimilable phosphate. Population dynamics of sulphate-reducing bacteria have similarly been quantified by quantification of their functional genes responsible for dissimilatory reduction of sulphates to adenosine 5'-phosphosulfate (APS), sulphites and eventually to gaseous hydrogen sulphide under anaerobic conditions.

Table 1: Marker genes used to monitor microbial activity affecting soil nutrient cycling

Biological function	**Targeted genes**	**References**
C-cycling	Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) (*cbbL*) Methane mono-oxygenase (*pmoA*) Methyl coenzyme M reductase (*mcrA*) Cellulase (*cel*) Chitinase (*chiA*) Alkane mono-oxygenase (*alkb*) PAH ring hydroxylating dioxygenase (PAH-RHD)	Powell *et al.*, 2006 Selesi *et al.*, 2007 Yergaeu *et al.*, 2009 Freitag *et al.*, 2010 Pereyra *et al.*, 2010 Yergeau *et al.*, 2012
N-cycling	Ammonia mono-oxygenase (*amoA*) Nitrogenase reductase (*nifH*), Nitrous oxide reductase (*nosZ*), Nitrite reductase (*nirS*, and *nirK*), Nitrate reductase (*narG* and *napA*), Nitric oxide reductase (*norB*)	Hai *et al.*, 2009 Hayden *et al.*, 2010 Bru *et al.*, 2011 Dose *et al.*, 2015, Dandie *et al.*, 2011
S-cycling	Dissimilatory sulfite reductase (*dsrA*) Adenosine 5′-phosphosulfate reductase (*aprA, ApsA*)	Ben-Dov *et al.*, 2007 Blazejak & Schippers, 2011
P-cycling	acid phosphatase (*phoC*) alkaline (*phoD*) phosphatase	Fraser *et al.*, 2017

2.6.3. Microbial symbionts contributing to crop nutrition

Arbuscular mycorrhizal fungi

Primers and probes to quantify abundance of different arbuscular mycorrhizal fungal (AMF) taxa in roots and in soil have been selected from taxon-specific markers in the nuclear large ribosomal subunit RNA genes (nrDNA) and the mitochondrial ribosomal mtDNA (König *et al*., 2010; Thonar *et al*., 2012; Voříšková *et al*., 2017). Interpretation of the qPCR results has been complicated by the multinuclear and multigenomic cellular organization of these fungi and the high DNA sequence diversity within the smallest biologically relevant units (i.e. single-spore isolates). Markers based on nrDNA, rather than mtDNA, are thought to be more suitable for the quantification of multiple AMF taxa as copy numbers of the former are better related to fungal biomass across taxa.

Nitrogen-fixing bacteria

Molecular markers used to quantify soil populations of nitrogen-fixing bacteria, such as *Rhizobium*, *Bradyrhizobium* and *Sinorhizobium* spp., include *rpoE1*, *nodC*, *nodD* and *nodZ* genes (Trabelsi *et al*., 2009; Boonen *et al*., 2010; Furseth *et al*., 2010; Macdonald *et al*., 2011). Nitrogen-fixing bacteria belonging to the genus *Frankia* have also been studied using markers within target genes *nifH* (Samant et al., 2012) and 23S rRNA (Samant *et al*., 2014; Ben Tekaya *et al*., 2017). Strain specific and general primer/probe sets are available for molecular qPCR analysis targeting these genes, offering a rapid and comparable alternative to the laborious procedure of most-probable number bioassays based on counting the number of nodules on the roots of test plants. Nodulation potential can be overestimated by the molecular methods due to presence of dead cells and DNA, which can remain in soil for up to 5 months after inoculation.

Plant growth promoting rhizobacteria (PGPR)

Several molecular markers have been used to detect and quantify bacteria considered to have some activity, which is plant growth promoting in the rhizosphere of different crops (Table 2). In most cases, strain-specific assays have been used to determine the fate of PGPR strains added to soils during assessment of their suitability for commercialisation. Functional genes of fluorescent *Pseudomonas* spp. have been used as markers for grouping strains with antibiotic or hydrogen cyanide activities which are dispersed across several different species (Kim *et al*., 2013).

