GREATSOILS

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Development and demonstration of robust DNA-based measures for routine monitoring of soil borne disease and identification of best management practices to minimise disease risk

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1. Abstract

As part of the work within the Soil Biology and Soil Health Research and Knowledge Exchange (SBSH) Partnership, this project evaluated the use of molecular approaches based on extraction of total soil DNA to determine how soil management practices can affect populations of soil-borne plant pathogens within the overall soil microbiome. Quantitative PCR (qPCR) was used to detect and quantify individual pathogens as well as biocontrol agents and populations of total bacterial and fungal communities in soil. Methods were optimised and standardised for sampling and extraction of soil DNA from the wide cross-section of soils studied in other projects within the SBSH Partnership. Over 20 different qPCR assays were validated as suitable for use and controls were developed that allow reproducible quantification of the target organisms in these soils. A second approach, involving the use of high throughput DNA sequencing technology to study the diversity of bacteria, fungi and mesofauna present in the range of soils according to their characteristic DNA barcodes (a process known as metabarcoding), is mainly reported under Project 6 of the SBSH Partnership.

These approaches have been used successfully to assess various soil management practices in long-term field trials within Projects 4, 6 and 7 of the SBSH Partnership. The qPCR approach used here confirmed that general bacterial and fungal activity was affected by soil pH, but that the cropping stage also played an interacting role. Total bacterial and fungal populations were also affected to some extent by repeated applications of farmyard manure, but not by inorganic fertiliser or by different tillage or drainage routines. Attempts to relate disease incidence to pre-planting levels of *Fusarium oxysporum* in onion and *Narcissus,* and *Verticillium dahliae* in raspberry trials within Project 7 were unsuccessful due to excessively high inoculum in the onion and very low detected inoculum levels in the other crops. Additional glasshouse trials are underway as part of an associated PhD study to investigate the relationship between soilborne inoculum levels and disease risk further.

However, the largest differences, in both bacterial and fungal populations, were observed between trial locations. Although soil management affects microbial activity, these effects are less than those resulting from natural geographic variation. Further investigation of the impact of soil type and sampling times, using the standard approaches developed here, will help to better establish the expected biogeography of soil biology.

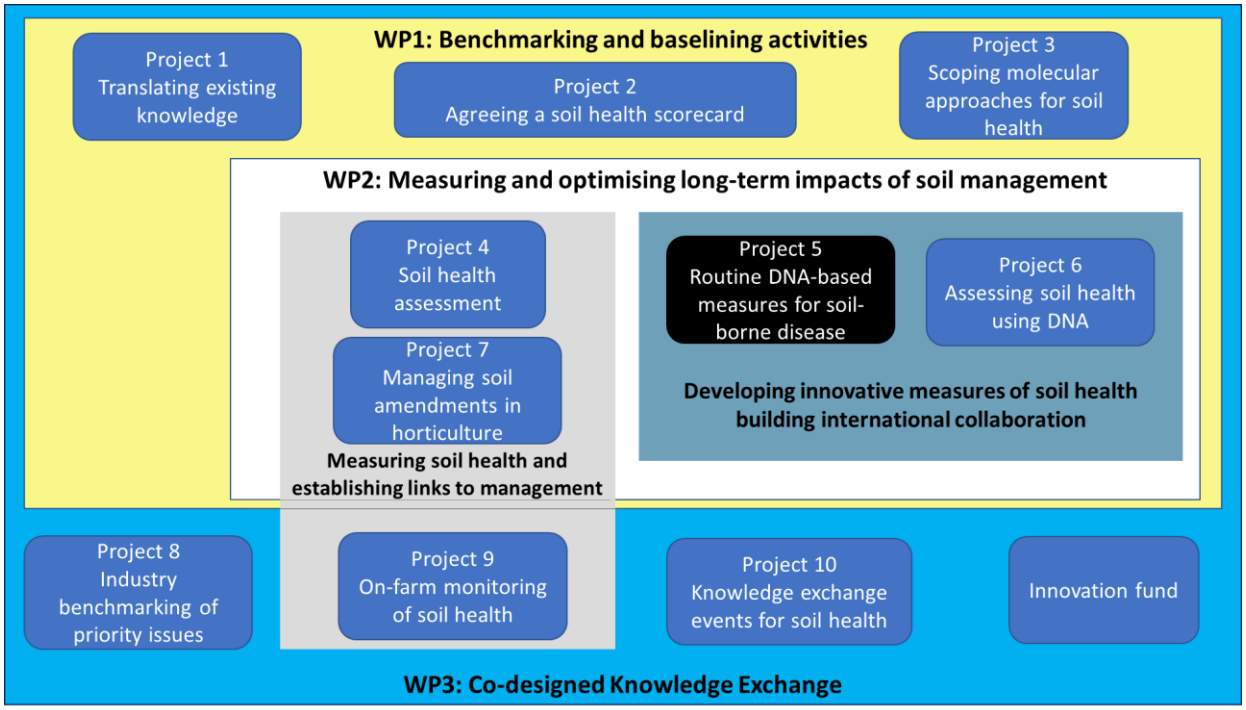
In collaboration with scientists from the South Australian Research and Development Institute (SARDI), developers of the industry award-winning PREDICTA® molecular soil testing service, we confirmed that molecular approaches in routine soilborne disease testing can reduce cost and increase speed and accuracy. However, due to the high variation in results from location to location, validation of DNA testing approaches for the UK would require extensive monitoring over many seasons and locations and could not rely on data obtained from isolated trials, such as those investigated in this project. As was the case for the PREDICTA® service, these data can only be accumulated through the formation of a routine testing service from which test results can be used

to further optimise the test procedures and fine-tune disease risk assessments. The development of such a service would depend on initial government and/or industry support.

2. Introduction

This project is part of a suite of integrated projects within the Soil Biology and Soil Health Research and Knowledge Exchange Partnership (see Diagram below showing how this project fits into the wider organisation of projects). The overall aim of Work Package 2 (Projects 4, 5, 6 and 7) was to improve understanding of the role of soil biology in overall soil health, with respect to the ability of soils to support and sustain healthy and productive crops. In particular, it aimed to establish any aspects of soil biology that are key to this function of soils and to explore practical and effective ways to measure and manage these key components alongside already established measurement and monitoring of soil physical and chemical properties. This project (Project 5), together with Project 6, develops innovative measures of soil health, largely based on molecular approaches, and builds on the review presented in Project 3.

Diagram to show how Project 5 (shown in black) fits within the integrated project delivery of the Soil Biology and Soil Health Research and Knowledge Exchange Partnership.



2.1. Aims and Objectives

The overall aim of Project 5 (led by Fera Science Ltd.) was to increase understanding of the effects of common agronomic soil management practices on soil health by selecting and developing available molecular methods able to quantify changes in population dynamics of soilborne pathogens and beneficial biocontrol agents, in relation to overall microbial diversity across a range of experimental trial sites. As a starting point, an initial literature review entitled “Molecular approaches

for routine soil-borne disease and soil health assessment – establishing the scope" (Elphinstone et al., 2017) was conducted within WP1 (Project 3) of the Partnership. This review was then used to select appropriate methodology for the sampling of soils, extraction and purification of total DNA and subsequent analysis of the DNA for quantification of individual pathogens and biocontrol agents using quantitative polymerase chain reaction (qPCR) assays. Optimisation and use of these technologies within Project 5 and across the other projects (4, 6 and 7) within WP2 was then intended to better understand the links between soil management approaches, soil-borne disease risk and overall soil biological diversity. Cross-linking findings from the molecular approaches could then be used to support findings from traditional field-based assessments of biological, as well as physical and chemical, soil properties being used to monitor soil health status across the other projects.

2.1.1. Specific objectives of Project 5

1. Validate a suitable toolkit of qPCR assays for quantitative detection of key intractable soil-borne pathogens in arable and horticultural rotations.
2. Establish relationships between pathogen distribution and concentration in soils and potential for disease development.
3. Evaluate the effects of soil management practices on the survival of specific soil-borne pathogens in relation to the soil microbiome and overall soil health.
4. Design and demonstrate the benefits of an appropriate soil health testing service for growers and agronomists.
5. Integrate results of molecular testing for soil-borne disease risk with associated chemical and physical data in support of a robust soil health scorecard developed within the SBSH Partnership.

Work in objective 1 was largely completed as an MSc thesis by Amy Kerr from Newcastle University, with additional validation and work under the remaining objectives completed as part of a PhD thesis by Emma Chapelhow funded by AHDB (year 1) and the Institute for Agri-Food Research and Innovation (IAFRI) at Fera and Newcastle University (subsequent years of the PhD).

2.2. Background

2.2.1. Effect of soil management on soil microbiology

Two conventional key soil management interventions revolve around manipulating soil chemistry and hence crop nutrient availability, either indirectly by altering pH or directly via fertiliser addition. Soil pH is known to be one of the strongest abiotic factors driving bacterial and fungal community assemblages in multiple studies (Borneman and Hartin, 2000; Fierer *et al*., 2005; Fierer and Jackson, 2006; Rousk *et al*., 2010) including in agricultural soils (Wang *et al*., 2019). Fertiliser application has also been shown to affect the composition of bacterial and certain groups of fungal communities

depending on the type of phosphate fertilisation (Silva *et al*., 2017). In addition, there is evidence for the inhibitory effect of triple superphosphate fertiliser on mycorrhizal formation (Peine *et al*., 2019). However, to date, the general effect of fertiliser applications on soil bacterial and fungal diversity remains unclear.

Soil management practices have mostly developed with the aim of creating good soil physical structure and chemical fertility for crop growth, but less is known of how these practices affect soil biological activity. Such practices include managing organic matter input amounts and types, minimising soil structural disturbance (e.g. minimum or no tillage cultivations), and maintaining and diversifying plants through mixed cropping, crop rotations and/or cover cropping (Larkin, 2015). However, the relative effects of soil management practices across different regions and soil types on biological communities and their functions remain unclear due to the lack of a standardised approach for accurately measuring soil biodiversity. As a result, the relationships between soil biodiversity, soil management and crop health and productivity remain poorly described.

2.2.2. Management of intractable soil-borne plant pathogens

Numerous intractable soilborne pathogens represent a major constraint to UK arable and horticultural production (see Table 1). In this project we have mainly focussed on two important fungal pathogens that cause wilt diseases of a wide range of agricultural and horticultural crops: *Verticillium dahliae* and *Fusarium oxysporum*.

Verticillium wilt is probably the most devastating of all the soil-borne strawberry diseases in England and Wales (Raffle and O'Neill, 2006) and is also increasing in raspberry and blackberry fields in England (Raffle and O'Neill, 2010). The pathogen survives in soil for years as hardy resting spores (microsclerotia). The cost of establishing new strawberry plantations, after incurring the disease, was estimated at £15,000 per field hectare in 2006. *V. dahliae* also causes disease on *Acer*, *Cercis*, *Cotinus*, *Helichrysum*, *Chrysanthemum*, potato, rose, tomato, and linseed.

Fusarium oxysporum causes wilt, basal rot and root rot of many important crop plants, including asparagus, basil, beans (*Phaseolus* and *Vicia* spp.), *Callistephus*, *Cyclamen*, *Dianthuss* pp., *Gladiolus*, *Lisianthus*, *Narcissus*, onion, peas, stocks and tomato. *F. oxysporum* is a species complex made up of many host-specific *formae speciales* (f.spp.; special forms). They survive in soil for many years as hardy resting spores (chlamydospores). In addition, there are also non-pathogenic isolates of *F. oxysporum*, some of which have been exploited as biological control agents. The following investigations focus on *F. oxysporum* f. sp. *cepae*, the cause of basal rot of onion, an increasing problem for UK growers, and *F. oxysporum* f. sp. *narcissi,* the most important pathogen of UK daffodils, also causing basal rot of the bulbs similar to that of onion. UK onion growers have been estimated to lose an average of 2-6% of the bulb crop each year corresponding to average economic

losses of £7.6M in 2017, although basal rot incidence of 10% or greater is becoming more common and further losses of 3-10% occur during storage (Clarkson, 2019). UK *Narcissus* growers have been estimated to commonly lose 10% of their crop, equating to some £4.5M per annum (Clarkson, 2019).

There are few specific recommendations or approvals for fungicide treatments to control these pathogens. Various soil fumigants have been used but they penetrate only to a limited depth and are expensive and being increasingly de-registered. Furthermore, fumigation is non-selective, also reducing beneficial and saprophytic soil microorganisms. Disinfectants and hot water treatments have been used to reduce infection in bulbs before planting (Clarkson, 2014) but do not affect residual pathogen populations already present in the soil. A review by Noble and Coventry (2005), described the biological suppressive effect of composts on soil-borne diseases including wilts caused by *Fusarium oxysporum* and *Verticillium dahliae*, although the effects in greenhouse studies were less variable than in the field. They concluded that the mechanisms and antagonistic micro-organisms involved in disease suppression required further investigation and that the inoculation of composts with biological control agents may improve the efficacy and reliability of disease control obtained.

To select the most appropriate control strategies, including the most suitable crop rotations and additional soil management practices that reduce or eliminate residual pathogen populations between susceptible crops, reliable and practical methods to monitor pathogen levels in the soil are needed. At present, there are no tests commercially available to growers that will detect and quantify specific *Fusarium oxysporum* pathogens in soil. Bioassays, involving the growing of susceptible plants in samples of soil under greenhouse conditions can be useful for research purposes but are too laborious and space-consuming and take too long to complete (1-2 months) to be commercially viable. There is a currently available test for *Verticillium dahliae*, based on the method described by Harris *et al*. (1993), that involves culturing soil suspensions on agar plates and estimating the number of resting spore propagules that grow into colonies. The current cost of this test at Fera Science Ltd. is £220 (+VAT) per sample and results are available within 7-12 weeks, depending upon the number of samples submitted. Again, it is difficult to scale up this kind of testing for routine and affordable analysis of replicated samples from multiple fields to provide results for on-farm decision-making prior to planting new crops.

2.2.3. Role of molecular biology in monitoring soil microbiology

New molecular tools are allowing a step-change in the study of microbial populations associated with soil and roots (Elphinstone *et al*., 2017). DNA extracted from soil can be used to detect and quantify specific target organisms present in the same sample, including pathogens and beneficial organisms. Furthermore, high throughput DNA sequencing and metabarcoding technology now offer

the potential to simultaneously identify the diversity of whole microbial communities present in soil, according to specific DNA barcode sequences that are unique to each individual organism.

Quantitative polymerase chain reaction (qPCR)

Quantitative real-time PCR (qPCR) provides a single platform for assessment of multiple target pathogens present in a single soil sample. There are many individual qPCR assays that have been developed for specific detection of soil-borne plant pathogens (see Table 1). These include specific assays for *Verticillium dahliae* and related pathogens such as *V. albo-atrum* and *V. longisporum* and a generic species-level qPCR assay for *Fusarium oxysporum*. Further, qPCR assays that detect individual pathogenic *F. oxysporum* f. spp. are also nearing development, including assays for *F. oxysporum* f. sp. *cepae* and *F. oxysporum* f. sp. *narcissi* (Clarkson, 2015; 2019), of specific interest to this project. While most of these assays have been tested on infected plant material, there remains a need for a standardised and validated approach to ensure that they are fully functional for quantification of each target pathogen across the range of agricultural and horticultural soils that will need to be assessed. Furthermore, where testing has been performed on soil, the quantities of soil used in each test have usually been small (0.5-10 g) and therefore unlikely to be representative of large field areas. A key aim of this project was therefore to optimise sampling and extraction methods to allow reliable qPCR testing across a range of different soil types and using as large a quantity of soil as possible per sample.

Since different cropping systems and their rotations will maintain different combinations of soilborne pathogens in the soil, the ability to simultaneously test for series of pathogens in the same soil sample is essential when establishing the most effective rotations and selecting the most appropriate fields and varieties to limit the risk of disease for each crop. The process of soil sampling and extracting and purifying the DNA is by far the most costly part of qPCR testing. It is therefore most cost effective to maximise testing of each extracted DNA sample for the full range of pathogens relevant to all crops in the rotation. This project therefore attempts to validate qPCR tests for a full range of soil-borne pathogens in addition to those of specific interest to the project.

High throughput sequencing and metabarcoding

Modern high-throughput DNA sequencing and associated bioinformatic tools have the potential to comprehensively characterise microbial communities (George *et al.*, 2019; Tedersoo *et al.*, 2020). Lauber *et al.* (2009) were one of the first to use such an approach to describe a correlation between soil bacterial community structure and pH from a wide array of ecosystem types. DNA sequencing studies have not achieved an agreement on the effect of fertiliser application on bacterial and fungal communities: some report fertiliser application increases richness and diversity (Pan *et al.*, 2020; Wang *et al.*, 2017) while others found no significant influence of fertilisers (Yao *et al.*, 2018). More generally, recent soil DNA sequencing studies have shown significant but small and inconsistent

differences between fungal (Hannula *et al.*, 2021; Morrison-Whittle *et al.*, 2017) and bacterial (Hendgen *et al.*, 2018) diversity in soils under conventional versus conservation agricultural management approaches. These include simultaneous analyses of bacteria and fungi (Hartmann *et al.*, 2015), and total soil biology across time and space (Giraldo-Perez *et al.*, 2021), and include attempts to analyse the functions of these communities (Harkes *et al.*, 2019). Recent studies have also shown how fungal community structure and functionality (Hannula *et al.*, 2021), as well as bacterial diversity (Hartmann et al., 2015), have been affected by different long-term agricultural practices such as tillage, cover cropping and organic amendment. Moreover, Hannula *et al.* (2021) and Giraldo-Perez *et al.*, (2021) concluded that different components of soil biodiversity responded differentially to agricultural practices depending on geographic location and time of year. In general, to date studies have focused on different agricultural system types, and only evaluated soils in one or a few locations and timepoints and have lacked methodological and analytical standardisation making it very hard to cross-compare studies to evaluate any general effects on soil biology. There is an urgent need for a standardised approach for measuring soil biodiversity to allow meaningful comparisons and to quantify the effects of soil management practices across agricultural systems, climates, and soil types. This approach has been further investigated under Project 6 of the Soil Biology and Soil Health Partnership.

3. Materials and methods

3.1. Objective 1: Validation of a suitable toolkit of qPCR assays for quantitative detection of key intractable soil-borne pathogens in arable and horticultural rotations.

3.1.1. Selection of qPCR assays

A series of qPCR assays were selected for specific detection and quantification of important soilborne fungal plant pathogens of agricultural and horticultural importance. Assays were chosen from publications identified during review of the literature (Elphinstone *et al*., 2017) or which have been developed for diagnostic purposes in the Fera plant clinic but had not yet been fully validated. A full list of the pathogen assays, with primer and probe sequences, is shown in Table 1. Additional assays were selected for detection and quantification of total bacterial and fungal DNA, based on specific 16S and 18S rRNA sequences respectively (Table 2). These assays were employed as controls to verify successful DNA extraction and amplification from soils. Further assays (Table 3) were selected for detection and quantification of a biocontrol fungus *Gliocladium catenulatum* (syn. *Clonostachys rosea*), active ingredient of the commercial biofungicide Prestop® (Lallemand plant Care, Danstar Ferment, A.G.Poststrasse 30, Zug CH-6300 Switzerland), and three species of arbuscular mycorrhiza fungi (AMF), constituents of one commercial formulation for field application (Plant Works Ltd., Sittingbourne, Kent, UK). These assays were validated for use in subsequent field and glasshouse experiments.

Table 1: Selected qPCR assays for soilborne pathogen detection and quantification.