Table 2: Marker genes used to monitor plant growth promoting bacteria (PGPR) in soils

PGPR	Marker	Reference
Azospirillum lipoferum	CRT1 (RFLP product SCAR marker)	Couillerot *et al.*, 2010
Azospirillum brasilense	*nifA* (nitrogen fixation)	Faleiro *et al.*, 2013
Bacillus		
Herbaspirillum seropedicae	HERBASI1 (prophage sequence)	Pereira *et al.*, 2014
Enterobacter radicincitans	16S rRNA	Schreiner *et al.*, 2009
Paenibacillus polymyxa	16S rRNA	Timmusk *et al.*, 2009
Fluorescent *Pseudomonas spp.*	Multiple strain specific makers (RAPD product SCAR markers) *phlD* (2,4-diacetylphloroglucinol) *phzCD* (phenazine-1-carboxylic acid) *hcnBC* (hydrogen cyanide)	Von Felten *et al.*, 2010 Kim *et al.*, 2013
Pseudomonas brassicacearum	OPA2-73 (RAPD product SCAR marker)	Holmberg *et al.*, 2009
Pseudomonas sp. (DSMZ 13134)	*dnaX* (housekeeping gene)	Mosiman *et al.*, 2016

2.6.4. Biocontrol agents

Molecular markers have been used for detection and quantification of commonly used fungal biocontrol agents to assess their establishment, distribution and longevity in different soils. General ITS markers have often been used for fungi such as the mycoparasitic *Trichoderma harzianum* (Lopez-Mondéjar *et al*., 2010). RNA detection using reverse transcriptase amplification of the same markers has enabled estimation of viable populations (Beaulieu *et al*., 2011). Strain specific markers within the housekeeping gene *aox1* allow differentiation of commercial biocontrol agent strains of *T. harzianum* from other soil inhabiting strains (Horn *et al*., 2016). Other markers have been used for analysis of *T. atroviride* (Cordier *et al*, 2007; Savazzini *et al*., 2008). Vallance *et al*. (2009) used ITS markers to study the biocontrol agent *Pythium oligandrum* in the rhizosphere. ITS markers have also been used to study the nematophagous fungus *Plectosphaerella cucumerina* used for control of potato cyst nematodes (Atkins *et al*., 2003) and the entomopathogenic fungi *Entomophaga maimaiga* (Castrillo *et al*., 2007) and *Beauveria bassiana* (Bell *et al*., 2009; Garrido-Jurado *et al*., 2016). SSR (single sequence repeat) microsatellite markers have also been used to distinguish between biocontrol strains of *Beauveria bassiana* and *B. brongniartii* (Canfora *et al*., 2016). SCAR (sequence-characterised amplified region) markers, based on unique RAPD amplified sequences, have also been used to recognise the biocontrol agents *B. bassiana* (Castrillo *et al*., 2008) and *Chaetomium globosum* (Aggarwal *et al*., 2014).

Detection and identification of soilborne bacterial biocontrol agents, such as those belonging to the *Bacillus subtilis* group (including *Bacillus subtilis* and *B. amyloliquefaciens*), have also been based on SCAR markers, identified from unique PCR amplicons generated after RAPD or rep-PCR analysis. Strain specific differentiation has been based on sequence variation within housekeeping genes, including a tryptophan biosynthesis gene *trpE* (G) (Johansson *et al*., 2014) and RBAM 007760 (Gotor-Vila *et al*., 2016), a gene involved in surface adhesion and biofilm formation. This has allowed identification of strains, which better colonize the rhizosphere. Strains of *Pseudomonas fluorescens* that produce the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) have been monitored in cereal rhizospheres using *phlD* gene sequences as markers (Mavrodi *et al*., 2007).

2.6.5. Nematode assemblages

For community analysis of nematodes extracted from soils, markers within the small sub unit (SSU) of the ribosomal DNA (rDNA) have been most widely exploited to identify nematode feeding guilds (Griffiths *et al*., 2006). Donn *et al*., (2012) developed a directed terminal restriction fragment length polymorphism (dT-RFLP) method whereby PCR primers are used to amplify the SSU rDNA, followed by restriction of the amplicons with selected enzymes and comparison of the resulting fragment sizes with those in sequence databases produced from fully characterised species. A series of rDNA barcodes have been selected that allow identification to family and genus levels (Floyd *et al*., 2002; Holterman *et al*., 2006; Vervoort *et al*., 2012), which can then be allocated to feeding guilds. These have been used experimentally to show effects of long term soil management practices on the frequency of occurrence of each taxon following qPCR analyses with up to 51 primer sets (Quist *et al*., 2016 and 2017). Wang (2012) proposed a simpler system based on qPCR of 18S rDNA targets, but which only identifies certain guilds of bacterial-feeding, fungal-feeding, omnivorous and predatory nematodes but not herbivorous nematodes. A range of bespoke markers have also been designed for specific detection of different plant pathogenic nematode species (e.g. Madani *et al*., 2005; Holeva *et al*., 2006; Nakhla *et al*., 2010).