Pathogen	Primer/probe	Sequence	Reference
Colletotrichum coccodes	CcTqF1 CcTqR1 CcTqP1	TCTATAACCCTTTGTGAACATACCTAACTG CACTCAGAAGAAACGTCGTTAAAATAGA FAM-CGCAGGCGGCACCCCCT-TAMRA	Cullen *et al*., 2002
Fusarium culmorum	Cul370(F) Cul437(R) Culm(P)	TTGGTGTTGGGAGCTGCA CTATGGAAGCTCGACGTGACC FAM-CCTGCTGCACTCCCCAAATACATTGG-TAMRA	Cullen *et al.,* 2005
Fusarium oxysporum (generic)	Foxy_F Foxy_R Foxy_P	AAAGCATCGTCGCCATGAG CTGCCAACACACCGACATGT FAM-ATAAAATGGAAGGTAGGTGCGGGTGCATAG-TAMRA	J Woodhall, pers. comm
Gaeumannomyces tritici fsp. tritici	GgtEFF1 GgtEFR1 GgtEFPR1	CCCTGCAAGCTCTTCCTCTTAG GCATGCGAGGTCCCAAAA FAM-ACTGCACAGACCATC-TAMRA	Keenan *et al*., 2015
Phytophthora asparagi	Forward Reverse Probe	TGAACCGTATCAACCCAATTAGTTG CAGCCGTCAGCCCATTACAG FAM-CTTGCTCTGGCGTGCGGCTGTT-BHQ	Chimento, 2007
Phytophthora rubi	Forward Reverse Probe	TTTATTATTAACTTTACCCGTATTGGCA CCCCCGGAAGGATCATAAA FAM-AATGTTATTAACTGATAGAAATTTAAATAC-MGB	Woodhall and Peters 2014
Plasmodiophora brassicae	DC1F DC1mR PB1	CCTAGCGCTGCATCCCATAT CGGCTAGGATGGTTCGAAA FAM-CCATGTGAACCGGTGAC-NFQ-MGB	Deora *et al*., 2015
Pythium violae	AT_ITS FOR AT_ITS REV Probe	TGGTGTTTTCGGACGCTGCGCTG TCCGCACACACACATTGCTG FAM-CGGAGGAGGAACGAAGGTTGGTCTTGT-TAMRA	Clarkson 2015
Rhizoctonia cerealis	RcF RcR RcP	AAAGCATCGTCGCCATGAG CTGCCAACACACCGACATGT FAM-ATAAAATGGAAGGTAGGTGCGGGTGCATAG-TAMRA	Woodhall *et al*., 2017
Rhizoctonia solani AG2-1	AG2-1_F AG2-1_R AG2-1_P	CTTCCTCTTTCATCCCACACA TGAGTAGACAGAGGGTCCAATAACCTA FAM-AAGTAAATTCCCCATCTGT-TAMRA	Budge *et al*., 2009; Woodhall *et al*., 2013
Rhizoctonia solani AG8	AG-8_F AG-8_R AG-8_P	AGTTGGTTGTAGCTGGTCCATTAAT AGTAGACAGAGGGGTCCAATAAATGA FAM-TGT GCA CAC CTC CTC- NFQ-MGB	Budge *et al.*, 2009
Sclerotinium sclerotiorum	TMSCL2F TMSCL2R TMSCL2P	CCCAGTTCGACTCTCCTCTTTTAT AACTCAGACTCGGAAGGGTTTTG HEX-AGACATCTTGACCGACACCGCCCC-IBFQ	Kim and Knudsen, 2008
Sclerotium cepivorum (Stromatinia cepivora)	Z996-340F Z996-450R Z996-382T	CCATGTCAGCAATGGCAGA AAAATTTAGGTTTTGACAGAAGCACAT FAM-TTGGGTCCTGAACGTAG-MGB	Woodhall *et al*., 2012
Spongospora subterranea	SponF SponR SponP	CTTTGAGTGTCGGTTTCTATTCTCCC GCACGCCAATGGTTAGAGACG FAM-TCTTTCAAGCCATGGACCGACCAGA-BHQ	Qu *et al*., 2011
Stemphylium vesicarium	Forward Reverse Probe	AGGGTCGCTACAGA CTGGGTCACT GCACTCATAAGGTTAGTAATAACTGTAGC FAM-CTGCTTAATGTACAGGCGAAAC-BHQ	Graf *et al*., 2016
Verticillium albo-atrum	Vaa-CF Vaa-CR Vaa-CP	CATCGCCAATCGACAACATG CATCGTCCAGGTGACCTGAA FAM-CACCCGAACCTCTGTCTCGCTTTT-TAMRA	Peters, 2012
Verticillium dahliae	Vd-F929-947 Vd-R1076-1094 Vdhrc	CGTTTCCCGTTACTCTTCT GGATTTCGGCCCAGAAACT FAM-CACCGCAAGCAGACTCTTGAAAGCCA-BHQ	Bilodeau *et al*., 2012
Verticillium longisporum	Vd EF2 Fwd VdEF2Rev VdEF2Probe	TGGCTATCCGGACCTCTGTCT GGCCAGCGACATCATCTATCTAC FAM-TTTTTGTCGTTCAGGTCACCTGGACGA-TAMRA	J Woodhall, pers. comm

TAMRA: Tetramethylrhodamine quencher dye

FAM: 6-carboxyfluorescein reporter dye

NFQ: Non-fluorescent quencher dye

BHQ: Black hole quencher dye

MGB: Minor groove binder

HEX: Hexachlorofluorescein dye

IBFQ:Iowa black fluorescent quencher dye

Table 2: Selected qPCR assays for detection and quantification of total bacterial and fungal DNA

Universal Bacteria (16S rRNA)	U16SF U16SR U16SPe	TGGAGCATGTGGTTTAATTCGA TGCGGGACTTAACCCAACA FAM-CACGAGCTGACGACARCCATGCA-TAMRA	Yang *et al*., 2002
Universal Fungi (18S rRNA)	FungiQuant_F FungiQuant_R FungiQuant_Pr	GGRAAACTCACCAGGTCCAG GSWCTATCCCCAKCACGA FAM-TGGTGCATGGCCGTT-NFQ	Liu *et al*., 2012

Table 3: Selected qPCR assays for detection and quantification of fungal constituents of biofungicides and arbuscular mycorrhizal soil additives.

Gliocladium catenulatum (*Clonostachys rosea*)	VTTact-forward VTTact-reverse VTTact probe	GGCCAGAGATTGTGTTGATGA ACAGGTTAGGCTCAATGCTC GAGGCTGGCAAGAGAGGTCAGTCAC	Gimeno *et al*., 2019
Funneliformis mosseae	Forward Reverse Probe	GGAAACGATTGAAGTCAGTCATACCAA CGAAAAAGTACACCAAGAGATCCCAAT FAM-AGAGTTTCAAAGCCTTCGGATTCGC-BHQ	Thonar *et al*., 2012
Rhizophagus irregularis	Forward Reverse Probe	TTCGGGTAATCAGCCTTTCG TCAGAGATCAGACAGGTAGCC FAM-TTAACCAACCACACGGGCAAGTACA-BHQ	Thonar *et al*., 2012

3.1.2. qPCR reaction conditions

All qPCR reactions contained 1 x PCR Environmental Master Mix 2.0 (Applied Biosystems), primers and probes were added at concentrations of 7.5 µM and 5 µM (Eurofins, Integrated DNA Technologies) and 5 µl of extracted DNA was added in a total reaction volume of 25 µl, the remaining volume being made up with molecular grade water. An Applied Biosystems 7500 real-time PCR system was used throughout the study with cycling conditions of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 min. The cycle threshold (Ct) value for each reaction was assessed using the default threshold setting of 0.2 ΔRn units. An average Ct value was determined from two replicate reactions in each case. Target DNA in soil samples was quantified by including five DNA standards in each run. The standards consisted of a DNA sample of known concentration determined using a Nanodrop fluorometer (ThermoFisher) taken from appropriate reference cultures which was used to produce a dilution series of five ten-fold dilutions. Target DNA concentration was then determined by linear regression.

3.1.3. Assay efficiency and analytical sensitivity

Preparation of reference DNA

Reference DNA was extracted from approximately 200 mg of mycelium from the surface of growing fungal cultures using a method outlined by Woodhall *et al*. (2012) and adapted from the standard instructions for the Wizard Magnetic DNA Purification System (Promega, UK). Reference cultures from the Fera culture collection (Table 4) were grown on Potato Dextrose Agar (PDA). For the non-culturable pathogens, freeze dried plant material infected with *Plasmodiophora brassicae* and

freeze-dried spore balls from potatoes infected with *Spongospora subterranea* were used as starting material. Mycelium or infected tissue was disrupted by shaking with 300 µl glass and silica beads in 1.25 ml Lysis Buffer A at 6800 rpm in 2 x 30 second periods in a Precellys 24 bead beater (Bertin Technologies SAS, France). Further lysis by vortexing in 250 µl Buffer B was followed by removal of protein and other cellular material by adding 750 µl precipitation buffer (Promega Wizard Food Kit) followed by centrifugation at 13000 g for 5 minutes. DNA was then bound by adding paramagnetic particles (50 µl MagneSil beads) and the bound DNA was further purified using a Kingfisher robotic sample purification system (ThermoFisher Scientific) for sequential washing in 600 µl isopropanol, 1 ml buffer B and 2 x 1 ml of 70% ethanol before eluting the purified DNA in 200 µl TE buffer.

Table 4: Reference cultures of soilborne pathogens used to prepare purified reference DNA of known concentrations.

Pathogen	Culture Fera code	Alternative code	Host of origin	Country of origin
Rhizoctonia solani AG2-1	cc1946	FWHC1 (3)12	Cauliflower	UK
Rhizoctonia cerealis	254		Wheat	UK
Pythium violae	cc74	CBS 102609	*Daucus carota*	UK
Fusarium culmorum	cc2633	2804-3f	Potato	UK
Fusarium oxysporum	cc260	Fus2 scs 9=18	Onion	UK
Plasmodiophora brassicae	Freeze Dried		*Brassica*	UK
Sclerotinia sclerotiorum	cc1183	SCI	Oilseed rape	UK
Sclerotium cepivorum	cc2579	276.93	Onion	Netherlands
Verticillium longisporum	cc2825	L/a3 BX09/002	*Brassica napus*	UK
Verticillium dahliae	cc29	79/4990 244717	*Chrysanthemum*	UK
Verticillium albo-atrum	cc435		Soil	UK
Phytophthora rubi	cc380	CBS 967.95	Raspberry	UK
Colletotrichum coccodes	cc1499	CABI	Potato	UK
Spongospora subterranea	spore balls		Potato	UK

Determination of assay sensitivity and performance.

Assay sensitivity was determined on 10-fold serial dilutions of purified reference DNA to produce standard curves showing the logarithm of DNA concentration vs critical threshold (Ct) value, the qPCR cycle at which amplification of the DNA target was first measurable. The standard curves were then used to determine amplification efficiency (Svec, *et al.*, 2015). Ct value vs the logarithm of target DNA was plotted and expected to be linear with a negative slope (a slope of -3.33 represents 100% efficiency and assumes target DNA amount doubles after each reaction cycle). Amplification efficiency was calculated from the standard curves according to the following formula: E = (10^(-1/slope)-1)*100. The theoretical limit of detection was also estimated from the standard curves produced. The lowest concentration of target DNA that could be reliably quantified was

determined as the lowest concentration of the standard dilution series with a corresponding Ct value <40 within the linear range of the curve.

3.1.4. Assay performance on spiked soils

Sampling of soils

At the start of the project (July 2017) soil was sampled from 21 sites representing all cropping rotations and soil types at trial sites across the UK that would be studied during the SBSH Partnership (Table 5). Composite samples of 2 kg of soil were collected from each site as multiple1.5 cm diameter cores collected from the top 10-15 cm soil, collected in a 'W' pattern transect to account for potential patchiness of pathogen distribution in the field. Samples were thoroughly mixed to homogeneity after collection. Samples were kept refrigerated until required for DNA extraction.

Inoculation of soils with pathogen cultures

Reference isolates (Table 4) were spiked into sub-samples of each soil as either mycelium from PDA cultures or freeze-dried infected tissues, as described previously. Mycelium was weighed into low, medium or high quantities (approximately 0.5-2 mg, 10 mg and 50-100 mg mycelium/tissue) and mixed directly into 250 g soil sub-samples, ready for DNA extraction.

DNA extraction from inoculated soils

Soil was air-dried before being weighed out into sub-samples with gravel and coarse organic debris first removed by hand. A standard soil sub-sample size of 250 g was used for artificially spiked samples. For extraction of DNA from all samples the method outlined by Woodhall *et al*. (2012) was used.

qPCR analysis

To indicate presence or absence of any background target pathogen populations, qPCR assessment was first performed on DNA extracted from 50 g non-spiked soil samples from each site. Appropriate qPCR assays were then performed on DNA extracted from the 250 g sub-samples of each inoculated soil. In addition to the specific assays for each spiked pathogen, the universal assays for total fungi and bacteria (FungiQuant ITS & Universal 16S rRNA respectively) were used as positive controls to ensure DNA extracted from each soil was of suitable quality for qPCR analysis. The performance of each assay was then determined according to the successful detection of the target pathogen spiked at different concentrations into the range of different soil types.

Table 5: Soil sampled from different trial sites under various crop rotations and management conditions

Sample I.D	Site name	Soil type	Rotation	Management
1	Project 7, Norfolk raspberry trial site 1 (Place)	Sandy silt loam	B, Rsp, Rsp, Rsp	Barley rotation
2	Project 7, Norfolk raspberry trial site 2 (Place)	Sandy silt loam	Rsp.	No barley
3	Project 4, Harper Adams	Sandy loam (12% clay)	G, W, P, W	Manure
4	Project 4, Harper Adams	Sandy loam (12% clay)	G, W, P, W	No manure
5	Project 4, Gleadthorpe	Loamy sand (6% clay)	M, SB, SB, W	Manure
7	Project 4, Gleadthorpe	Loamy sand (6% clay)	M, SB, SB, W	No manure
6	Project 4, Loddington	Clay (40% clay)	OSR, W, Cov/B, W	
8	Project 4, Craibstone	Sandy loam (12% clay)	**P,** W, Gx3, O, Sw, B	potato, pH 4.5
9	Project 4, Craibstone	Sandy loam (12% clay)	**P,** W, Gx3, O, Sw, B	potato, pH 6.5
10	Project 4, Craibstone	Sandy loam (12% clay)	**W,** Gx3, O, Sw, B, P	wheat, pH 4.5
11	Project 4, Craibstone	Sandy loam (12% clay)	**W,** Gx3, O, Sw, B, P	wheat, pH 6.5
12	SRUC clubroot trial		OSR	
13	Project 7, Bedford, onion trial site (Parish)	Clay loam	O	
14	Project 7, Norfolk *Narcissus* trial site (Eves)	Sandy silt loam	N	
15	Project 4, Terrington	Silty clay loam (28% clay)	W, W, OSR, SB	Manure
16	Project 4,Terrington	Silty clay loam (28% clay)	W, W, OSR, SB	No manure
17	Rectory Farm Oxfordshire (ADAS)			
18	Project 4, Boxworth 40 Acre	Clay (35% clay)	W & OSR	
19	AHDB Rotations Prtshp 3		P-SB	
20	AHDB Rotations Prtshp 1		P-SB	Compost
21	AHDB Rotations Prtshp 2 (Greenwell Farms, Poor Walk, Suffolk)		P-SB	Compost
22	AHDB Rotations Prtshp 2 (Greenwell Farms, Poor Walk, Suffolk)		P-SB	No compost

Rotation crops: Barley (B), raspberry (Rsp), grass ley (G), wheat (W), potato (P), maize (M), sugar beet (SB), oil seed rape (OSR), cover crop (cov), oat (O), swede (Sw), *Narcissus* (N).

3.2. Objective 2: Establish relationships between pathogen distribution and concentration in soils and potential for disease development.

This objective was mainly delivered as a PhD project (student - Emma Chapelhow) sponsored through AHDB (year 1) and the Institute for Agri-Food Research and Innovation at Fera and Newcastle University.

3.2.1. Systematic review of methods for extraction of DNA from soils

A meta-search was conducted to further explore methods used to extract total DNA from soil for subsequent PCR based detection of specific plant-related bacterial or fungal organisms. Key words for the search were identified from 5 key papers on the subject (Habib *et al*., 2017; Budge *et al*., 2009; Deora *et al*., 2015; Huang and Kang, 2010 and Nunes *et al*., 2010), which were always cited in subsequent searches. The keywords were Polymerase chain reaction, DNA, Soil, Real time qPCR, PCR, Detection, Crop, Field, Inoculated and Molecular. Two online search literature databases were searched: SCOPUS and Web of science (WOS) (Table 6).

Table 6: Search criteria adapted for use in both SCOPUS and Web of Science

Database	**Search criterion**	**Number of results**
SCOPUS	(TITLE-ABS-KEY (*"Polymerase chain reaction"*) OR TITLE-ABS-KEY (*qpcr*) OR TITLE-ABS-KEY (*pcr*) OR TITLE-ABS-KEY (*rtpcr*) OR TITLE-ABS-KEY (*rt-pcr*) AND TITLE-ABS-KEY (*soil*) AND TITLE-ABS-KEY (*detect**) AND NOT TITLE (*"gene" OR "genes"*) AND NOT TITLE (*compar**) AND NOT TITLE (*method**) AND NOT TITLE (*extract**) AND NOT TITLE-ABS-KEY (*human*) AND NOT TITLE-ABS-KEY (*animal*) AND NOT TITLE-ABS-KEY (*vir**) AND NOT TITLE-ABS-KEY (*parasit**) AND NOT TITLE-ABS-KEY (*nematode*) AND NOT TITLE-ABS-KEY (*insect*)) AND (LIMIT-TO (PUBSTAGE , *"final"*)) AND (LIMIT-TO (DOCTYPE , *"ar"*)) AND (LIMIT-TO (LANGUAGE , *"English"*)) AND (LIMIT-TO (SRCTYPE , *"j"*))	2233 (15/5/20)
WOS	(TS=("Polymerase chain reaction" OR qpcr OR pcr OR rtpcr OR rt-pcr) AND TS= ("soil") AND TS=(detect*) NOT TI= (method* OR extract* OR "gene" OR "genes") NOT TS= (human OR animal OR vir* OR parasit* OR nematode OR insect)) AND LANGUAGE: (English) AND DOCUMENT TYPES: (Article) Indexes=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, ESCI Timespan=All years	2354 (15/5/20)

From a total of 4587 search results, duplicates and those missing key information were removed, leaving 3030 results. A further 2593 were removed on the basis that they were not plant related (412), not soil related (361), did not consider downstream PCR detection (212), concerned community analyses by sequencing of functional genes (1512) or were review papers (96). All papers had been peer reviewed and published and were therefore scientifically robust. DNA extraction methods were categorised according to the starting volume of soil, method of sample disruption, and type of lysis buffer. Lysis buffers were sorted according to chelating agents, detergents, salts, enzymes and other key reagents used. It was recorded whether or not a commercial extraction kit was used. For commercial kits, patent-protected information on individual reagents was not always freely available. Results were recorded as frequency of use of each method. This data was also recorded per decade to monitor change in trends over time.

3.2.2. Method development for improved extraction of DNA from soil

Considering the review of procedures for DNA extraction, the extraction method used in 3.1.4. (Woodhall *et al*., 2012) was adapted to ensure consistent yield and purity of DNA extracted from soils taken from the range of field trials. The following extraction method was selected following preliminary investigations of a number of alternatives:

1. Soil samples were thoroughly mixed during which any stones and plant material were removed manually.
2. 50 g soil was added to 100 mL cetrimonium bromide (CTAB) lysis buffer (containing 120 mM sodium phosphate buffer pH 8.0, 2% CTAB, 1.5 M sodium chloride and 3% Antifoam B).
3. The sample was thoroughly homogenised in 250 mL Nalgene bottles in a Minimix auto paint shaker (Merris Engineering Ltd, Ireland) with 6 ball bearings (25 cm dia; grade 316) and shaken for 4 minutes.
4. The sample was transferred to a clean 50 ml tube and centrifuged at 5000 g for 3 minutes and 10 ml of the supernatant was then transferred to a clean 50 ml tube
5. Proteins were then precipitated by adding 9 ml of 7.5 M ammonium acetate at 4 °C to aliquots of the homogenised sample.
6. After centrifugation for 5 min at 12,000 x g to remove the precipitate, the DNA was precipitated from the supernatant by adding equal parts of isopropanol.
7. The DNA was concentrated to a pellet by further centrifugation at 12,000 x g and removal of the supernatant.
8. For further purification of the DNA part of the DNeasy PowerMax Soil Kit (Qiagen, Netherlands) was used according to the manufacturers' instructions. The DNA pellet was first suspended in 30 mL of high salt-containing buffer C4 to bind it to the silica membrane of a MB Maxi Spin Column during passage of the entire suspension through the column during centrifugation at 2500 x g for 2 minutes at room temperature. Guanidine salts in buffer C4 also disrupt DNases.

9. The bound DNA was then washed twice in 10 mL of an ethanol-based washing solution (buffer C5) by passing through the column to remove excess salt as well as additional humic acids and other PCR inhibitors.
10. Finally, the DNA was eluted from the silica membrane by passage of 5 mL of buffer C6 (10 mM Tris) through the column at 2500 x g for 3 minutes.
11. The eluted DNA was then stored at -20 °C before use in qPCR reactions.

Preliminary studies showed that addition of powdered active charcoal (PAC) or $MgCl_2$ to the lysis buffer, respectively to bind PCR inhibitors and protect DNA from DNases, did not increase the performance of the above procedure, in terms of total fungal or bacterial DNA extracted. To evaluate the above procedure, it was compared with the use of the full commercial DNeasy PowerSoil kit method, including the use of micro bead beating, lysis and protein removal stages included with the kit but with the 10 g maximum recommended starting amount of soil. Two soils (13 and 14), sampled earlier from Project 7 *Narcissus* and onion trial sites (Table 5), were chosen for the comparison.

3.2.3. Effect of sampling on pathogen distribution and soil microbial diversity

An experiment was conducted to investigate the effect of soil sampling intensity on the qPCR detection of target pathogens and on the metabarcoding estimations of total fungal and bacterial richness and diversity. A field trial installed in 2019, as part of Project 7 of the SBSH Partnership (trial layout shown in Annex 1 with full description in the Project 7a report), was sampled in December 2018, prior to planting onion in a field in Bedfordshire (F.B. Parrish & Son Ltd, Lodge Farm, Chicksands, Shefford, Bedfordshire), in which a previous onion crop had been heavily affected in 2016 with basal rot (*Fusarium oxysporum* fsp. *cepae*). Two crops of winter wheat had been planted and harvested since the previous onion crop. The trial was divided into six blocks with four plots per block. Half of the plots had been seeded into the wheat stubble with a cover crop of (80% rye, 15% vetch, 5% phacelia) in the previous August 2018. In December 2018, 2 samples of 1 kg of soil were randomly collected per plot in a W pattern, each with multiple 2.5 cm diameter cores from a depth of 15 cm. Further samples from each plot were similarly collected before harvest of the onion crop in August 2019. For qPCR analysis, DNA was extracted from 10 g sub samples of thoroughly mixed soil using the DNeasy PowerMax Soil Kit (Qiagen, Netherlands) according to the manufacturer's instructions. qPCR was performed as described in 3.1.4. using *Fusarium oxysporum*, *Sclerotium cepivorum*, bacterial 16S rRNA and fungal 18SrRNA assays to quantify populations of specific pathogens, total bacteria and total fungi, before planting and before harvest of the onion trial. For metabarcoding analysis, DNA was extracted using the full DNeasy Powersoil kit (Qiagen, Netherlands) procedure on 10 g subsamples of thoroughly mixed soil from each sample.

3.2.4. Effect of soil pathogen dynamics on disease development

Potential relationships between fungal pathogen populations detected in soil prior to planting and subsequent crop disease development were investigated in 3 field trials established within Project 7 of the SBSH Partnership.

(a) *Fusarium oxysporum* on onion

In the onion field trial (as described in full in project report 91140002-7a), soil was sampled in December 2018 prior to planting onion (cv. Rumba) to determine pre-planting soilborne *F. oxysporum* DNA as described in 3.2.3. These data were compared with the incidence and distribution of onion basal rot monitored before harvest in August 2019.

(b) *Fusarium oxysporum* on *Narcissus*

In the *Narcissus* field trial (as described in full in project report 91140002-7b), pre-planting soilborne populations of *F. oxysporum* DNA detected by qPCR were compared with subsequent development of basal rot of *Narcissus* at harvest in 2020.

(c) *Verticillium dahliae* on raspberry

In the raspberry field trial (as described in full in project report 91140002-7c), soilborne populations of *V. dahliae* DNA detected by qPCR were compared with subsequent development of *Verticillium* wilt on raspberry during two seasons in 2018 and 2019.

3.3. Objective 3: Evaluate the effects of soil management practices on the soil microbiome and survival of specific soil-borne pathogens in relation to overall soil health.

3.3.1. Effect of soil management treatments on soil-borne pathogen populations

Field trials

Where the pathogens were quantifiable using the qPCR procedures described above, population fluctuations were measured in response to different agronomic practices. In the raspberry trial, the target pathogen (*Verticillium dahliae*) was not detected using the specific qPCR assay, therefore it was not possible to assess the effect of treatments. Further qPCR analysis was also undertaken to investigate pathogen dynamics in two other long-term field trials with asparagus (in partnership with Cranfield University) and with combinable crops (in partnership with NIAB).

(a) *Fusarium oxysporum* in onion

The onion trial (as described in full in project report 91140002-7a) compared four soil treatments:

1. Untreated control
2. Cover crop (80% rye, 15% vetch, 5% phacelia) sown in August 2018 and incorporated before planting.
3. Green compost (30 t/ha) incorporated before planting in March 2019.

4. Cover crop + green compost

The soil was sampled before planting in December 2018 and again just before onion harvest in August 2019, 4 months after planting. DNA was extracted as described in 3.2.2. and *F. oxysporum* populations were quantified using the generic qPCR assay as described in 3.1.2.

(b) *Fusarium oxysporum* in *Narcissus*

The *Narcissus* trial (as described in full in project report 91140002-7b) compared four soil treatments:

1. Untreated control
2. Green compost (30 t/ha) incorporated during cultivation in August 2018.
3. Pig manure (35 t/ha) incorporated during cultivation in August 2018.
4. Inoculation of bulbs with Mycorrhizal fungi (PlantWorks Ltd., Sittingbourne, Kent, UK) at planting (1g per bulb) in August 2018.

The soil was sampled in the first and second year of the crop in August 2019 and again in June 2020. DNA was extracted as described in 3.2.2. Populations of *F. oxysporum* and mycorrhizal fungi were quantified using the generic qPCR assays as described in 3.1.2.

(c) *Fusarium oxysporum* and *Stemphylium vesicarium* in asparagus

The asparagus trial conducted by Cranfield University is described in full by Mašková *et al*. (2021). The trial was a split plot design with half of the plots being re-ridged (typical practice) at the start of each season and half without re-ridging (zero tillage) with six management treatments:

1. Bare soil
2. Bare soil with shallow soil surface disturbance.
3. Mustard (*Sinapis alba* L. var. Severka) companion crop (broadcast in late August and removed the following March).
4. Rye (*Secale cereale* L var. Protector) companion crop (broadcast in late August and removed the following March).
5. Mulch of PAS100 green compost with shallow soil surface disturbance.
6. Mulch of straw with shallow soil surface disturbance.

Asparagus (var. Gijnlim) was planted in April 2016. Re-ridging was performed in March/April each year and shallow soil disturbance was done spring each year by winged tine to 0.25-0.3 m depth. Companion crops were sown in August each year. For pathogen testing in March 2019, soil from inter-row treatments 2, 3 and 4 was sampled from 6 plots per treatment (3 with re-ridging and 3 without). In July 2020, soil was sampled from 6 plots (3 with re-ridging and 3 without) of each of the 6 inter-row treatments. DNA was extracted as described in 3.2.2. and *Fusarium oxysporum* and *Stemphylium vesicarium* (syn. *Pleospora herbarum*) populations were quantified using the qPCR assays as described in 3.1.2.

(d) *Gaeumannomyces tritici* in winter wheat

Soil was sampled from the long-term STAR (Sustainability Trial in Arable Rotations) trial on a heavy clay loam soil. This trial assesses the effects of rotation and cultivation treatments on soil health, agronomy and production (a full description is given by Morris, 2016). Four different methods of cultivation and four different types of rotation had been used for the previous 10 seasons. This forms a fully factorial design with16 treatments. Rotation treatments were:

1. Winter wheat cropping with a winter sown break crop
2. Winter wheat cropping with a spring sown break crop,
3. Continuous wheat every year
4. Alternate fallow/cover crop with wheat every other year.

Cultivation treatments were:

a. Annual ploughing
b. Shallow (c.10cm) non-inversion tillage
c. Deep (c. 20cm) non-inversion tillage
d. Managed system decided annually after field assessment.

As part of a MSc degree (Briggs, 2018), samples were collected in April 2018 from 3 replicated plots of each of the 16 rotation/tillage combinations. DNA was extracted as described in 3.2.2. and *G. tritici* populations were quantified using the relevant qPCR assay as described in 3.1.2.

3.4. Objective 4: Design and demonstrate the benefits of an appropriate soil health testing service for growers and agronomists.

A review of the Australian industry award-winning PREDICTA® DNA-based soil testing service for plant pathogens was undertaken by consulting relevant published scientific literature and factsheets, information on the service website (https://pir.sa.gov.au/research/services/molecular_diagnostics) and direct consultations with key scientists involved in the development and running of the service (Drs. Alan McKay, Daniele Giblot-Ducray, Michael Rettke and Kathy Ophel-Keller). The findings were then used to ensure that validated methodology was in place to allow compilation of a similar testing service adapted for UK pathogens and soils and to evaluate what further development would be needed before such a service could be fully implemented. Additional consideration was given to the possibility of widening such a service to also include a more general analysis of soil microbiology and its diversity, using DNA metabarcoding and bioinformatic analyses for general assessment of soil health.

3.5. Objective 5: Integrate results of molecular testing for soil-borne disease risk with associated chemical and physical data in support of a robust soil health scorecard developed within the SBSH Partnership.

3.5.1. Estimation of total bacterial and fungal DNA by quantification of 16S and 18S rRNA genes

Control data generated with 16S and 18S rRNA qPCR assays (see 3.1.1 and 3.1.2.) was used to estimate total bacterial and fungal populations in soils sampled from the long-term field trials studied in Project 4 of the SBSH Partnership. This data was used to compare general effects of the various soil management treatments used in each trial on the overall size of bacterial and fungal populations at the time of sampling.

3.5.2. Correlations between pathogen detection and other scorecard data

Where individual populations of *Fusarium oxysporum* and *Verticillium dahliae* were monitored using specific qPCR assays, estimated pathogen levels were compared to other field data collected during application of the scorecard tests, standardised in Project 4, at the time of sampling. Pathogen levels were compared with measurements of soil pH, % soil organic matter, and potentially mineralisable nitrogen (PMN).

4. Results

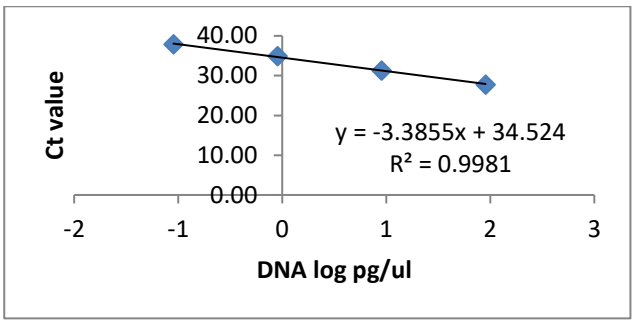
4.1. Objective 1: Validation of a suitable toolkit of qPCR assays for quantitative detection of key intractable soil-borne pathogens in arable and horticultural rotations.

4.1.1. Assay efficiencies and analytical sensitivities

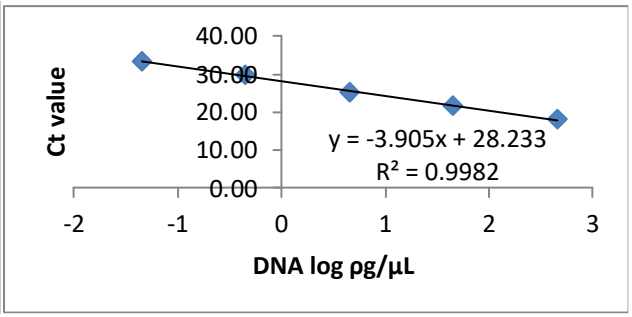
Assay sensitivities and efficiencies were calculated from the results of the qPCR analysis performed on 10-fold dilutions of purified reference DNA from each target organism. Linearity (R^2), theoretical limits of detection (LOD), and reaction efficiency (E) are shown for each assay in Table 7. Standard curves showing the linear relationship between Log_{10} DNA concentration and critical threshold (Ct) value are shown in Figure 1. Standard curves varied in linearity between assays with R^2 ranging from 0.92-1.0. The limit of detection also varied between assays, most were highly sensitive able to detect target DNA at the highest dilution tested (10^{-5}) and with low estimated LOD values. Sensitivity was lower and LOD higher for the *Rhizoctonia cerealis*, *Plasmodiophora brassicae*, *Sclerotium cepivorum* and *Colletotrichum coccodes* assays. Amplification efficiency, representing the amount of PCR product increase after each cycle, varied between assays, with a range of 79-157%. Ten of the fourteen pathogen assays were within the usually accepted range of efficiency (90-110%).

Table 7: Linearity (R^2), efficiency and LOD of each assay determined from standard curves.

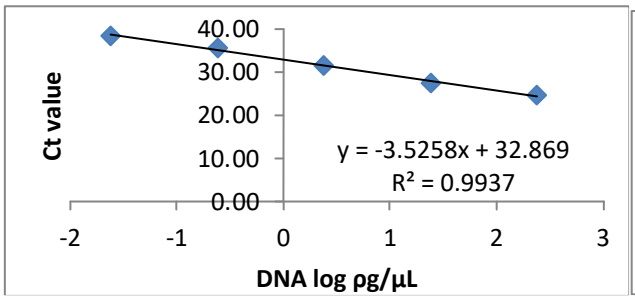
Pathogen	R^2	LOD (pg DNA/µl)	Efficiency
Colletotrichum coccodes	0.9981	0.09	97%
Fusarium culmorum	0.9982	0.045	80%
Fusarium oxysporum	0.9937	0.024	92%
Phytophthora rubi	0.9973	0.016	88%
Plasmodiophora brassicae	0.944	1.78	79%
Pythium violae	1	0.03	101%
Rhizoctonia cerealis	0.9854	0.14	92%
Rhizoctonia solani AG2-1	0.9972	0.015	98%
Sclerotinia sclerotiorum	0.9959	0.018	106%
Sclerotium cepivorum	0.9991	0.52	97%
Spongospora subterranea	0.9798	0.018	157%
Verticillium albo-artrum	0.9953	0.036	94%
Verticillium dahliae	0.993	0.019	100%
Verticillium longisporum	0.9961	0.339	97%



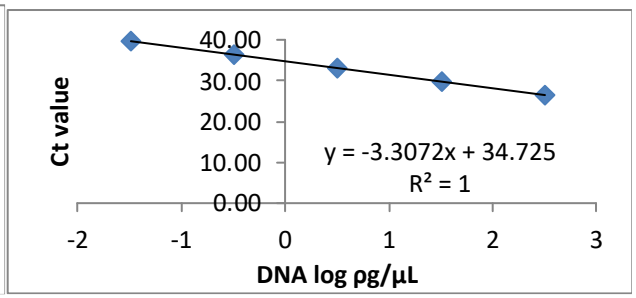
(a) *Colletotrichum coccodes*



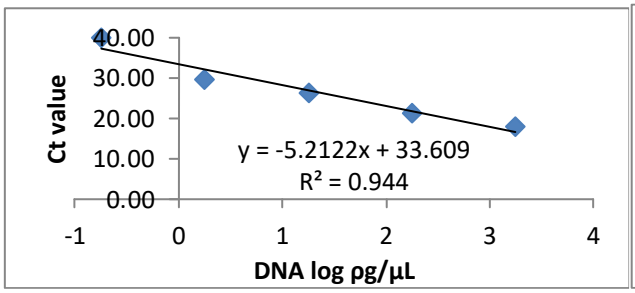
(b) *Fusarium culmorum*



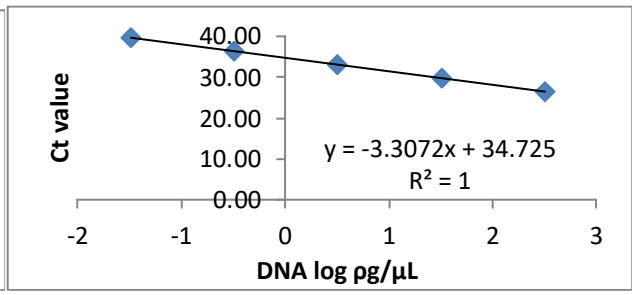
(c) *Fusarium oxysporum*



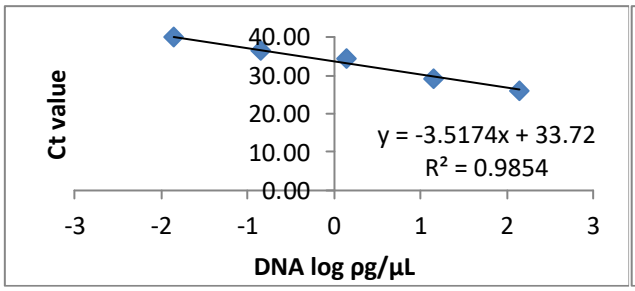
(d) *Phytophthora rubi*



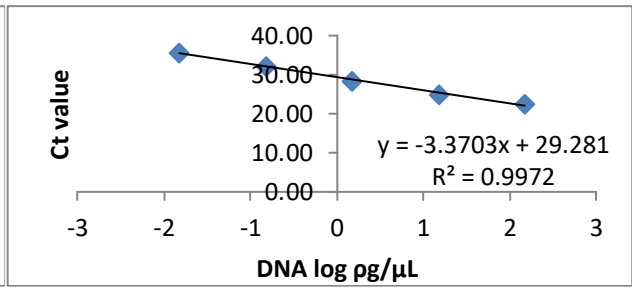
(e) *Plasmodiophora brassicae*



(f) *Pythium violae*

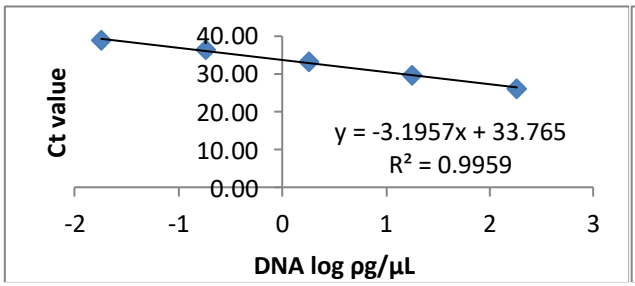


(g) *Rhizoctonia cerealis*

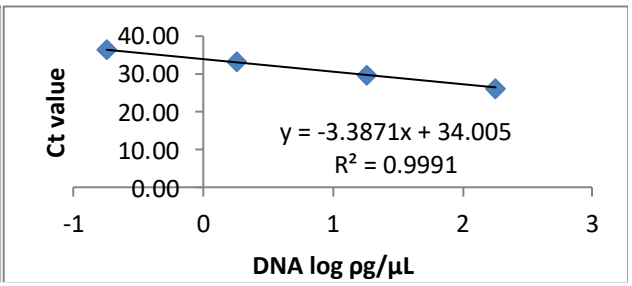


(h) *Rhizoctonia solani* AG2-1

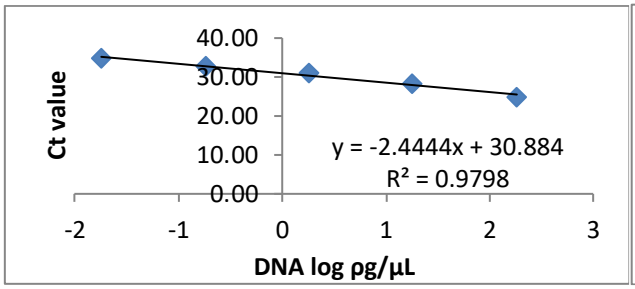
Figure 1: Standard curves for qPCR assays on purified target DNA with Log10 DNA concentration (ρg/µL) plotted against Critical qPCR threshold (Ct)



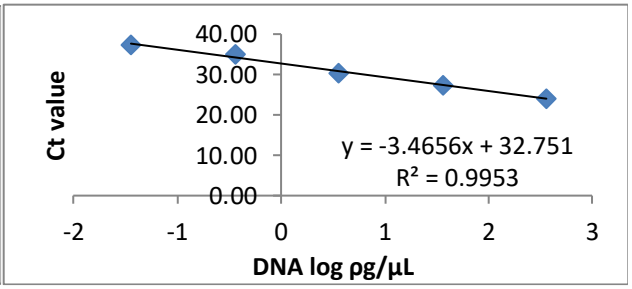
(i) *Sclerotinia sclerotiorum*



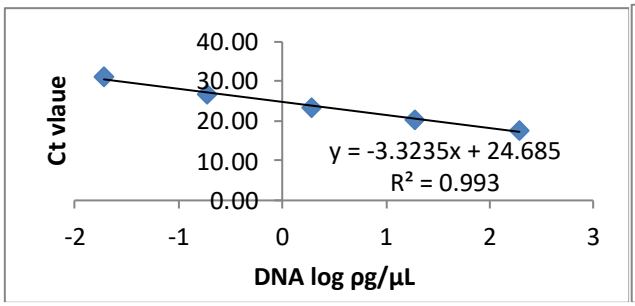
(j) *Sclerotium cepivorum*



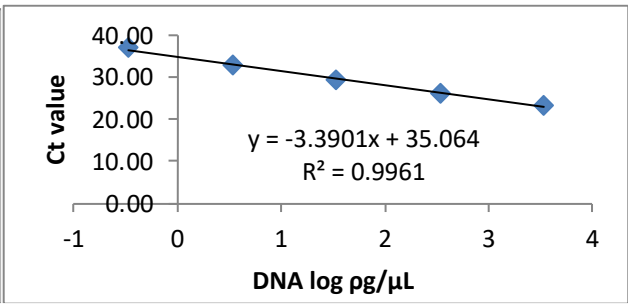
(k) *Spongospora subterranean*



(l) *Verticillium albo-atrum*



(m) *Verticillium dahliae*



(n) *Verticillium longisporum*

Figure 1 continued: Standard curves for qPCR assays on purified target DNA with Log10 DNA concentration (ρg/µL) plotted against Critical qPCR threshold (Ct)

4.1.2. Assay performances on spiked soils

Amplification of 16S and 18S rRNA targets for quantification of total bacterial and fungal DNA

Successful qPCR amplification of 16S and 18S rRNA control sequences was achieved from all soils with the exception of soil 21 where no qPCR amplification products were detected (Figure 2). A Ct value of 40 indicates no detection of the target DNA and Ct values then decrease as the concentration of target DNA increases in the extract.

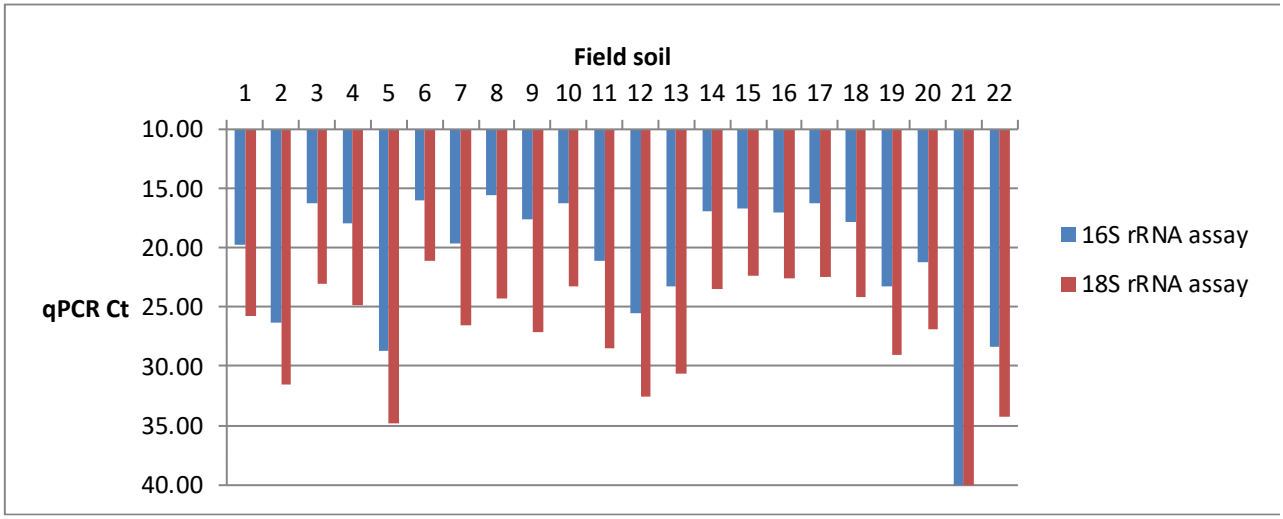
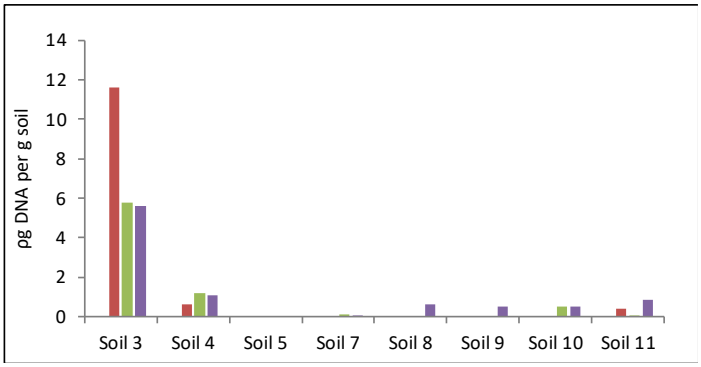


Figure 2: Amplification of fungal (18S) and bacterial (16S) rRNA in DNA extracted from 250 g per sample of 22 soils (mean of 3 extractions per soil).