2.6.6. Soil-borne plant pathogens

Bilodeau (2011) reviewed the use of qPCR methods for detection of soil-borne plant pathogenic micro-organisms and nematodes. An updated list of available qPCR assays suitable for detection of soil-borne pathogens is shown in Table 3.

Table 3: Markers used for detection and quantification of soil-borne pathogens

Pathogen	Marker	**Reference**
Aphanomyces cochlioides	rDNA (ITS)	Almquist *et al.*, 2016
Colletotrichum coccodes	rDNA (ITS)	Cullen *et al.*, 2002
Fusarium graminearum *Fusarium culmorum*	PKS13 Zearalenone biosynthesis	Atoui *et al.*, 2012
Fusarium avenaceum *Fusarium culmorum* *Fusarium graminearum* *Fusarium poae*		Waalwijk *et al.*, 2004
Fusarium culmorum *Fusarium avenaceum* *Fusarium coeruleum* *Fusarium sulphureum*	rDNA (ITS)	Cullen *et al.*, 2005
Fusarium oxysporum	rDNA (ITS1-5.8S-ITS2)	Jiménez-Fernández *et al.*, 2010
Fusarium oxysporum f. sp. *melonis*	translation elongation factor (*TEF-1α)*	Haegi *et al.*, 2013
Fusarium culmorum *Fusarium graminearum* *Fusarium pseudograminearum*	Trichodiene synthase gene (*tri5*)	Hogg *et al.*, 2010
Fusarium solani f. sp. *phaseoli*	rDNA (SSU)	Filion *et al.*, 2003
Globodera rostochiensis *G. pallida*	rDNA (ITS1) rDNA (ITS1)	Toyota *et al.,* 2008 Adams *et al.,* 2009
Gaeumannomyces graminis var. avenae *Gaeumannomyces graminis var. tritici*	rDNA (TS1)	Bithell *et al.*, 2012
Gaeumannomyces graminis var. *tritici*	translation elongation factor gene (*EF1-α*)	Keenan *et al.*, 2015
Helminthosporium solani	rDNA (ITS)	Cullen *et al.*, 2001
Heterodera avenae	mitochondrial cytochrome oxidase subunit 1 (COI)	Toumi *et al.*, 2015
Heterodera schachtii	rDNA (ITS)	Madani *et al.*, 2005
Phomopsis sclerotioides	rDNA (ITS)	Shishido *et al.*, 2013
Phytophthora kernoviae	rDNA (ITS)	Hughes *et al.*, 2011
Phytophthora rubi	*cox1* cytochrome oxidase sub unit 1	Woodhall & Peters, 2014
Plasmodiophora brassicae	rDNA (18S and ITS1) rDNA (18S) rDNA (ITS) rDNA (ITS) rDNA (ITS) rDNA (18S) rDNA (ITS1)	Sundelin *et al.*, 2010 Rennie *et al.*, 2011 Wallenhamer *et al.*, 2012 Li *et al.*, 2013 Kennedy *et al.*, 2013 Cao *et al.*, 2014 Deora *et al.*, 2015
Polymyxa betae *Polymyxa graminis*	rDNA (ITS)	Ward *et al.*, 2004

Table 3 (continued)