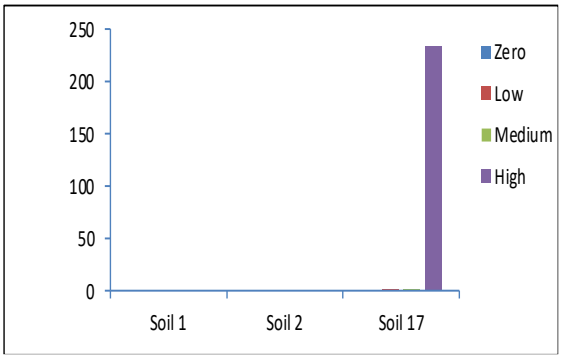
Variation in Ct values was observed from soil to soil with values for both assays following similar patterns. It is likely that this variation was due to differences in the total amount of DNA extracted per sample, rather than reflecting the natural populations of bacteria and fungi inhabiting each soil, which would be expected to vary independently. In each case, the 16S rRNA bacterial target was more abundant than the 18S rRNA fungal target.

Amplification of specific qPCR targets from soils spiked with varying amounts of relevant soilborne fungal pathogens.

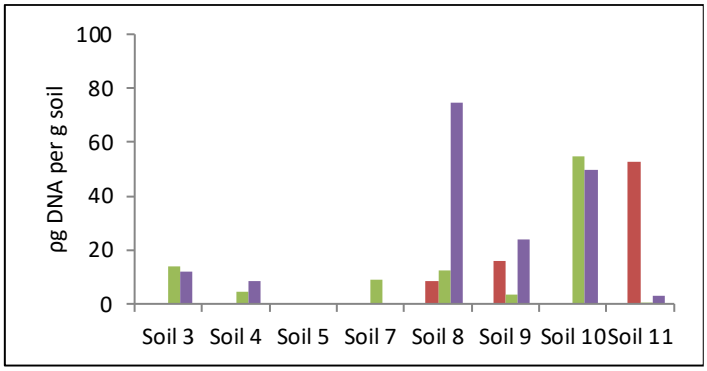
The reliability of qPCR for both detection and quantification of individual fungal plant pathogens from inoculated soils varied between assays and soils (Figures 3 and 4). Observed differences in quantification of target DNA between assays may have resulted from differences in the initial amounts of DNA added with the different fungal inocula used, which was difficult to standardise at the point of inoculation. In particular, the concentrations of detected DNA of *F. culmorum*, *P. rubi*, *R. cerealis* and *V. dahliae* appeared low, even at the highest inoculum levels. It was also clear that detection and quantification of pathogen DNA was successful in some soils but not others, despite the same initial inoculum concentrations being used to spike each soil. Amplification of target pathogen DNA consistently failed, independently of the assay used, from soils from the raspberry trial in Norfolk (2), farmyard manure-amended soil from Gleadthorpe (5), from a clubroot trial at Craibstone (12) and from potato crops in Suffolk (20-22). Interestingly, the lowest levels of fungal 18S rRNA targets were also detected in these soils.



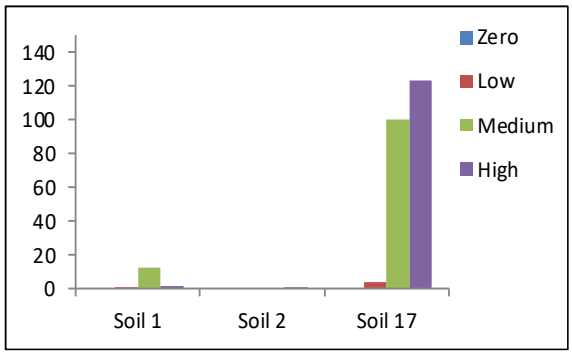
(a) *Fusarium culmorum*



b) *Phytophthora rubi*



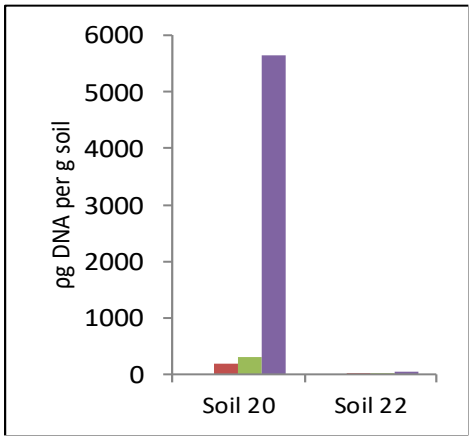
(c) *Rhizoctonia cerealis*



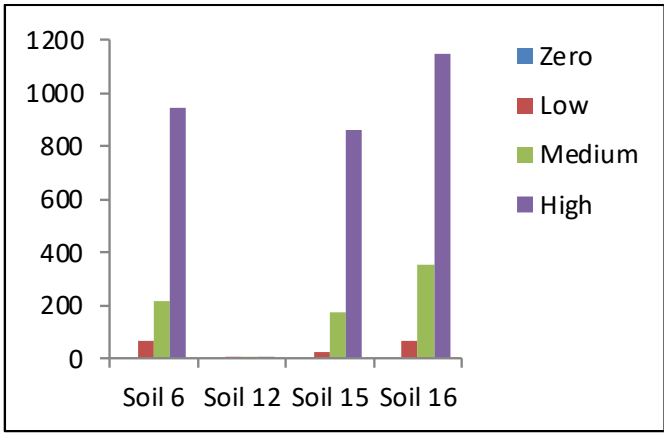
(d) *Verticillium dahliae*

Figure 3: Detection and quantification of key soil-borne pathogens in different soils using specific qPCR assays. Low inoculum levels aimed to be roughly at the theoretical limit of detection for each pathogen. In these four assays, concentrations of detected DNA were very low even at high inoculum levels. High levels aimed to exceed the theoretical maximum levels of detection. Medium levels were set midway between low and high levels.

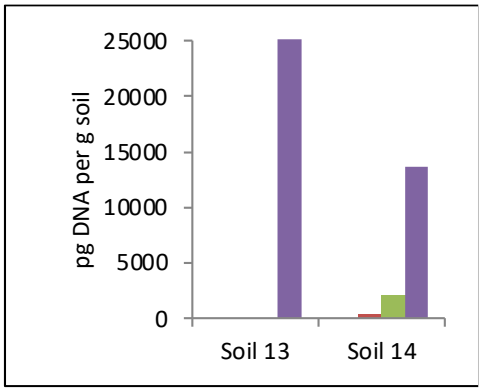
From most of the other soils it was possible to detect at least the highest pathogen inoculum doses (Figure 4). However, quantification of pathogen DNA varied greatly with the source of soil. With the exception *F. culmorum* assay,which failed to detect the reference isolate used, it was possible to quantify low, medium and high inoculum levels with all qPCR assays, but only in some of the soils, which also corresponded with those from which high levels of fungal 18S rRNA target were recorded (Figure 4).



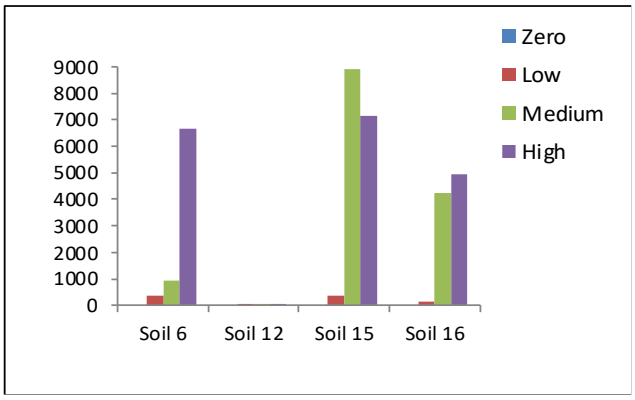
(a) *Colletotrichum coccodes*



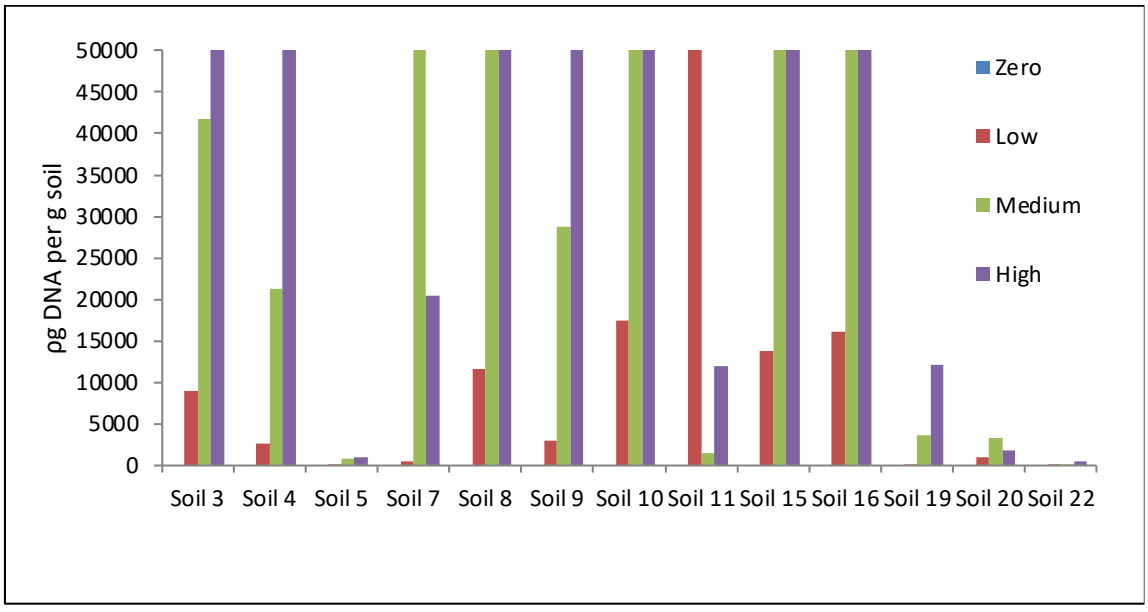
(b) *Rhizoctonia solani* AG-1



(c) *Fusarium oxysporum*

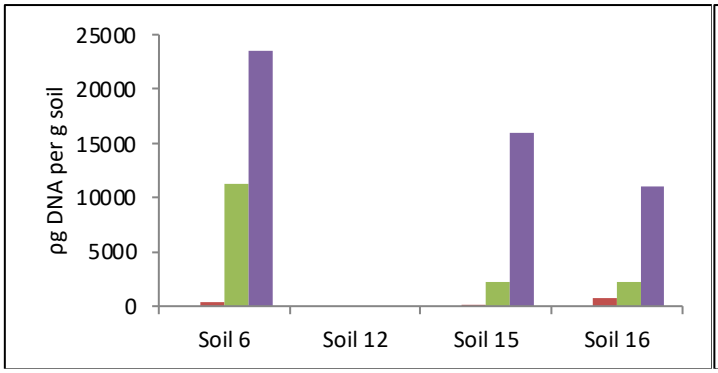


(d) *Plasmodiophora brassicae*

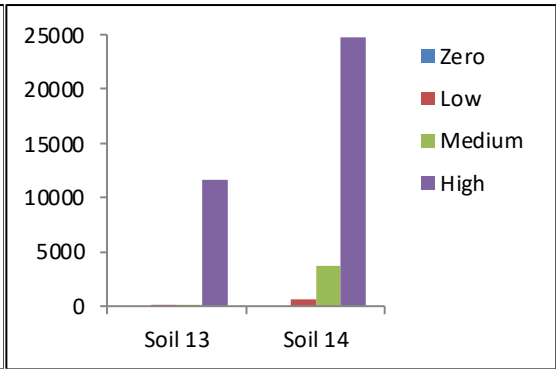


(e) *Pythium violae*

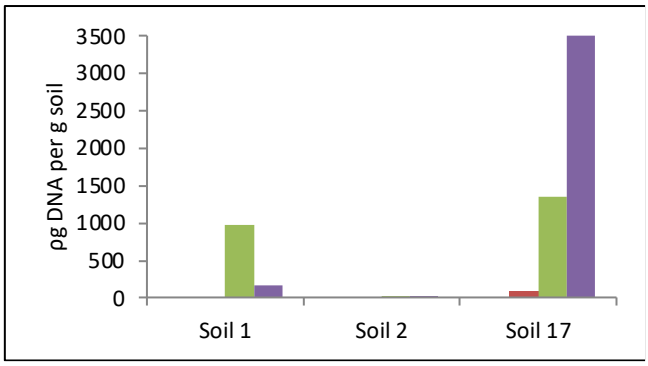
Figure 4: Detection and quantification of key soil-borne pathogens in different soils using specific qPCR assays. Low inoculum levels aimed to be roughly at the theoretical limit of detection for each pathogen. High levels aimed to exceed the theoretical maximum levels of detection. Medium levels were set midway between low and high levels.



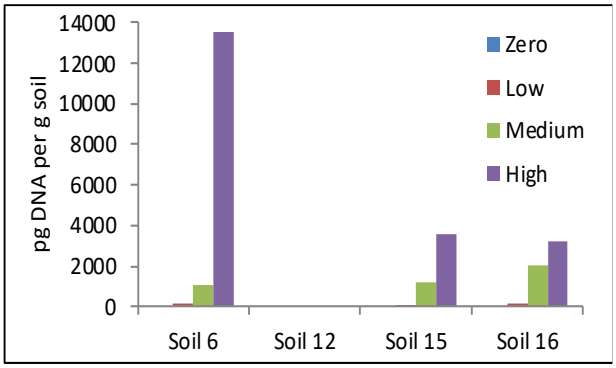
(f) *Sclerotinia sclerotiorum*



(g) *Sclerotium cepivorum*



(h) *Verticillium albo-atrum*



(i) *Verticillium longisporum*

Figure 4 continued: Detection and quantification of key soil-borne pathogens in different soils using specific qPCR assays. Low inoculum levels aimed to be roughly at the theoretical limit of detection for each pathogen. High levels aimed to exceed the theoretical maximum levels of detection. Medium levels were set midway between low and high levels.

4.2. Objective 2: Establish relationships between pathogen distribution and concentration in soils and potential for disease development.

4.2.1. Systematic review of methods for extraction of DNA from soils

The systematic review highlighted changes in procedures, optimised over time by the scientific community, that are most likely to accurately quantify the DNA of target organisms in soil. Key elements found essential for method standardisation were identified as follows:

(a) *Sampling:* Despite most studies using soil sample sizes of < 1g, it is generally recommended to use large samples of up to 250g, especially for targeted detection of individual pathogen species that may be widely dispersed in field soils (Herdina and Roget, 2000; Ellingsøe and Johnsen, 2002; Ranjard *et al*., 2003; Budge *et al*., 2009; Taberlet *et al*., 2012 and Woodhall *et al*., 2012).

(b) *Sample storage:* DNA should be extracted as soon as possible after soil collection. Refrigeration of samples between field and laboratory is recommended. If further storage is required, then freezing (-20 or -80 °C) preserves total DNA (Lee *et al*., 2007; Wallenius *et al*., 2010; Peoples and Koide, 2012; Martí *et al*., 2012) but reference samples should be stored to quantify effects of long-term storage (Frostegård *et al*., 1999).

(c) *Sample homogenisation:* Mechanical sample homogenisation methods have changed over time from grinding and shaking or freezing/thawing to current practice that now almost exclusively uses bead beating. Bead-beating involves mechanically shaking in vessels with quartz or metal beads. Depending on bead size, shaking speed and duration, recalcitrant cellular material (including fungal resting spores and Gram-positive bacteria) can be disrupted and individual cells or their contents released into a buffered suspension. Bead-beating should be optimised to maximise yield of target DNA with minimal co-extraction of PCR inhibitors and shearing of target DNA molecules (van Elsas *et al*., 1997; Kuske *et al*., 1998; Miller *et al*., 1999; Bollmann-Giolai *et al*., 2020; Guerra *et al*., 2020).

(d) *Cell lysis:* To increase yields of DNA released from target organisms, a wide range of lysis buffers have been used to increase cell disruption. Lysis buffers usually contain chelating agents, detergents, salts and sometimes enzymes. At least 2 or 3 of these components were commonly used in reported procedures. The most used chelating agents (EDTA and/or Tris) sequester metal ions that are required for activity of DNases that degrade DNA. The most commonly used detergents (SDS or CTAB) dissolve lipids in cell membranes, releasing the DNA. CTAB also binds polysaccharides, removing them from solution. By buffering pH, added salts (usually phosphate buffers or phosphate buffered saline) neutralise DNA molecules making them hydrophobic and preventing binding to proteins and other cellular material. The most commonly added enzymes are lysozymes that assist with cell lysis or proteinase-K that degrades DNases. Guanadinium isothiocyanate is also often used to lyse cells and also denatures DNases.

(e) *DNA purification:* Numerous approaches have been used to purify the extracted soil DNA to ensure that it is sufficiently free from inhibitory compounds, such as proteins, phenolic compounds, humic and fulvic acids, and heavy metals, to allow reliable PCR amplification. Whilst early studies described many *ad-hoc* laboratory protocols, most recent studies have now adopted the use of commercial kits, which although more expensive, are convenient and standardised across studies. The two most-used commercial purification kits are currently Fast DNA™ spin kit for soil (MP Biomedicals LLC, California, USA) and the DNeasy PowerSoil Kit (Qiagen NV, Netherlands) formerly sold by Mo Bio Inc., California, USA as PowerSoil DNA Isolation Kit). Both kits, use bead beating to homogenise samples in proprietary lysis buffers and free DNA is electrostatically bound onto a silica membrane in a spin column format. Bound DNA is then washed by spinning proprietary solvents through the columns and purified DNA is eluted from the membrane. Washing buffers often contain soluble PVP (polyvinylpyrrolidone) or insoluble PVPP (polyvinylpolypyrrolidone) polymers that bind phenolic PCR inhibitors. As an alternative to spin columns, Budge *et al*. (2009) and Woodhall *et al*. (2012) incorporated an automated magnetic capture system (into the DNA purification stage, whereby the DNA is bound to silica-coated magnetic particles (Wizard® Magnetic DNA Purification System for Food; Promega, UK) that can be moved through a series of solvent washes using a magnet using a robotic KingFisher system (ThemoFisher Scientific, UK).

4.2.2. Comparison of DNA extraction protocols

No significant effects of extraction method on qPCR results (CT) were observed (Figures 5 and 6). Higher variation in CT values for both 16S and 18s rRNA assays were observed using the full PowerMax Soil kit protocol with 10 g subsamples of soil (method 1) compared with the adapted method with 50 g subsamples (method 2). The coefficient of variation was much larger for the 18s rRNA (fungi) assay compared with the 16S rRNA assay. Since detection of target pathogens in low abundance is critical, the method extracting from the larger amount of soil was selected for all further analysis.