Pathogen	Marker	**Reference**
Polyscytalum pustulans	rDNA (ITS)	Lees *et al.*, 2009
Pratylenchus penetrans	β-1,4-endoglucanase gene	Mokrini *et al.*, 2013
Pythium violae	rDNA (ITS) rDNA (ITS and 5.8S)	Cullen *et al.*, 2007 Schroeder *et al.*, 2006
Rhizictonia cerealis	β-tubulin gene Unique SCAR sequence	Guo *et al.*, 2012 Woodhall *et al.*, 2017
Rhizoctonia solani AG1-1 AG2-1 AG2-2 AG3-PT AG4 AG5 AG8	rDNA (ITS), β-tubulin	Lees *et al.*, 2002 Budge *et al.*, 2009 Woodhall *et al.*, 2013
Rhizoctonia solani AG2-2 IIIB	rDNA (ITS1, 18S and 5.8S)	Abbas *et al.*, 2014
Sclerotinia sclerotiorum	mitochondrial small subunit rRNA intron and ORF1	Rogers *et al.*, 2009 Kim & Knudsen, 2008
Sclerotium cepivorum	rDNA (ITS)	Woodhall *et al.*, 2012
Spongospora subterranea	rDNA (ITS) rDNA (ITS2)	van de Graaf *et al.*, 2003 Qu *et al.*, 2011
Streptomyces spp.	*nec1* virulence gene *txtAB* thaxtomin toxin synthetase gene 16S rRNA	Qu *et al.*, 2011 Cullen & Lees, 2007 Schlater *et al.*, 2010
Synchytrium endobioticum	rDNA (ITS)	Van Gent-Pelzer *et al.*, 2010
Verticillium dahliae *Verticillium longisporum* *Verticillium tricorpus*	β-tubulin gene rDNA (ITS)	Debode *et al.*, 2011
Verticillium albo-atrum	rDNA (IGS)	Bilodeau *et al.*, 2012 Peters, 2012 Maurer *et al.,* 2013
Verticillium dahliae	β-tubulin gene rDNA (IGS)	Duressa *et al.*, 2012 Bilodeau *et al.*, 2012
Verticillium dahliae *Verticillium longisporum*	rDNA (18S) rDNA (ITS-5.8S)	Banno *et al.*, 2011

3. Conclusions

The analysis of biological community structure and function in soil is benefiting from the availability of an ever-expanding assortment of molecular tools. Although complex and expensive, and therefore mostly confined to use in research, some molecular procedures are rapidly evolving and may become available as affordable procedures for routine analysis of biological indicators of soil health. The use of qPCR to detect and quantify specific organisms from total soil DNA and eDNA is already starting to be used on a routine basis. For example, the Predicta® soil testing service offered by the South Australian Research and Development Institute (SARDI) is already offering quantitative analysis of the distribution of some soil-borne pathogens of broadacre and potato crops in relation to the risk of disease development (Ophel-Keller *et al*. ,2008). The first uses of high throughput next generation sequencing in national and international surveys are suggesting that there is a core microbiome in geographically distant and disparate soils (Orgiazzi *et al*., 2013), but that there are also major differences in taxonomic and functional representation (Noronha *et al*., 2017). Metagenomic analysis of soils is also showing that the microbiome is dynamic, varying with season (Jumpponen *et al*., 2010) and agricultural land use (Fierer *et al*., 2012; Souza *et al.,* 2015; Zhalnina *et al*., 2015; Jenkins *et al*., 2017; Manoharan *et al*., 2017).

A number of technical challenges remain to be fully overcome in the application of molecular analyses for reliable characterisation of the taxonomy and function of soil communities (Delmont *et al*., 2012). It is very difficult to suggest standardised practices to sample and extract unbiased and representative samples of DNA from organisms with very different cell membranes and accessible DNA and across the full variability of soil types. This problem is exacerbated by the uneven spatial distribution of microbial communities in soil. Furthermore, the yield and quality of extracted DNA varies with the chemical and physical properties of different soils (Feinstein *et al*., 2009) as well as the extraction method used (Petric *et al.,* 2011). Although attempts have been made to standardize methods for DNA extraction from soil (Phillipot *et al*., 2010), their validation has been restricted to certain analytical methods. Since different analytical methods have different DNA quality requirements, there remains a need for further validation and standardisation of methods suitable for use with each new analytical method of choice. In addition to sampling and extraction, biases in soil community analyses can be attributed to differences in the molecular markers targeted as well as in the methods used for their analysis and the databases used for their identification. Heterogeneity of PCR templates amplified from different target organisms can create artefacts and biases (Goyer and Dandie, 2012). The accuracy of quantification is also influenced by the number of copies of the target gene in the genome of each organism.

Whilst standardization of sampling, extraction and analytical methods is highly desirable, it is unlikely that a single standardised procedure will be suitable for all types of molecular soil analysis. It is much more likely that a series of standardised procedures will be needed that are optimised for several parameters, including the types of organism to be studied, the numbers of soil samples to

be tested, the amounts of soil in each sample and the type of analysis to be performed. Of key importance will be the introduction of suitable internal controls that ensure efficient extraction of high quality DNA and the accuracy of its detection, quantification and representation within the soil community. For routine comparisons of soil health, the costs of sampling, DNA extraction and molecular analysis are also likely to be highly influential. High-throughput PCR and sequencing methods contribute to a significant increase in testing efficiencies, allowing simultaneous investigation of multiple targets and whole communities from a single DNA extract. Developments in technology are also moving towards the performance of molecular analyses *in situ*, rather than having to transport samples for laboratory analysis. Nevertheless, the cost of molecular analysis remains a key constraint to its routine application in monitoring soil health.