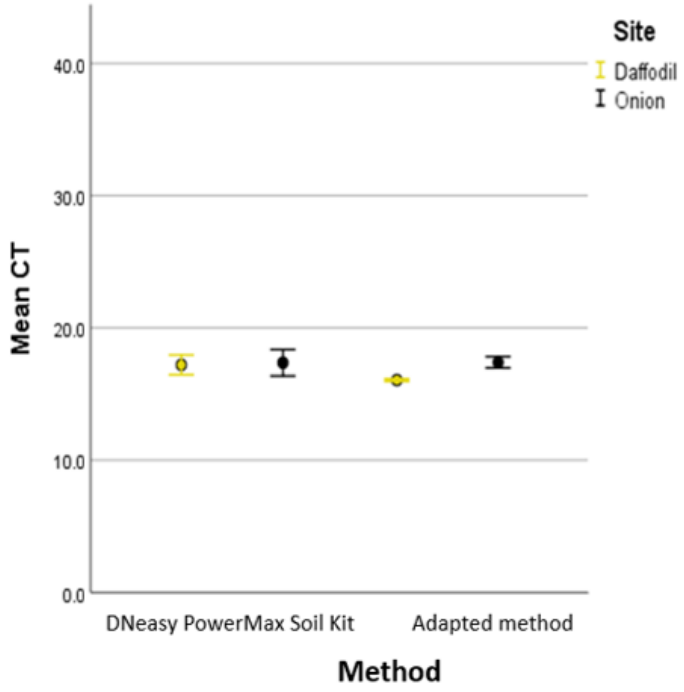


Figure 5: CT values obtained with bacterial 16S rRNA positive control assay. A 3-fold increase in CT denotes an approximately 10-fold decrease in DNA target concentration. Error bars= +/- 2 SD

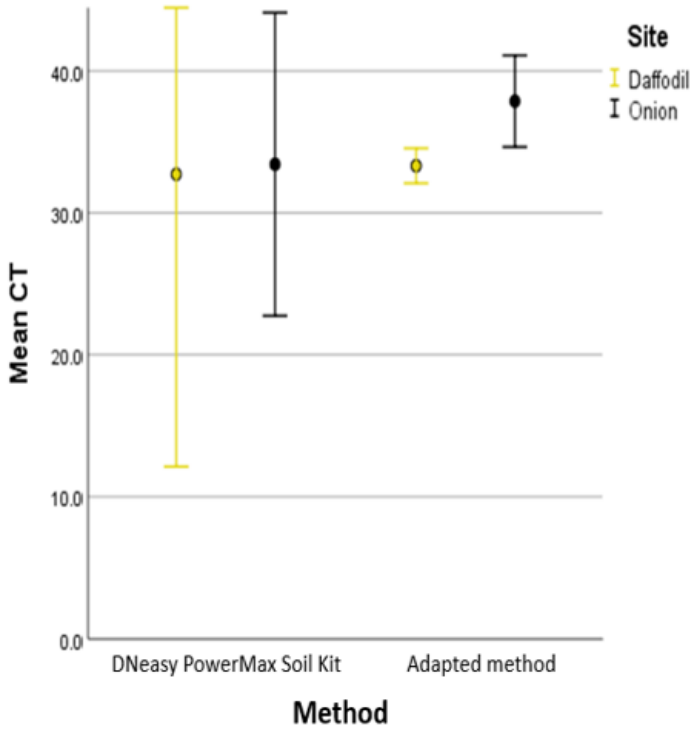


Figure 6: CT values obtained with fungal 18S rRNA positive control assay. A 3-fold increase in CT denotes an approximately 10-fold decrease in DNA target concentration. Error bars= +/- 2 SD

4.2.3. Effect of sampling on pathogen distribution and soil microbial diversity

At the onion trial site, *F. oxysporum* was detected in all samples collected either before planting or before harvest (Figure 7). The amount of target pathogen DNA detected in the soil increased between 2- and 9-fold during the onion cropping season and *F. oxysporum* was consistently detected from all plots on both sampling occasions. Similarly, 18S rRNA assays were successfully used to quantify total fungi in all samples (Figure 8).

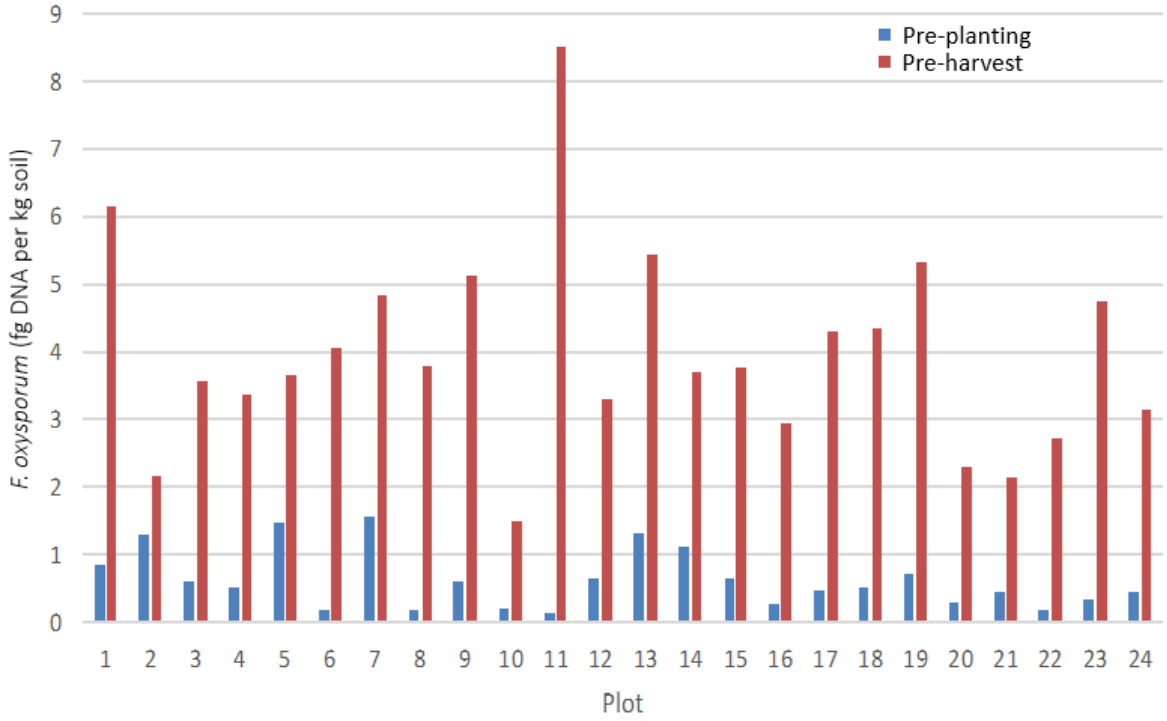


Figure 7: Detection of *F. oxysporum* DNA in multiple soil samples collected across the onion trial in Bedfordshire before planting in December 2018 and before harvest in August 2019.

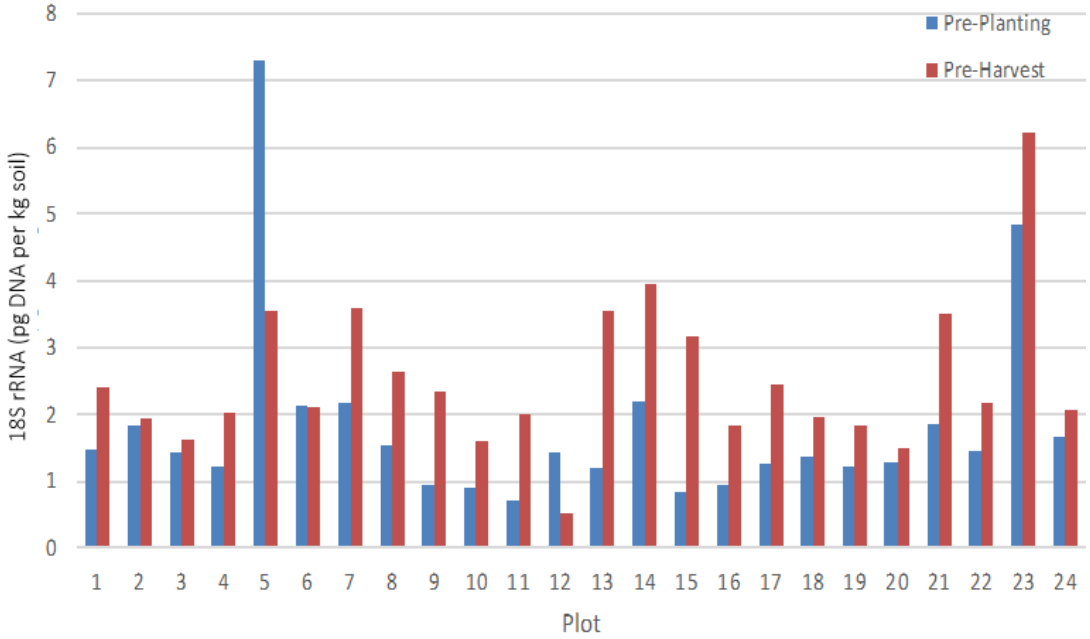


Figure 8: Quantification of total fungal DNA in multiple soil samples collected across the onion trial in Bedfordshire before planting in December 2018 and before harvest in August 2019.

Comparison of the relative abundances of amplicon sequence variants (ASVs) identified by metabarcoding of bacterial 16S rRNA and ITS amplicon barcodes is shown in Figure 9. These show variation in bacterial and fungal diversity across the site but no clear patterns with blocks or treatments. Within the different taxa identified at species level, no significant differences were found between duplicate soil samples from the 24 plots.

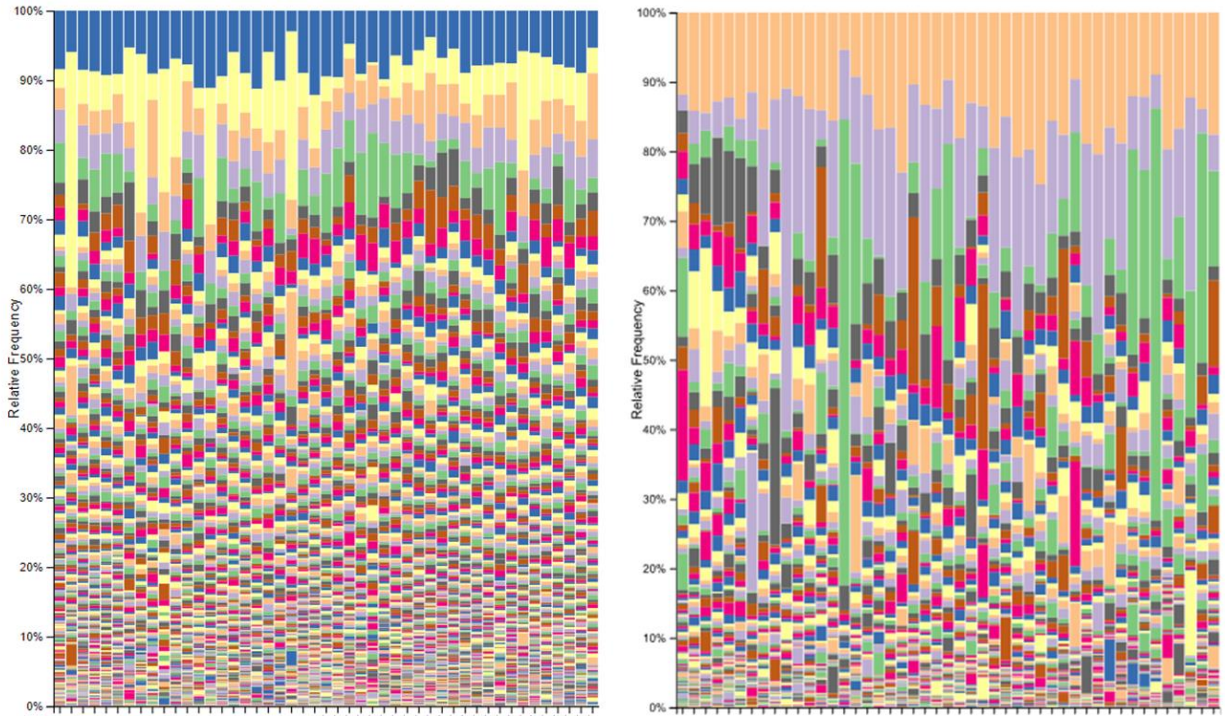


Figure 9: Relative abundance of different (a) 16S rRNA and (b) ITS amplicon sequence variants (ASVs) representing taxa, of soil bacteria and fungi respectively, at species level in replicated soil samples (1-48) sampled from the onion trial in Bedfordshire.

4.2.4. Effect of soil pathogen dynamics on disease development

(a) *Fusarium oxysporum* on onion

F. oxysporum was detected in all plots of the trial in December 2018 prior to planting onion (Figure 7), despite the fact that two seasons of winter wheat had been grown since the last infected onion crop. A subsequent increase of *F. oxysporum* DNA was then detected in all plots prior to harvest of the onion trial, corresponding with widespread disease development in the trial (Figure 7). *Fusarium* basal rot of onion developed extensively in all plots, regardless of initial pathogen population or soil treatments applied. No correlation was observed between *F. oxysporum* DNA levels detected before sowing and the disease incidence recorded at harvest (R^2 = 0.0066; Figure 10). It was therefore not possible to relate the initial DNA levels detected to the risk of disease due to the high disease incidence occurring in all plots. Unfortunately, a qPCR assay for specific detection of *F. oxysporum*

fsp. *cepae* did not become available within the life of the project and it was not therefore possible to measure the exact population dynamics of the onion pathogen, which may have been over estimated due to presence of other *F. oxysporum* variants.

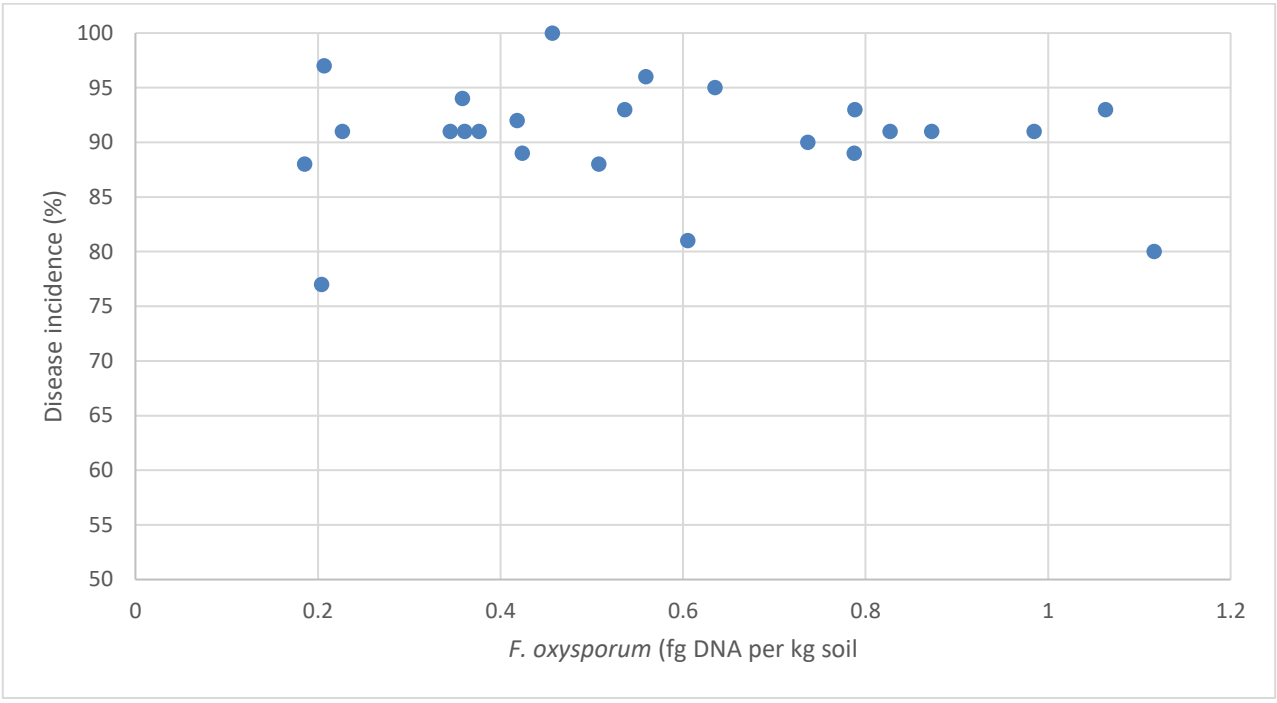


Figure 10: Relationship between detection of *F. oxysporum* DNA prior to planting and basal rot disease incidence at onion harvest.

(b) *Fusarium oxysporum* on *Narcissus*

A low average level of *F. oxysporum* was detected in soil sampled in the previous crop at the trial site in 2017, prior to planting *Narcissus* bulbs. *F. oxysporum* DNA was again detected in most plots in the growing crop prior to harvest of the trial (Figure 11) but differences in the amounts of DNA detected in August 2019 could not be correlated with the amount of basal rot recorded after harvest in June 2020 (R^2 = 0.52; Figure 11). Unfortunately, a qPCR assay for specific detection of *F. oxysporum* fsp. *narcissi* did not become available within the life of the project, it was not therefore possible to measure the exact population dynamics of the *Narcissus* pathogen, which may have been over estimated due to presence of other *F. oxysporum* variants.

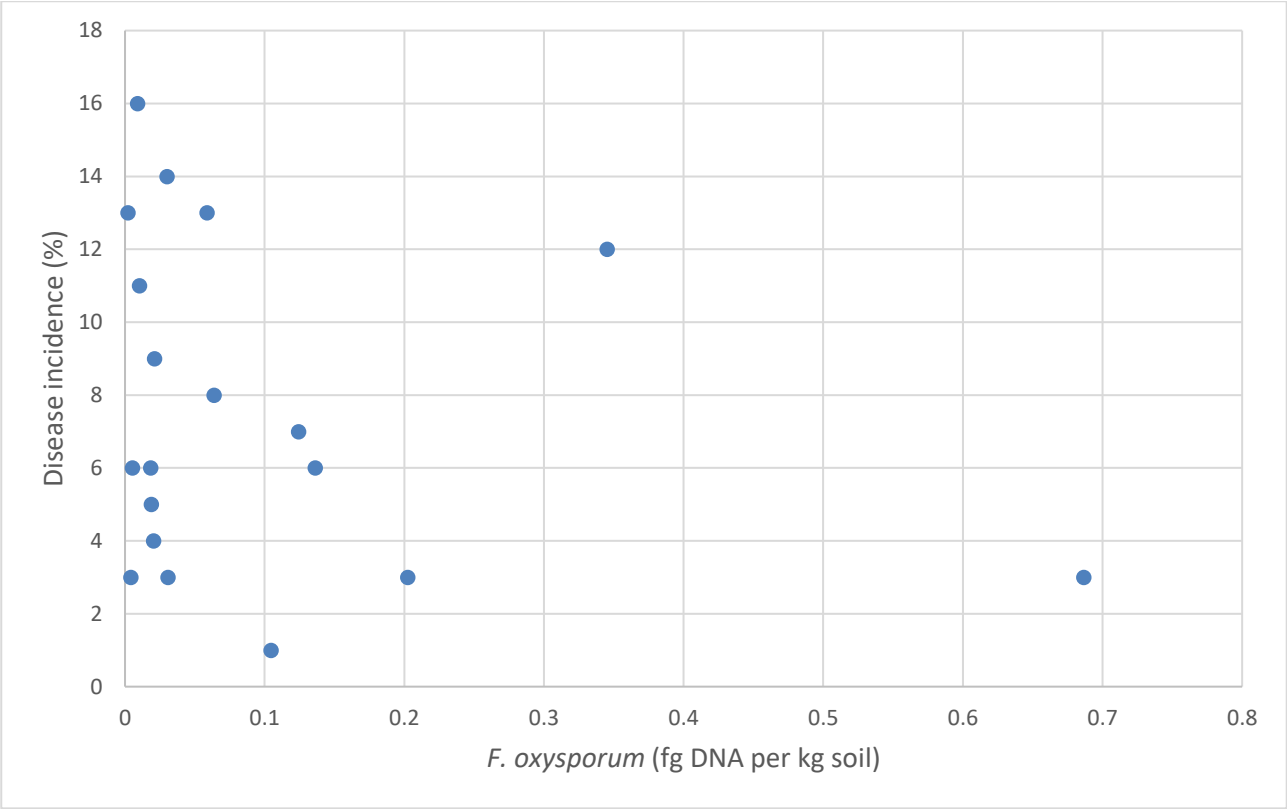


Figure 11: Relationship between detection of *F. oxysporum* DNA prior to planting and basal rot disease incidence at *Narcissus* harvest in year 2.

(c) *Verticillium dahliae* on raspberry

Traces of *Verticillium dahliae* were detected by qPCR prior to installation of the raspberry trial in Norfolk. Low concentrations of pathogen DNA were also detected from the soil sampled subsequently during the 2019 and 2020 seasons with lower concentration in 2020 than in 2019. Some limited *Verticillium* wilt incidence was recorded in 2019 but was very low and no wilt was recorded in 2020. Estimated pathogen populations were consistently too low in both seasons to relate pathogen populations to disease development. Results of an alternative method for assessment of *V. dahliae* microsclerotia, by sieving and plating on agar plates (Harris *et al*., 1993), identified the pathogen in soil samples from the trial at each sampling occasion.

4.3. Objective 3: Effects of soil management practices on the soil microbiome and survival of specific soil-borne pathogens

4.3.1. Effect of soil management treatments on soil-borne pathogen populations

(a) *Fusarium oxysporum* in onion trial

No significant effect of cover crop or green compost either alone or in combination was observed on the level of *F. oxysporum* DNA detected in treated compared with untreated soil prior to harvest of the onion crop (Figure 12).

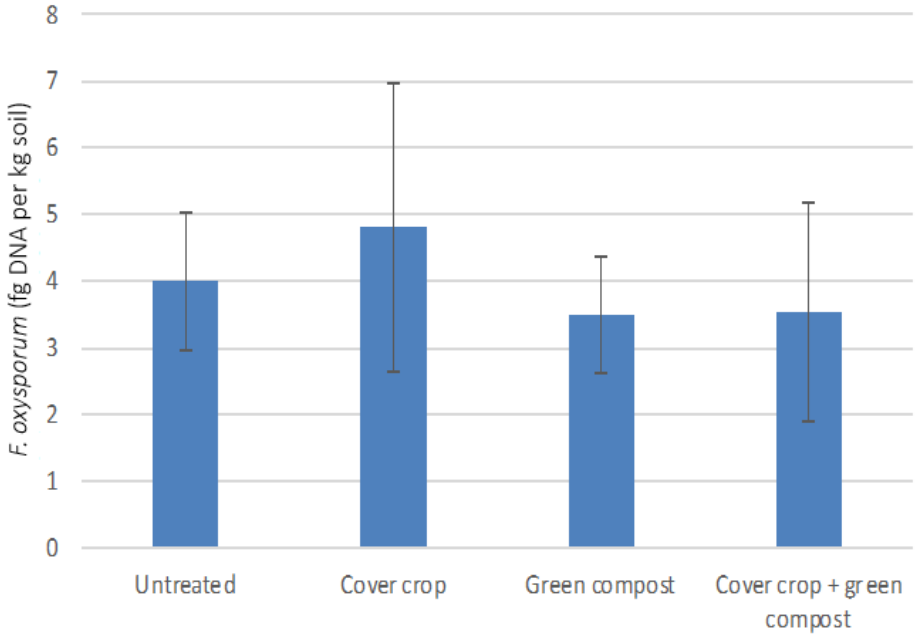


Figure 12: Effect of soil treatments on detection of *F. oxysporum* DNA (mean from 6 plots with standard deviations) prior to onion harvest.

(b) *Fusarium oxysporum* in *Narcissus* trial

No significant effects of pig manure, green compost or mycorrhizal fungi treatments on the level of *F. oxysporum* DNA detected were observed compared with untreated soil in either year (Figure 13). However, *F. oxysporum* was not detected in the second season in plots when bulbs had been treated with mycorrhizal fungi at planting.

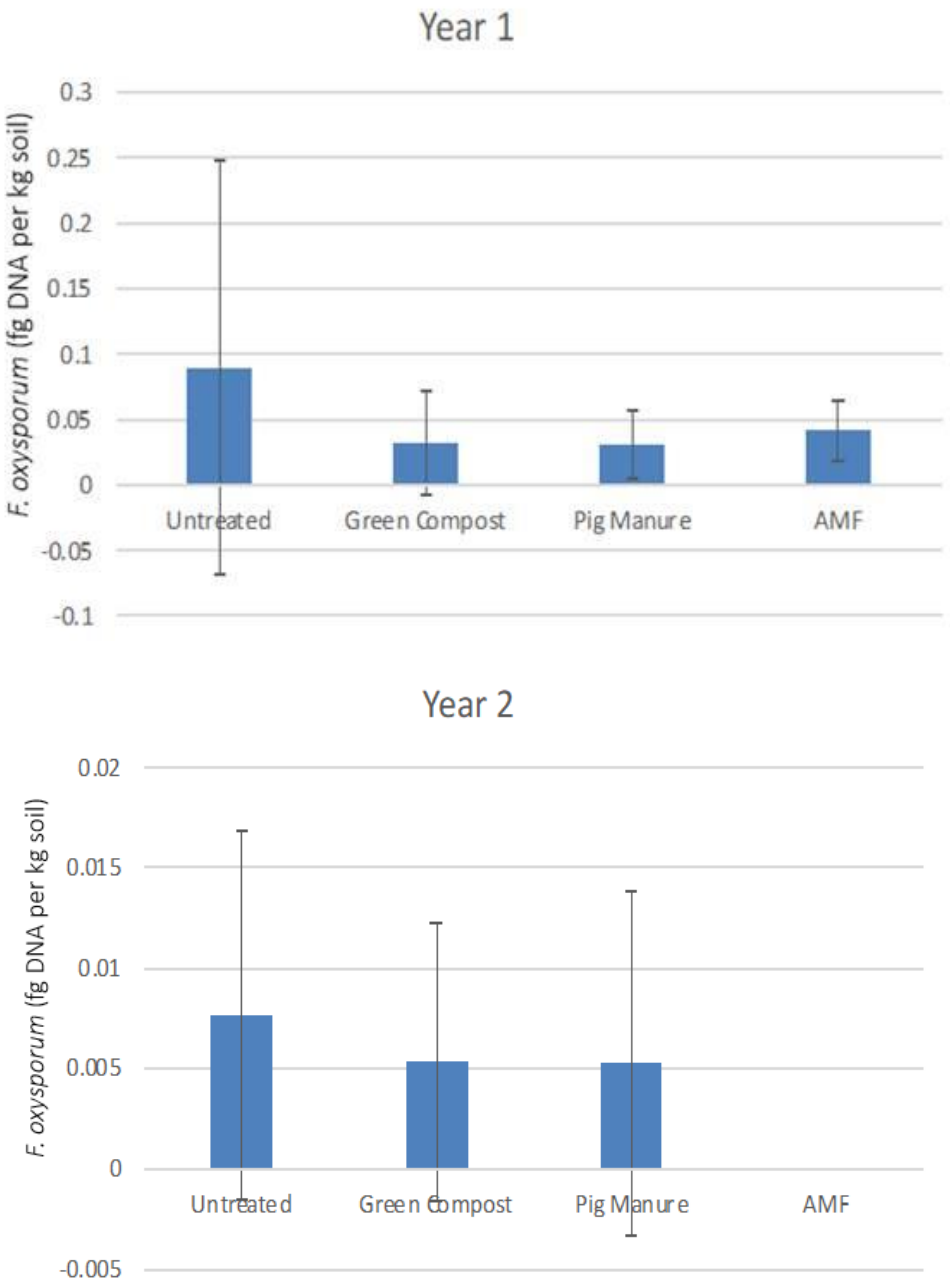


Figure 13: Effect of soil treatments on detection of *F. oxysporum* DNA (mean from 6 plots with standard deviations) in two cropping seasons.

(c) *Fusarium oxysporum* and *Stemphylium vesicarium* in asparagus trial

No significant effects of annual re-ridging of the asparagus beds on either *Fusarium* or *Stemphylium* pathogen populations in the soil were detected in either year (Figures 14 and 15). Furthermore, there were no significant differences in populations of either pathogen when soil from plots with mustard or rye companion crops was compared with soil from plots without companion crops, provided the bare soil plots had undergone shallow soil disturbance to mimic cultivations to remove the companion crops. However, when bare soil plot without shallow soil disturbance (minimum till plots)

were also sampled in 2020, *F. oxysporum* populations were significantly higher in these plots than in both the mustard and rye companion crop plots. Similarly, *S. vesicarium* populations were also higher in the minimum till plots than in the mustard plots. *Fusarium* populations were particularly variable in plots treated with the two mulches in 2020, whereas there was evidence that green compost mulching resulted in significantly higher *Stemphylium* populations than were detected in plots with companion crops or in the control plots with shallow surface disturbance.

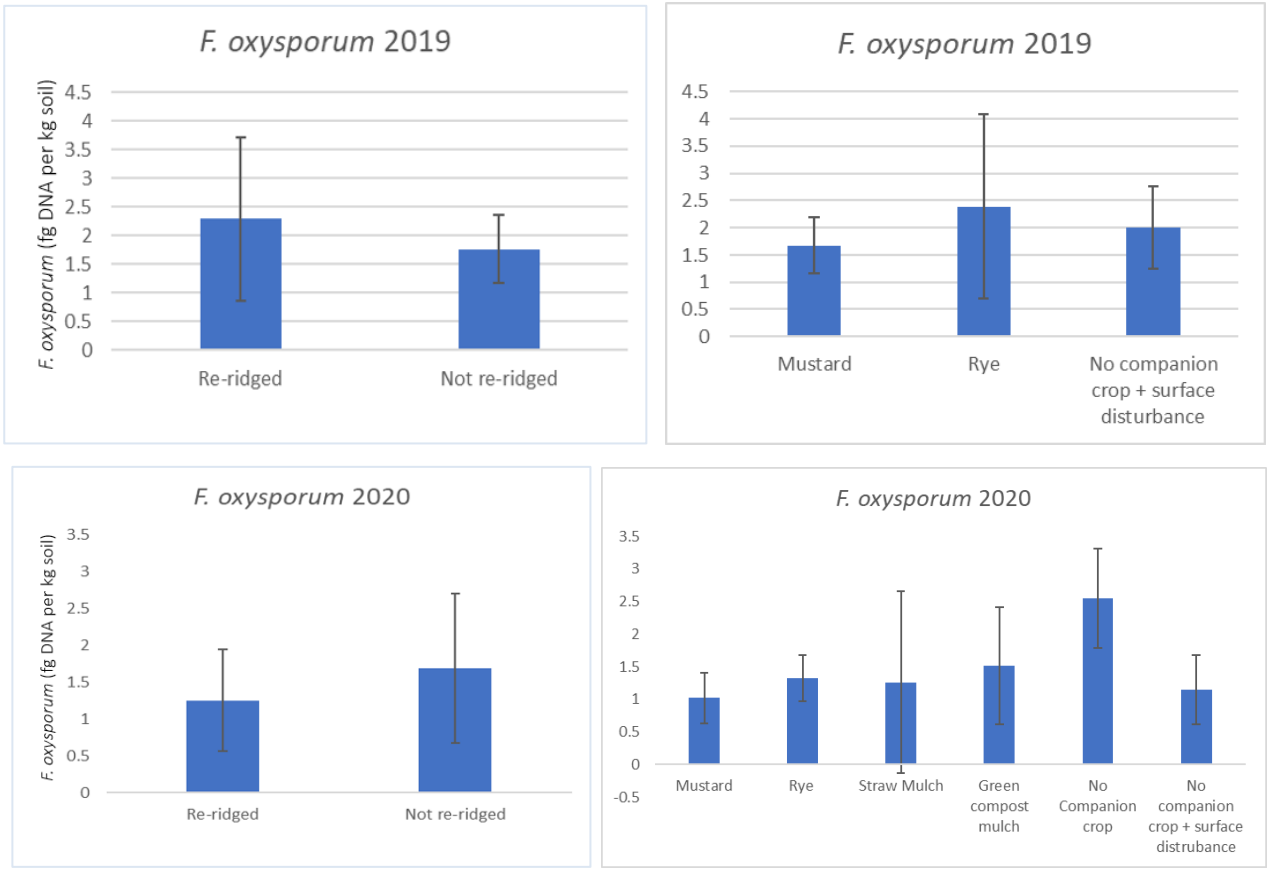


Figure 14: Quantification of *F. oxysporum* DNA in plots with different soil treatments (mean from 9 plots for re-ridging treatments and from 6 plots for all other treatments, showing standard deviations). Soil surface disturbance was carried out in controls to mimic disturbance during incorporation of the cover crops.

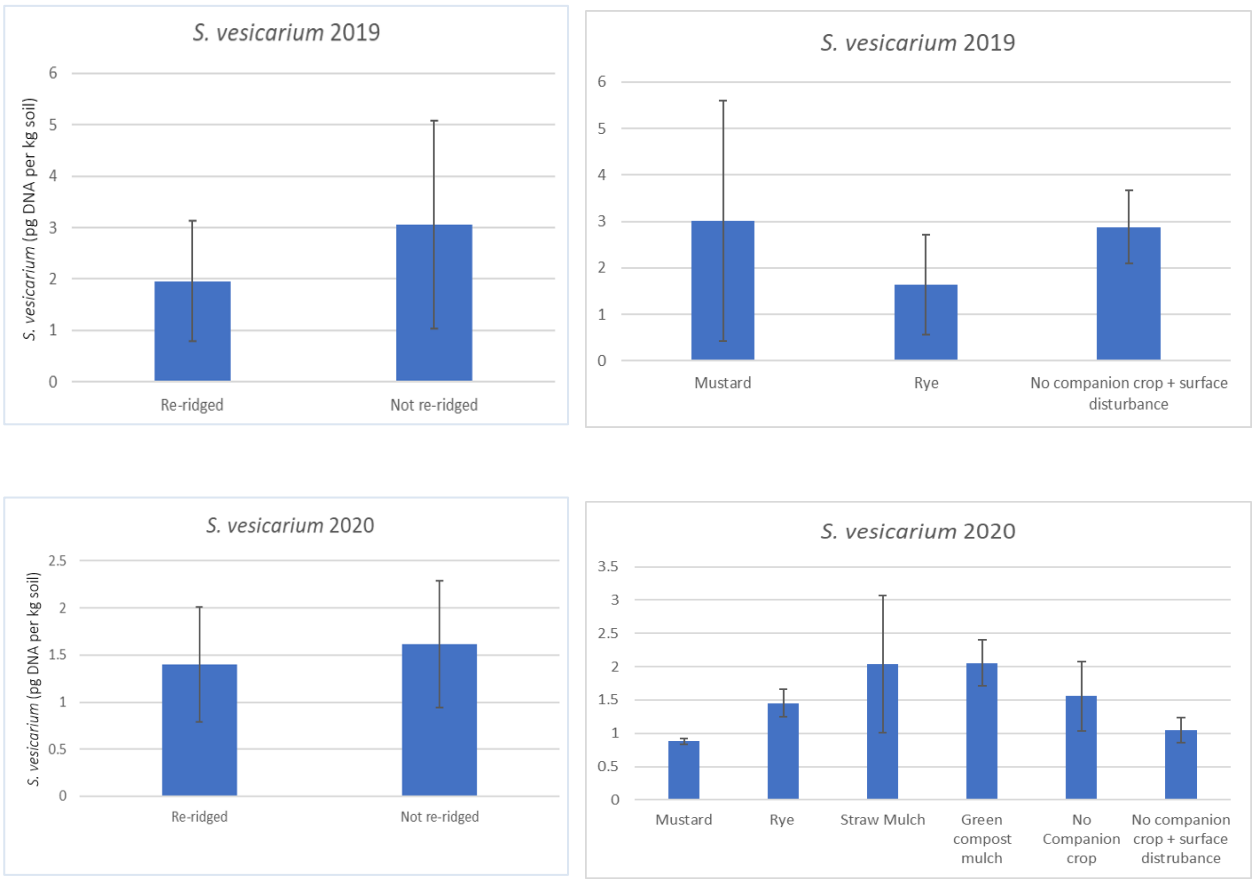


Figure 15: Quantification of *S. vesicarium* DNA in plots with different soil treatments (mean from 9 plots for re-ridging treatments and from 6 plots for all other treatments, showing standard deviations). Soil surface disturbance was carried out in controls to mimic disturbance during incorporation of the cover crops.

(d) *Gaeumannomyces tritici* in winter wheat trial

The only soil treatments to clearly affect populations of *G. tritici* were the use of break crops either in spring or autumn where detectable populations of the take all fungus were significantly lower compared with continuous wheat (Figure 16). The combination of deep ploughing with either break crop resulted in the lowest populations of *G. tritici*. There was no effect of tillage method on population of the pathogen under continuous wheat. However, compared with all other treatments, significantly higher *G. tritici* populations were observed with the combination of deep ploughing and alternate fallow.

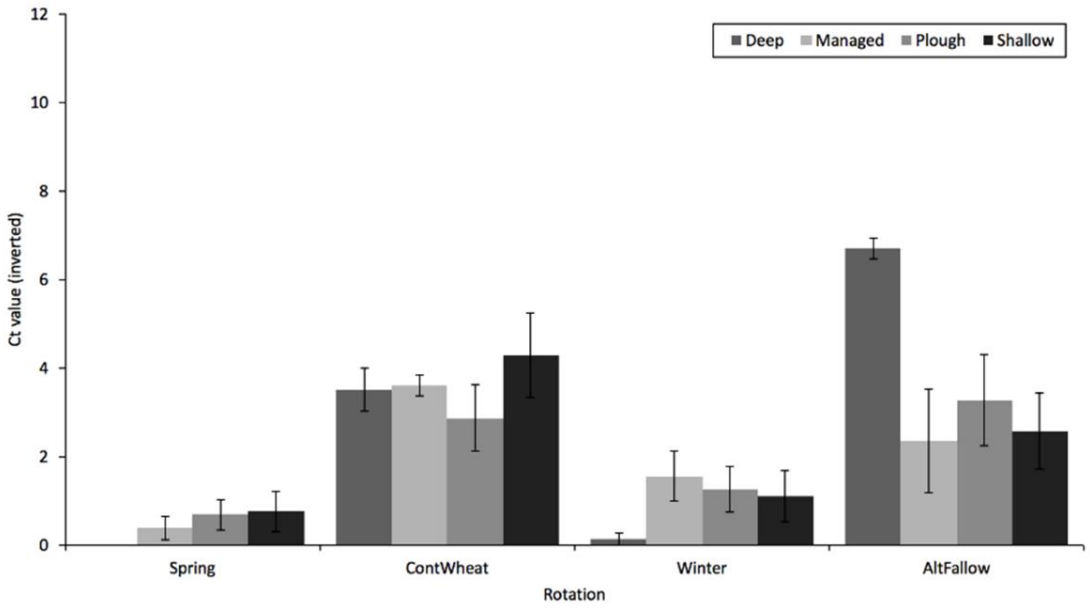


Fig. 16: Detection of *G. tritici* DNA in plots with different soil treatments (mean from 3 plots, showing standard deviations). The qPCR Ct (critical threshold) value was inverted (40-Ct) for ease of interpretation. A difference in Ct of 3 is roughly equivalent to a 10-fold difference in target DNA concentration (Briggs, 2018).

4.4. Objective 4: Design and demonstrate the benefits of an appropriate soil health testing service for growers and agronomists.

4.4.1. Review of PREDICTA® soil testing service

Overview

The South Australian Research and Development Institute (SARDI) has developed a DNA-based soil testing technology that quantifies the level of DNA of specific disease-causing pathogens in a sample of soil. SARDI is the principal research institute of the Government of South Australia. The industry award-winning service is marketed to growers and researchers throughout Australia under the PREDICTA® trademark, which has been owned by collaborators Bayer Crop Science (Lyon, France) since October 2000. The testing service helps growers to identify which soilborne pathogens pose a significant risk to their crops before planting, so steps can be taken to minimise production losses. There are currently three main PREDICTA® services available: PREDICTA®-B for broadacre crops, PREDICTA®-Pt for potatoes and PREDICTA®-Research for use by researchers. PREDICTA®-Pt also includes a suite of tests performed on potato tuber peel samples to assess post-harvest disease risk. A suite of tests is also available for vegetable growers and is most suited to use in brassica, carrot, capsicum, sweet potato and onion production. Suites of tests specifically suited to pulse and oilseed crops are also under development. The tests are based on quantitative real-time PCR (qPCR) methodology and, for some key soil-borne pathogens, allow the amount of pathogen DNA detected to be related to the risk of disease and yield loss. SARDI also provide training courses,

accreditation, manuals and disease factsheets (including on-line versions) to government and industry agronomists across Australia. This aims to ensure that a consistent standard of soil sampling is maintained and that test results are correctly interpreted, taking into account other factors that affect disease risk such as seasonal conditions, soil type, agronomic practices and the crop and variety previously planted. Accredited agronomists also give advice on management options to reduce the risk of yield loss.

Sampling and DNA extraction

For each sample, 30 individual cores (not exceeding 500 g) are collected per 1.0 ha area, using a standard supplied soil corer with a 15 cm depth by 1 cm diameter tip, into supplied sampling bags. Sampling follows a W pattern across each area, targeting productive cropping areas and avoiding field margins, waterlogged areas, areas close to trees or other atypical areas of the field. Samples are kept cool (<10°C) and delivered to the testing laboratory within 1-2 days, avoiding exposure to direct sunlight. The method used to extract DNA for the PREDICTA® tests is protected under commercial confidence, although it is reported that the method was developed to extract DNA from 500 g samples of soil, with a throughput of at least 160 samples per day (Ophel-Keller *et al*., 2008). Sub-samples of the DNA are used to conduct qPCR assays that test for each target organism in separate reactions in 384-well plates using robotic pipetting stations. For qPCR analysis, standard TaqMan assays are performed using a ABI HT 7900 real-time PCR system (Applied Biosystems, Foster City, CA). Return of the results to the grower is guaranteed within 2 weeks and is therefore much quicker than traditional pathogen culturing or bioassay methods that can take months to complete and are more expensive.

Test results

Test results are reported under one of two categories:

(a) Disease Risk: Where the risk of yield loss associated with a particular level of pathogen DNA has been substantiated over a wide geographical area, the results are displayed using a graphic to indicate a ‘low’, ‘medium’ or ‘high’ disease risk. Where a disease risk is found, advice is given regarding suitable control measures and choice of rotation crops. Developing disease risk categories can take several years, requiring extensive validation through testing of samples from multiple locations, target crops and over several seasons. Since Australia covers a vast array of cropping systems, climates and soil types, risk categories can vary from area to area.

(b) Population Density: Where a relationship between yield loss and pathogen DNA level has not been determined, the results are simply reported as levels of inoculum detected in each sample, usually as log (pg DNA/g soil). In this case, testing can simply be used to confirm diagnosis of a disease observed in the field or to monitor changes in soil populations over time as a result of different cropping sequences or disease management strategies (including

choice of varieties). Results can also be ranked and mapped over a given area to show inoculum hot spots and seasonal variation for a given pathogen in response to climate or other conditions (e.g. soil type or cropping system). Population density categories enable new tests to be reported to growers faster. Results can be used to rank levels of inoculum in different paddocks, monitor changes in inoculum during different phases of the cropping sequence and confirm disease diagnoses.

Currently available tests

A large number of qPCR tests are available through the PREDICTA® services but only relatively few have been validated for specific crops and over wide growing areas, such that the level of DNA detected in soil can be used to predict expected disease incidence/severity and resulting yield loss. At present, these include validated tests for a variety of plant pathogenic nematodes as well as for eight soilborne fungal diseases (Table 8). The PREDICTA®-Pt suite of tests also include three post-harvest pathogens for which the risk of storage blemishes can be predicted by testing samples of peel from harvested potato tubers.