The wide range of taxonomic and functional markers that are available for probing soil DNA, together with the high throughput methods that are available for automated analysis, mean that multiple analyses can now be easily and simultaneously performed on a single soil DNA extract. Since DNA extraction is the most expensive part of the analysis, it is cost effective to test each extract for multiple markers. The challenge over the current Research Partnership is to identify the most appropriate biological soil health indicators to include in this type of analysis. Knowledge exchange workshops are already in progress, together with growers and agronomists, to explore the most useful targets for routine testing. The challenge will be to link the results of such testing to yield and quality benefits across entire cropping systems. Future workshops will also include demonstrations of the benefits of testing and will involve agronomists in the sampling procedures and, eventually, in interpretation of the results of DNA analyses. Future research within the Partnership will concentrate on validating and standardising the most appropriate molecular methods and building data on the effects of long-term soil management practices on key soil health indicators, including both beneficial and pathogenic organisms.

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5. Appendix 1

Method for total DNA extraction from soil samples of up to 500g (after Woodhall *et al*., 2012)

Scope: This method is suitable for direct extraction and purification of total DNA from composite soil samples of up to 500g. The procedure below is described for samples of 250g.

Materials:

1. Minimix auto paint shaker (Merris Engineering Ltd., Ireland)
2. Kingfisher ML magnetic particle processor (Thermo Fisher Scientific).
3. Grinding buffer (120 mM sodium phosphate buffer pH 8.2, 2% centrimonium bromide, 1.5M sodium chloride)
4. Antifoam B (Sigma-Aldrich)
5. 5M potassium acetate
6. Isopropanol
7. Silicon dioxide (Sigma-Aldrich)
8. Wizard® Magnetic DNA Purification System for Food (Promega)

Procedure:

1. Sieve soil to remove stones.
2. Place 250 g soil into 1000 ml Nalgene wide mouth environmental bottles with 20 stainless steel ball bearings (25.4 mm), 500 ml grinding buffer and 15 ml Antifoam B.
3. Shake for 4 min. in a minimix auto paint shaker (Merris Engineering Ltd., Ireland).
4. Centrifuge a 50 ml sub-sample at 5,000g for 5 min.
5. Transfer 20 ml of the supernatant to a clean tube containing 2 ml of 5M potassium acetate and incubate on ice for 10 min.
6. Centrifuge at 12,000 g for 5 min.
7. Transfer the supernatant to a clean tube containing 15 ml isopropanol and 800 µl silicon dioxide suspension.
8. Shake on flat bed shaker at 100 rpm for 15 min.
9. Centrifuge at 12,000 g for 5 min.
10. Discard supernatant and add 2ml Buffer A (Promega Wizard® Food Kit) to the pelleted silica particles.
11. Shake tubes on their side for 10 min, at 65 °C and 100 rpm.
12. Centrifuge at 12,000 g for 5 min.
13. Extract DNA from 1000 µl of the supernatant by magnetic capture using the Wizard® Magnetic DNA Purification System for Food (Promega) in a Kingfisher ML magnetic particle processor (Thermo Fisher Scientific) according to the manufactuers' instructions.

6. Appendix 2

Method for extraction of extracellular eDNA from soil samples of up to 500 g (after Taberlet *et al*., 2012)

Scope: This method is suitable for direct extraction and purification of eDNA from composite soil samples of up to 500g. The procedure below is described for samples of 250g.

Materials:

1. Sodium phosphate buffer pH 8 (1.97 g NaH_2PO_4 and 14.7 g Na_2HPO_4 per l sterile distilled H_2O).
2. NucleoSpin Soil kit (Macherey-Nagel, Düren, Germany)

Procedure:

1. Sieve soil to remove stones
2. Add an equal weight of phosphate buffer (pH 8) to the soil in a wide neck bottle.
3. Mix thoroughly by gentle orbital shaking for 15-30 min (e.g. at 50-100 rpm) to homogenise the sample.
4. Centrifuge aliquots of the resulting soil suspension at 10,000 rcf for 10 min.
5. Purify DNA from 500 µl of the supernatants using a Nucleospin Soil commercial kit (Macherey-Nagel, Düren, Germany) following the manufacturers' instructions but skipping their lysis step.