Table 8: Pathogens for which an indication of the risk of yield loss can be predicted in some regions of Australia (SARDI)

Pathogen	**Disease**	**Risk assessment validated for:**
Gaeumannomyces graminis var. *tritici* *Gaeumannomyces graminis* var. *avenae*	Cereal take-all	PREDICTA®-B, PREDICTA®-Research
Fusarium pseudograminearum	Cereal crown rot	PREDICTA®-B, PREDICTA®-Research
Rhizoctonia solani AG8	Cereal root rot Onion stunt	PREDICTA®-B, PREDICTA®-Research Onion
Phoma koolunga *Phoma pinodella* *Didymella pinodes*	Blackspot of field pea	PREDICTA®-B, PREDICTA®-Research
Colletotrichum coccodes	Potato black dot	PREDICTA®-Pt
Verticillium dahliae	Verticillium wilt of potato	PREDICTA®-Pt
Spongospora subterranea	Potato powdery scab	PREDICTA®-Pt
*Helminthosporium solani**	Silver scurf	PREDICTA®-Pt
Rhizoctonia solani AG2.1 and AG3*	Rhizoctonia disease	PREDICTA®-Pt
Streptomyces spp.*	Common scab	PREDICTA®-Pt
Plasmodiophora brassicae	Club root	Brassicas

*Potato peel tests only

All other available tests (Table 9) are used to monitor soilborne pathogen levels but do not yet estimate the risk of yield loss. Annual data are used to amend the categories of predicted risk (high, medium or low) and mean pathogen population densities (high, medium or low) to take into account variation in the cumulative results obtained from testing multiple sites, crops and seasons. Tests also available for detection and quantification of beneficial organisms in the soil for monitoring of soil health. Targets include Free Living Nematodes (FLN, Arbuscular Mycorrhiza Fungi (AMF) and *Trichoderma* spp.

4.4.1. Validation of molecular test methods appropriate for soil health testing for UK growers and agronomists.

Using the PREDICTA® service as a model, it was possible to compile a series of similar testing procedures that would be suitable for use in the UK. Under sections 4.1, 4.2 and 4.3 of this report, a suite of qPCR tests for 18 soil-borne pathogens of importance to UK arable and horticulture industries (Table 1) was validated under laboratory conditions. Soil sampling methods and procedures for DNA extraction were optimised and demonstrated on a wide range of UK soils, allowing replicated testing of sub-samples of 50 g of soil. Internal control methods were also developed that ensure adequate extraction of amplifiable DNA across all samples whilst comparing total populations of bacteria and fungi present. Other controls were constructed from pathogen-specific DNA sequences (gBlocks) and used at known concentrations as qPCR controls to ensure accuracy of pathogen quantification for each reaction. The combined results from these validation studies suggest that the qPCR assays are suitable for comparing population densities of pathogens of UK importance between samples of representative soils. Furthermore, as a first step in developing disease risk categories, investigations have been initiated to study the relationship between pathogen DNA quantification in field and glasshouse soils and the risk of disease development and yield loss for two important soilborne pathogens (*Fusarium oxysporum* and *Verticillium dahliae*). As qPCR tests only indicate the presence of pathogens that are specifically targeted by the assays used, the potential was considered to also include standardised metabarcoding analyses, as described in the final report for Project 6, in a UK molecular testing service.

Table 9: Pathogens for which qPCR tests are used to monitor population densities in the soil without relating to the risk of disease and yield loss, in Australia (SARDI)

Pathogen	**Disease**	**Test validated for:**
Fusarium graminearum & F. culmorum	Cereal crown and root rot	PREDICTA®-B, PREDICTA®-Research
Rhizoctonia solani AG-8 AG2.1, AG3, AG4	Cereal root rot Potato stem canker	PREDICTA®-B, PREDICTA®-Research PREDICTA®-Pt
Bipolaris sorokiniana	Cereal common root rot	PREDICTA®-B, PREDICTA®-Research
Pythium clade f*		PREDICTA®-B, PREDICTA®-Research
Pythium clade I**		PREDICTA®-Pt
Pyrenophora tritici-repentis	Wheat yellow leaf spot	PREDICTA®-B, PREDICTA®-Research
Eutiarosporella tritici-australis E. darliae and E. pseudodarliae	White grain disorder	PREDICTA-B®, PREDICTA®-Research
Phoma rabiei	Chickpea *Ascochyta* blight	PREDICTA-B®, PREDICTA®-Research
Phytophthora medicaginis	Chickpea root rot	PREDICTA-B®
Macrophomina phaseolina	Soybean charcoal rot	PREDICTA-B®, PREDICTA®-Research + beans, cucurbits, sweet corn, sweet potato and strawberries.
Oculimacula yallundae	Eyespot	PREDICTA®-B, PREDICTA®-Research
Sclerotinium sclerotiorum/*S. minor*	*Sclerotinia* rot	PREDICTA-B®, PREDICTA®-Pt + beans, brassicas, carrots, celery and lettuce
Helminthosporium solani	Potato silver scurf	PREDICTA®-Pt
Phytophthora erythroseptica, P. crytogea, P. dreschleri	Potato pink rot	PREDICTA®-Pt
Pythium sulcatum Pythium violae	Carrot cavity spot	Carrot
Aphanomyces euteiches	*Aphanomyces* root rot	Peas and beans
Thielaviopsis basicola	Black root rot	Beans, lettuce, carrots and cucurbits
Setophoma terrestris	Pink root	Onion
Verticillium dahliae	*Verticillium* wilt	Brassicas, lettuce and strawberry

**Pythium* species in clade F include *P. irregular*, *P. sylvaticum*, *P. debaryanum*, *P. spinosum*, *P. paroecandrum* and *P. mamillatum*.

***Pythium* species in clade I include *P. ultimum*, *P. splendens* and *P. heterothallicum*.

4.5. Objective 5: Integrate results of molecular testing for soil-borne disease risk with associated chemical and physical data in support of a robust soil health scorecard developed within the SBSH Partnership.

4.5.1. Estimation of total bacterial and fungal DNA by quantification of 16S and 18S rRNA genes.

Differences between trial sites

Comparison of qPCR results highlighted significant differences between the levels of bacteria and fungi estimated in samples from each field trial site (Figure 17). Bacterial DNA was always more abundant than fungal DNA irrespective of the site. Estimated mean bacterial DNA levels were significantly higher in samples collected from the trials at Terrington, Harper Adams, Gleadthorpe and the fertiliser trial at Craibstone, than those collected from Boxworth, Loddington and the pH trial at Craibstone. Conversely, estimated fungal DNA levels were significantly higher in samples collected from Loddington and the Craibstone pH trial than in samples collected from the other sites.

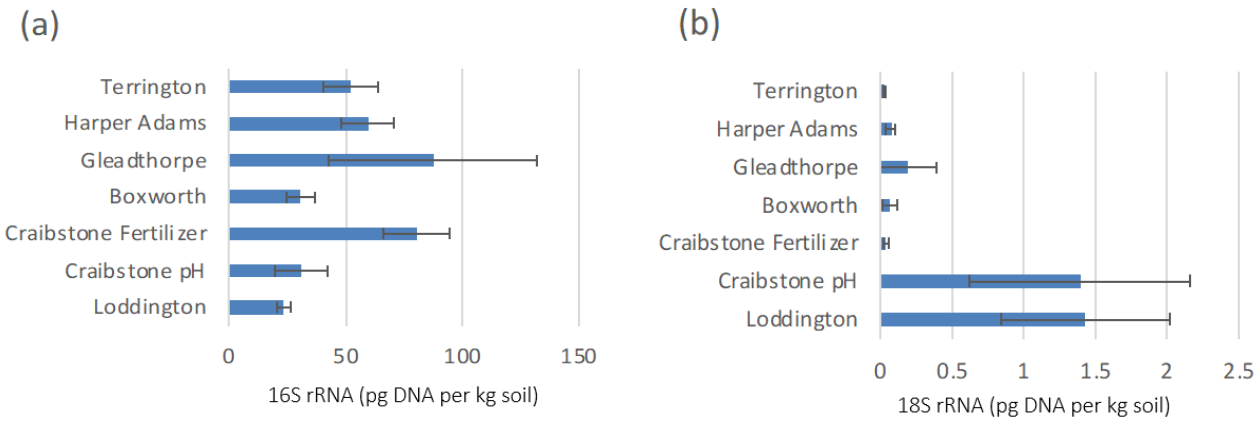


Figure 17: Quantification of (a) bacterial (16S rRNA) and (b) fungal (18S rRNA) DNA at each field trial site. The error bars show standard deviation from the mean value for each site.

Effects of pH and stage of crop rotation

In the pH trial at Craibstone, bacterial and fungal levels appeared to be influenced by both pH and stage of the cropping rotation (Figure 18). Except at pH 7.5, bacterial levels showed differences between crops with grass-clover ley (pasture) = wheat > potato > oats. Differences in bacterial levels between pH for the same crop were smaller and not always significant but there is some indication of a pH optimum for bacterial levels at pH 6.5. There was more interaction between pH and cropping for fungal levels, with higher fungal levels at higher pH for the grass-clover ley (pasture) and at pH 4.5 for the potato.

For samples taken after wheat, bacterial levels at pH 4.5 were significantly lower than at pH 6.5 and fungal levels were significantly lower at pH 4.5 than at pH 6.0 or 6.5. For samples taken in the grass-clover ley, bacterial levels at pH 4.5 were again significantly lower than at pH 6.0 whereas fungal levels appeared to be less affected by pH. Samples taken after oats had significantly lower bacterial levels at pH 4.5 than at pH 6.0 or at pH 7.5, whereas fungal levels were significantly lower at pH 4.5 than at pH 7.5. Furthermore, for all samples taken after the oat crop, bacterial levels were significantly lower than for those taken after wheat, pasture or potato crops at all pH levels except for pH 7.5. Similarly, fungal levels were significantly lower in samples taken after oats than in those taken after wheat at pH 6.0 and 6.5, after pasture at pH 4.5, 6.5 and 7.5 and after potato at pH 4.5 and 6.0. For samples taken after potato, bacterial levels were significantly lower than in samples taken after pasture at pH levels 4.5, 6.0 or 6.5 and were also lower than in samples taken after wheat at pH 6.0 and 6.5. However, bacterial levels in samples collected after potato did not appear to be significantly affected by pH levels, whereas fungal levels were significantly higher after potato at pH 4.5 than at pH 7.5. In fact, samples taken after potato at pH 4.5 had significantly higher fungal levels than samples taken after wheat, pasture or oat at the same pH. At the other pH levels, fungal levels in samples taken after potato did not differ from those taken after, wheat, pasture or oat.

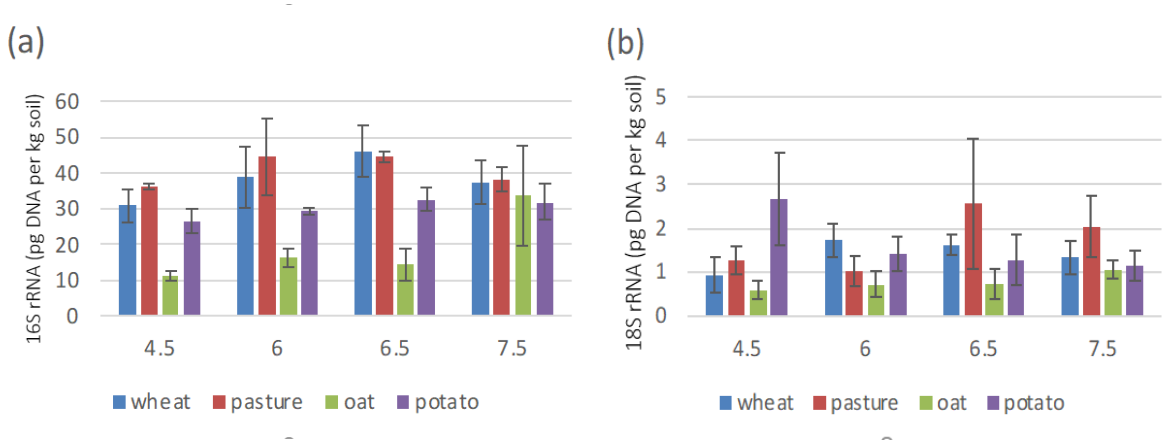


Figure 18: Quantification of (a) bacterial (16S rRNA) and (b) fungal (18S rRNA) DNA sampled from the Craibstone pH trial for soil samples collected after crop harvest (wheat, oat, potato) or within the grass-clover ley (pasture). The error bars show standard deviation from the mean value from three samples per treatment.

Effect of fertilisation treatments

No significant effects of fertiliser application, or stage of the cropping rotation, were observed on estimated total bacterial levels in the Craibstone fertiliser trial (Figure 19). Estimated total fungal levels were not affected by fertiliser treatment when samples from the same stage of the cropping

rotation were compared. However, for fertilised plots, fungal levels in the grass-clover ley (pasture) were the lowest measured and were significantly lower than after oats.

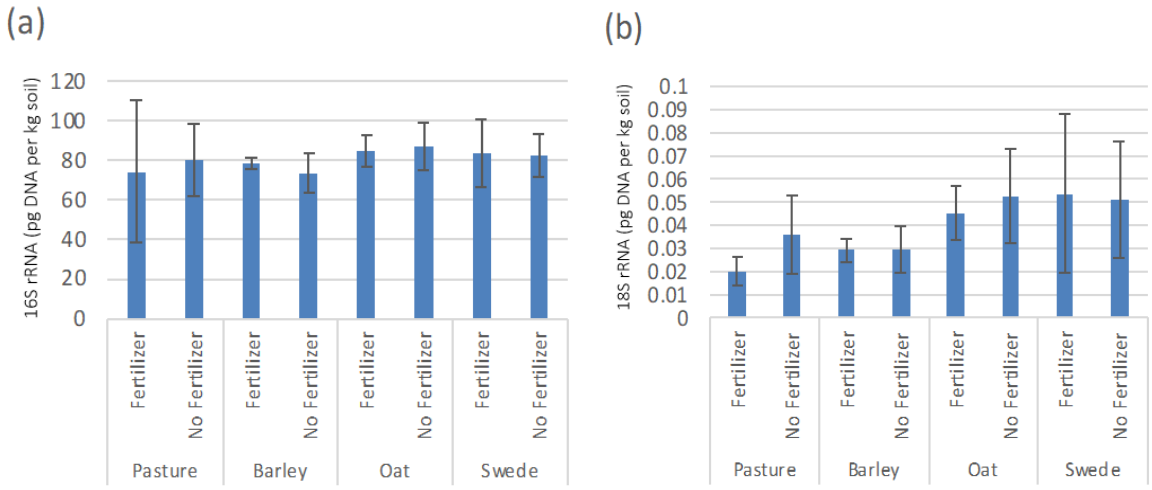


Figure 19: Quantification of (a) bacterial (16S rRNA) and (b) fungal (18S rRNA) DNA sampled from the Craibstone fertiliser trial. The error bars show standard deviation from the mean value from three samples per treatment.

Effect of organic amendment treatments

Subtle differences were observed between organic amendment treatments in trials at three locations (Figures 20, 21 and 22). Soils regularly amended with farmyard manures had the highest estimated bacterial levels at all three sites and these differed significantly from non-amended controls at two of the sites (Harper Adams and Terrington). No significant differences in bacterial levels were recorded when treatments with other types of organic amendments were compared with the non-amended controls. The only effect of organic amendment on estimated fungal levels was observed at Gleadthorpe where significantly higher fungal levels were recorded in soil which had been repeatedly treated with farmyard manure compared with soil treated with green compost.

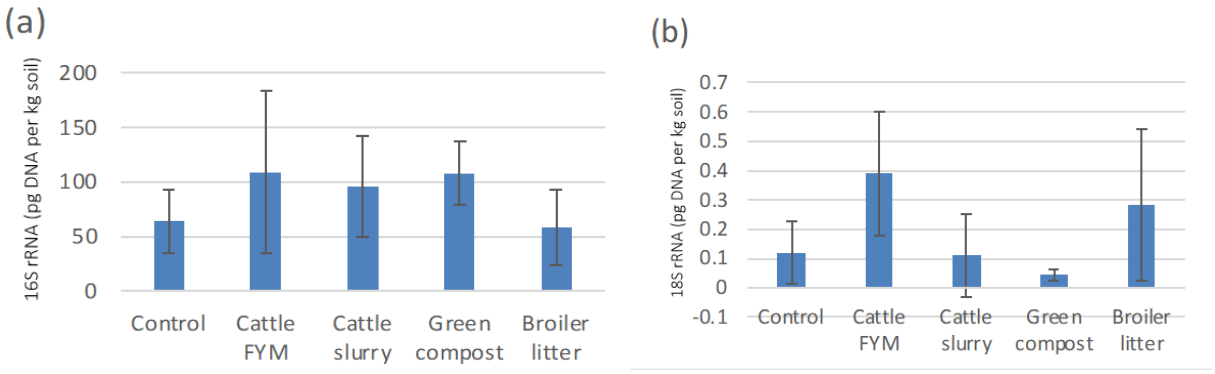


Figure 20: Quantification of (a) bacterial (16S rRNA) and (b) fungal (18S rRNA) DNA sampled from the Gleadthorpe organic amendments trial. The error bars show standard deviation from the mean values per treatment.

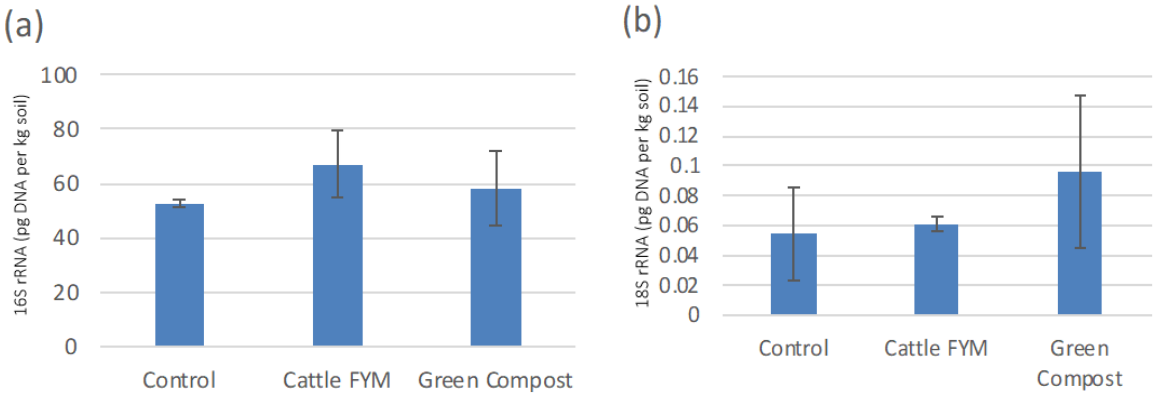


Figure 21: Quantification of (a) bacterial (16S rRNA) and (b) fungal (18S rRNA) DNA sampled from the Harper Adams organic amendments trial. The error bars show standard deviation from the mean values per treatment.

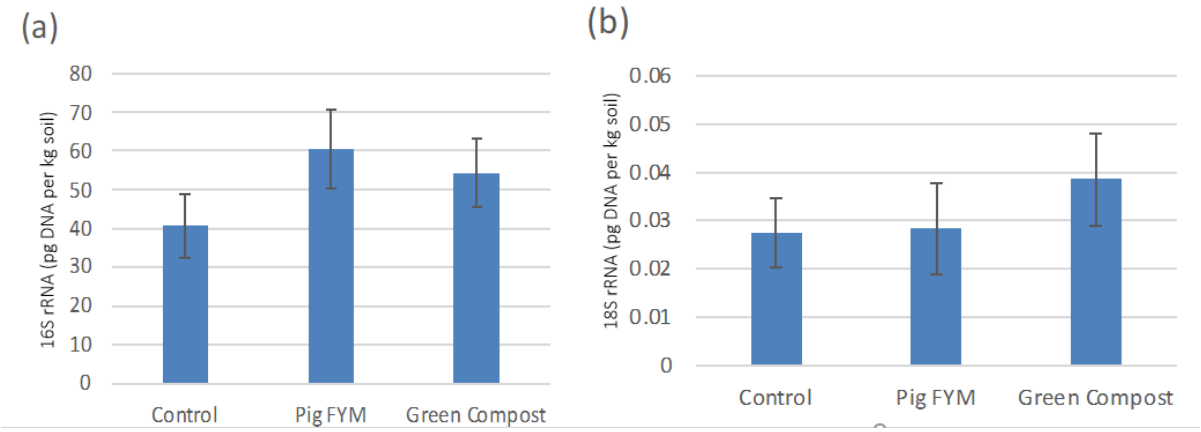


Figure 22: Quantification of (a) bacterial (16S rRNA) and (b) fungal (18S rRNA) DNA sampled from the Terrington organic amendments trial. The error bars show standard deviation from the mean values per treatment.

Effect of drainage treatments

No differences were observed between estimated total bacterial or fungal levels in soil samples from drained and undrained treatments at ADAS Boxworth (Figure 23).

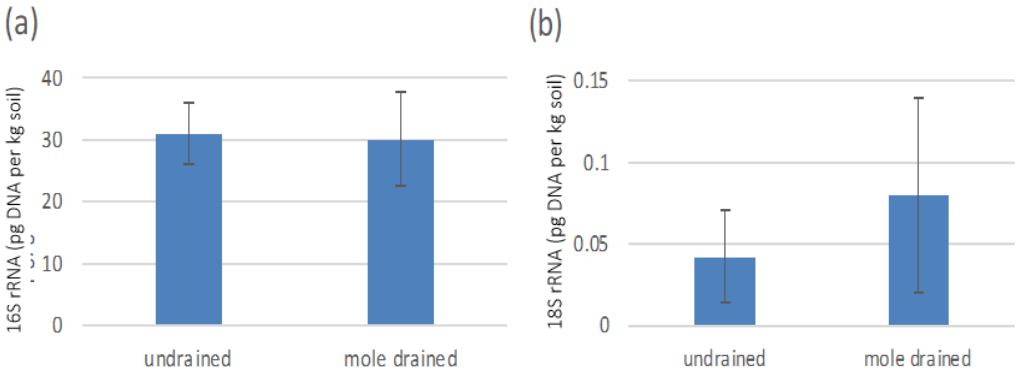


Figure 23: Quantification of (a) bacterial (16S rRNA) and (b) fungal (18S rRNA) DNA sampled from the Boxworth drainage trial. The error bars show standard deviation from the mean values per treatment.

Effect of tillage treatments

No differences were observed between estimated total bacterial or fungal levels in soil samples from ploughed or direct-drilled plots at Loddington (Figure 24).

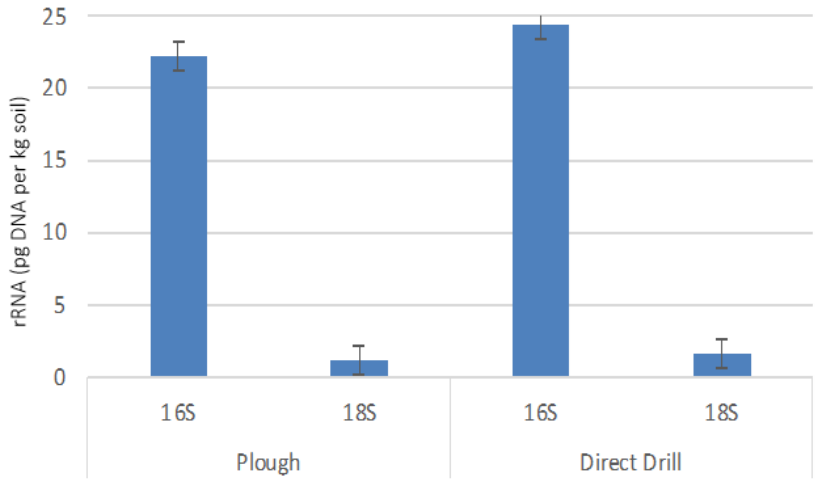


Figure 24: Quantification of bacterial (16S rRNA) and fungal (18S rRNA) DNA sampled from the Loddington cultivation trial. The error bars show standard deviation from the mean values per treatment.

4.5.2. Correlations between pathogen detection and other scorecard data

Quantities of *Fusarium oxysporum* and *Verticillium dahliae* DNA detected in the onion, *Narcissus* and raspberry field trials were compared with other field data collected in Project 7 of the SBSH Partnership. However, within trial variation between measurements of potentially mineralisable nitrogen (PMN), CO_2-burst, % soil organic matter and pH were too low to indicate any meaningful correlations with pathogen DNA levels detected by qPCR. Similarly, no correlations were observed between quantities of 16S or 18S rRNA targets with any of the other data sets.

The relationships between quantities of 16S or 18S rRNA targets (described in Section 4.5.1) with the other soil data collected from some of the other long term soil management trials are reported in the final report for Project 4.

5. Discussion

5.1. Selection of qPCR assays for quantitative detection of key intractable soil-borne pathogens.

A series of 18 qPCR assays (Table 1) were investigated for detection and quantification of key soilborne pathogens of arable and horticultural crops. Most were successfully validated for detection and quantification of purified pathogen DNA in the laboratory, but a number of issues were encountered during their validation for detection and quantification of pathogen DNA extracted from different soils using previously reported procedures. Primarily, the amount of total extractable DNA appeared to vary between soils from different sources and this inevitably affected the sensitivity of detection of individual pathogens, even when they had been previously added in equal measure to the different soils. Both yield and quality of extracted DNA is known to vary with the chemical and physical properties of different soils (Feinstein *et al.,* 2009) as well as the extraction method used (Petric *et al*.,2011). It is also possible that differences in soil type and agronomic management affected the chemistry of the qPCR reaction to different extents for different soils, rendering quantification of the extracted DNA unreliable. It is known that the PCR reaction can be inhibited to different extents by common soil components such as humic acids and phenolics as well as by changes in magnesium and calcium levels (Wilson, 1997).

It was possible to use qPCR assays to detect high inoculum concentrations in some soils freshly inoculated with most key soilborne pathogens. However, the methods used were not yet sufficiently sensitive to detect lower residual pathogen populations that would persist naturally in soils, especially for those pathogens with hardy resting spores from which it was difficult to extract DNA. It was therefore concluded that further optimisation of DNA extraction and purification methods would be needed before qPCR could be used for reliable quantification of plant pathogens across a range of naturally infested soils.

5.2. Relationships between pathogen distribution and concentration in soils and potential for disease development.

The scientific literature on methods used to extract total DNA from soil was fully reviewed and used to assemble an improved protocol suitable for use with starting quantities of 50 g soil. Since available commercial extraction kits can only be used on a maximum of 10 g of sampled soil, the improved protocol allowed at least 5x more starting material to be analysed for total bacterial and fungal DNA yield. Nevertheless, testing of DNA extracts from different field trials indicated that certain soils were still unsuitable for accurate quantification of individual target organisms, especially if present in low populations in the soil sample. Further investigation is needed to determine whether procedures can be further improved for use with problem soils such as those with high DNA-binding clay content or with high content of PCR inhibiting compounds, such as humic acids.

Both fungal and bacterial soil microbiomes appeared highly consistent across multiple sampling points in the onion field trial. The results of qPCR testing also showed that *F. oxysporum* was uniformly distributed across the trial, even 2 years after the preceding onion crop. It was therefore concluded that the soil sampling strategy used was adequate. Future refinements of the qPCR assays for detection of individual *F. oxysporum* f. spp. will be needed to confirm this. Similar data on the field distribution of different fungal pathogens across varying cropping systems and soil types will be needed to refine sampling strategies for specific combinations of pathogens.

Testing of soils sampled from three individual field trials, each with different soil-borne pathogens present, did not provide sufficient relevant data to be able to relate pathogen levels pre-planting to risk of disease development. *F. oxysporum* was detected in soil before planting in both onion and *Narcissus* trials and was shown to increase during the season in both cases. Furthermore, *Fusarium* basal rot disease did subsequently develop in both onion and *Narcissus* crops. However, the disease levels were consistently too high in the onion trial and too variable in the *Narcissus* trial to be able to relate disease incidence or severity to quantification of pathogen DNA before planting. Detection of *Verticillium* in the third field trial was unreliable, probably as a result of inhibition of the qPCR assay used by compounds coextracted with the DNA from the soil. Additional glasshouse experiments are underway to further explore this relationship under more controlled conditions as part of the PhD programme associated with this project. It will be necessary to accumulate data from a much wider range of soils and crops before a more reliable determination of the influence of soilborne populations of each pathogen on the risk of disease development can be undertaken.

5.3. Effects of soil management on survival of specific soil-borne pathogens

Soil management approaches tested in this research included organic amendments (green compost, FYM), inoculation with mycorrhizal fungi, cultivations, cover and companion cropping. Neither of the onion or *Narcissus* trials installed through Project 7 showed any obvious effects of soil organic

amendments on *Fusarium oxysporum* concentrations in the soil at the time of sampling. Growing of a cover crop before planting onion also had no effect on soil-borne *F. oxysporum* levels in the season it was applied. The observed lack of detection of *F. oxysporum* in plots where mycorrhizal fungi had been applied to *Narcisuss* bulbs at planting in the season before, perhaps warrants further investigation of long-term effects of the use of mycorrhizae supplements for suppression of this pathogen.

Disease incidence in the onion trial was extremely high regardless of soil treatment and multiplication of *F. oxysporum* on the crop occurred regardless of any effects of soil treatment. Conversely, soilborne *F. oxysporum* populations detected in the *Narcissus* trial were very low and decreased further in the second year, corresponding with low observed disease incidences in this trial. It should however be stressed that the available qPCR method detected all *F. oxysporum* variants and therefore may have overestimated the populations pathogenic to each specific crop. Further testing of the extracted DNA with assays specific for *F. oxysporum* f. sp. *cepae* and f. sp. *narcissi*, once available, may provide a clearer picture of any effects on these particular pathogens.

Investigation of *F. oxysporum* in the Cranfield University asparagus trial showed no differences in populations detected in the soil regardless of whether the plots had been annually re-ridged or not. Re-ridging has previously been reported to increase susceptibility to *Fusarium oxysporum* f. sp. *asparagi* (Elmer, 2015), the cause of root and crown rot of asparagus, leading to yield decline and direct economic losses. No effects of any other soil treatments on soilborne populations of *F. oxysporum* were detected when compared with untreated controls in which the soil surface had been disturbed to mimic cultivations in the treated plots. However, when tested in 2020, untreated control plots without shallow soil disturbance (minimum till plots) were found to have significantly higher *F. oxysporum* soil populations than was detected in controls that had undergone shallow soil disturbance. It would therefore seem that the shallow cultivations in the top 15 cm had either reduced the *F. oxysporum* populations in this upper sampled soil layer, or that populations of the pathogen had increased in the absence of cultivation. Shallow soil disturbance had also been applied to plots receiving either straw or green mulch treatments. In these cases, high variation in qPCR results from the mulched plots resulted in no significant differences between *F. oxysporum* levels estimated in these plots compared with those from the minimum till controls. However, the minimum till plots did show significantly higher *F. oxysporum* populations than in plots where mustard and rye companion crops had been grown. As opposed to mustard and other cruciferous crops, rye is known to promote arbuscular mycorrhizal fungi (White and Weil, 2010) and has been reported to reduce the severity of Fusarium crown and root rot in asparagus (Matsubara *et al.*, 2001). Mustard, on the other hand, is known for its bio-fumigation potential, which has been previously shown to reduce *Fusarium* levels (Cresswell and Kirkegaard, 1995; Sarwar *et a*l., 1998). From the results of this investigation, it was not possible to postulate whether there were any direct effects of the companion crops on *Fusarium*, or whether the observed effect resulted, at least in part, to cultivation during planting and/or removal of the companion crops.

Estimation in the same Cranfield plots of soil-borne populations of the asparagus purple spot pathogen, *S. vesicarium*, showed that there was again no effect of re-ridging between asparagus harvests. Reduced compaction due to re-ridging is commonly thought to reduce wet conditions which carry the risks of increased incidence of *Stemphylium* purple rot (Saude *et al*., 2008). Significantly higher pathogen populations were found in soil from minimum till control plots compared with mustard companion crops. In fact, soil from the mustard plots contained significantly less *Stemphylium* than that from plots with all other treatments except for control plots with shallow soil disturbance. It was therefore again not possible to conclude whether the mustard effect was due to biofumigation or simply to increased cultivation. Mulching with green compost resulted in higher detectable levels of *Stemphylium* compared with control plots only when both treatments included shallow soil disturbance. Levels of *Stemphylium* were also variably high in plots treated with straw mulch. It is likely that mulching maintained moisture levels suitable for *Stemphylium* multiplication/survival.

Data from the NIAB STAR trial confirmed that the use of non-cereal break crops between successive wheat crops significantly reduced detectable populations of the take-all fungus (*G. tritici)*, especially for systems incorporating annual cultivation by ploughing. It is well known that due to a build-up of antagonistic bacteria such as *Pseudomonas* spp. under continuous wheat, there is a decline in the impact of take all on wheat yield (Weller, 2015). However, the take-all decline alone does not sufficiently recuperate yield loss and break crops are recommended to further reduce the economic impact of this disease.

It is apparent from the results of these field studies that different agronomic practices interact to affect soil-borne pathogen dynamics. It is therefore difficult to make general conclusions on the effects of soil management on survival of different soilborne pathogens based on the results from individual field trials obtained over only one or two seasons. However, some observations from these trials are worthy of further investigation across different geographical locations and seasons. In particular, the role of shallow soil cultivation in reducing populations of *Fusarium* and *Stemphylium* and the use of break crops to control *Gaeumannomyces* and other pathogens deserves further investigation whilst considering wider geographical and seasonal variations.

5.4. Potential for a molecular soil health testing service for UK growers and agronomists.

5.4.1. *qPCR* testing for pathogen quantification

The review of the Australian PREDICTA® service presented in Section 4.4 of this report outlined the benefits of a co-ordinated service for molecular testing of arable and horticultural soils, able to compare population densities of soilborne pathogens across different seasons, agro-ecological zones, cropping systems and disease management interventions. Even in the absence of a defined

link between pathogen DNA level in soil and the risk of disease and yield loss, there are distinct benefits for this kind of rapid testing:

1. Regular standardised testing can determine the effectiveness of rotations and cover crops at managing the level of pathogen inoculum and ensure that the correct system is in place to reduce or eliminate inoculum between successive susceptible crops.
2. The effectiveness of disease management treatments can be assessed, even in the absence of high levels of disease.

Results of method development and laboratory and field validations presented in Sections 4.1., 4.2. and 4.3. of this report suggest that sampling, DNA extraction and qPCR methods are suitable for quantification of most of the important fungal pathogens found in UK soils. Indeed, the PREDICTA®-B system has already been successfully used to quantify populations of the take all fungus (*Gaeumannomyces graminis* var. *tritici*) in soils at two field trials at Rothamsted Experimental Station and one in Suffolk (Gutteridge *et al*., 2008). Furthermore, some of the qPCR assays used in the PREDICTA®-Pt suite of tests, were originally developed in the UK, including assays for *Colletotrichum coccodes* (Lees *et al*., 2010), *Rhizoctonia solani* (Lees *et al*., 2002) and *Spongospora subterranea* (van de Graaf *et al*., 2003). Disease risk prediction for potato has already been established in the UK for *C. coccodes* and to a lesser extent for *S. subterranea* and *Rhizoctonia solani* through AHDB Project 2008/6 (Brierley *et al*., 2008). As with PREDICTA® testing in Australia, qPCR quantification of *Plasmodiphora brasiccae* resting spores in soil prior to planting of commercial oilseed rape fields in England and Scotland was positively correlated with infections by the clubroot pathogen (Kennedy *et al*., 2016).

Whilst it should be possible to use validated qPCR tests for monitoring pathogen population densities, data from limited field trials is currently insufficient to establish the pathogen levels that would constitute high, medium or low risk categories across UK agriculture and horticulture cropping systems and agro-ecological conditions. Similarly, the establishment of disease risk categories will only be possible once data collected over several seasons, soils and cropping systems becomes available. There will therefore be an initial requirement to perform testing over several years to permit sufficient data accumulation for full validation of each test. At present, no such testing service is active in the UK. The PREDICTA® service in Australia is not run on a commercial basis and relies on both Government and Industry support. Similar initial support would be critical to establishing a usable service in the UK. In addition to pathogen DNA levels, other factors such as climate, management practices, soil type, crop type, variety, seasonal conditions and seedling health (if transplanted) should be considered when interpreting test results, assessing disease risk and recommending appropriate management strategies. As with the PREDICTA® service, it will be necessary to build and train a support network of agronomists as an integral part of a similar UK service.

5.4.2. Metabarcoding for diversity testing

Standardised methods evaluated within Project 6 have also demonstrated that it is possible to use metabarcoding to compare the diversity of bacteria, fungi and, to a more limited extent, mesofauna across samples of soil. As a trial venture, these approaches have already been used to assess samples of arable soils submitted from growers (including samples from the field sites selected in this project) through the Big Soil Community initiative at Fera Science Ltd. (https://www.fera.co.uk/news/big-soil-community). This involves a community effort between growers, agronomists and Fera scientists to investigate the diversity of UK soils and understand how it can affect crop production and long-term soil health. Since the launch in 2018, over 400 samples have been analysed for fungal and bacterial diversity and an additional nematode screen was also introduced in 2021. Samples have mostly come from country-wide arable growers, enabling comparisons to be made between different variables, including locations, soil types, organic and conventional systems and crop yields.

Participating farmers have submitted samples in October/November and received results in January as two reports; the first detailing the diversity in each soil sample and highlighting the most abundant taxa of interest (including beneficial organisms and pathogens), and the second anonymously benchmarking each result against the wider community picture. Initially costed at £250 per sample, participating growers are aware that they are contributing to the development and interpretation of the tests as well as understanding the diversity of their soils. It is anticipated that the cost per sample will fall as interest in the scheme increases. Sustainability of the scheme is dependent on the usefulness of the information to each grower. Automated methods that identify key taxa and their relative abundances from the soil DNA, developed in this project, are key to increasing the value of information that can be fed back to growers. For example, methods that can automatically predict functions associated with taxa identified within the soil microbial communities will help to add practical value to the results by not only identifying the most abundant organisms but also estimating whether they are providing key ecological services and whether they may be harmful or beneficial to crops. It is expected that this approach will facilitate the identification of bioindicators common to all samples, allowing the development of more targeted field tests that could monitor changes in the behaviour of these indicators as influenced by factors such as cropping practices, soil management, soil types and climate. Furthermore, standardised analytical procedures described under Project 6, that allow analysis of merged DNA sequencing data, will permit comparisons of field assessments from multiple sources, even when there is variation in the methods used to collect the data. This will open the investigation of factors contributing to biological soil health to a much wider breadth of soils and agroecological systems both across the UK and internationally.

5.5. Integration of molecular testing in support of a robust soil health scorecard developed within the SBSH Partnership

Within the SBSH Partnership, both qPCR and metabarcoding procedures have been applied alongside field data collected in Projects 4 and 7 during testing of the standard soil health scorecard approach. Field data accumulated using qPCR, on the population and distribution of the specific plant pathogens *Fusarium oxysporum* and *Verticillium dahliae* across individual field trials is, as yet, insufficient to allow meaningful benchmarking of these levels across soils and cropping systems. These approaches have therefore not been further integrated as part of the soil health scorecard approach.

Nevertheless, it has been possible, through the analysis of soil from the range of selected long term field trials with varying soil types, to demonstrate that robust molecular technology is now available and can be used to identify the effects of soil type and agronomic management on soil biology. Quantification of specific target organisms or of total populations of all bacteria and fungi present in soils has been achieved using qPCR and comparison of the diversities of these bacterial and fungal communities has been demonstrated using a standardised metabarcoding approach. Changes in both numbers and diversity of these communities have been demonstrated in response to agronomic soil management practices, including crop rotation and cover-cropping, pH manipulation, as well as organic and inorganic fertilisation. However, these changes were relatively small when compared with the microbiological changes that were measured between different locations, or between seasons at the same location. In this respect, it will be difficult to formulate general effects on soil biology across all agricultural and horticultural holdings and soil types since different communities may respond to differently to the same management. It is therefore clear that there is a need to systematically collect and analyse data from as wide a range of soils and sampling times as possible before any general management advice can be formulated. More widespread use of the soil health scorecard as an initial screening tool may help to identify specific situations where the molecular data will be most useful in optimising more localised management practices for maintaining or improving soil health.

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Annex 1 – Treatments and layout of onion trial conducted under Project 7a.

The treatments were an autumn sown cover crop, with and without a following spring application of green compost. The remaining plots were left without any organic matter incorporation. Onion sets were planted in Spring 2019, directly after the green compost addition. There were six replicate blocks of four treatments (**Table A1A1 & Figure A1**).

Table A1: Organic matter treatments incorporated before onion planting in April 2019 on Claypits field.

Trt. Number	Treatment
1	Untreated
2	Cover Crop sown in August 2018
3	Green Compost applied in March 2019
4	Cover Crop and Green Compost

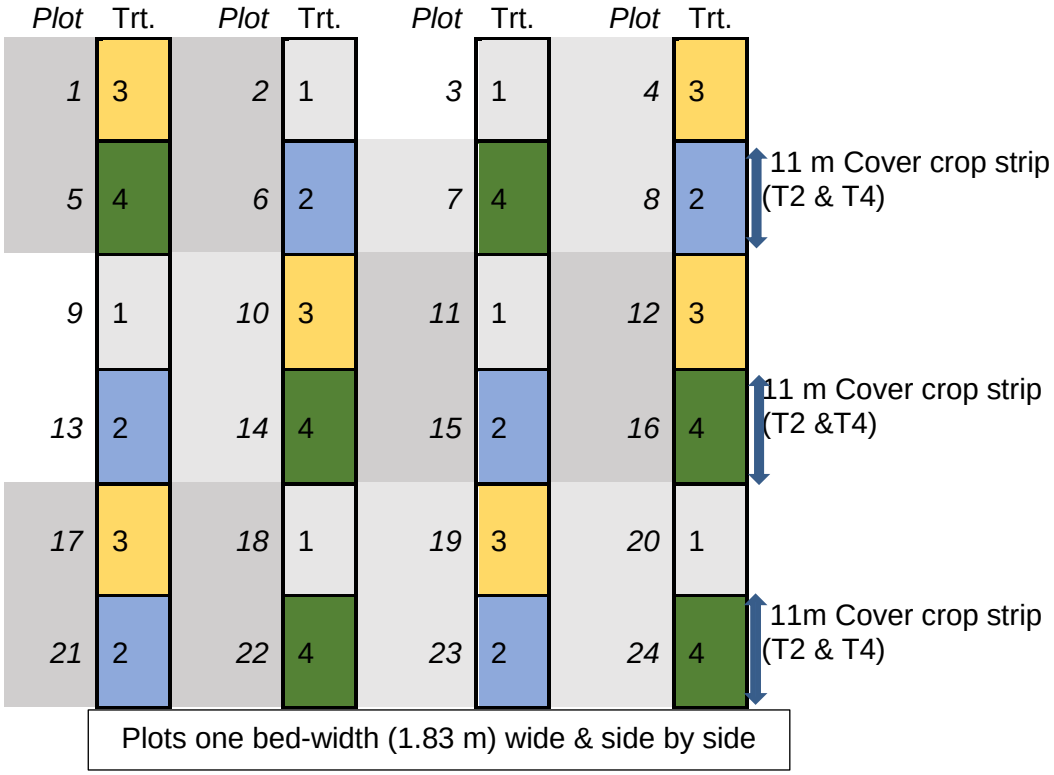

Figure A1: Layout of alternating cover crop strips in cereal stubble Autumn 2018, followed by incorporation of green compost to half the plots in Spring 2019 before onion planting. Plots 1 to 4 were at the southern end of the field, with both the previous cereal crop and the subsequent onion rows running from south to north, while the cover crop strips ran east to west.