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Candidate Causal Organisms for Apple Replant Disease in the United Kingdom

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ABSTRACT

Continuous planting of apple in the same area leads to reduced growth vigor and subsequent crop losses, i.e., apple replant disease (ARD) syndrome. Several soilborne plant pathogens including *Pythium*, *Fusarium*, and *Cylindrocarpus* spp. are often proposed as candidate causal organisms for ARD. In addition, the presence of root lesion nematodes and the lack of beneficial groups of bacteria are believed to exacerbate or ameliorate the effects of the plant pathogens. The importance of these proposed causal and auxiliary agents seems to vary with site. Using a spatially explicit sampling strategy to minimize spatial variability we collected rhizosphere soil samples from neighboring pairs of healthy and putative ARD trees to identify candidate causal organisms of ARD. Amplicon-based metabarcoding was used to obtain community-level profiles of the bacteria, fungi, oomycetes and nematodes in the soil samples. Total bacterial and fungal biomass in each sample was estimated using qPCR to adjust the raw sequence reads data. The results suggested that ARD is not ameliorated by enhanced rhizosphere biodiversity per

se. We identified 25 bacterial operational taxonomic units (OTUs), 16 fungal OTUs, 18 oomycetes OTUs, and one nematode OTU group with differential abundance between healthy and putative ARD trees. All 25 bacterial OTUs had lower abundance in samples from ARD symptomatic trees than from healthy trees. One AMF OTU had lower abundance in samples from ARD symptomatic trees. None of 13 fungal OTUs that had higher abundance in samples from ARD symptomatic trees is a known plant pathogen; but at least one *Pythium* OTU (probably *Pythium intermedium*) is a candidate for causing ARD. The abundance of one nematode OTU was much higher in samples from ARD symptomatic trees than in healthy trees. The results suggest that, apart from the use of broad spectrum fumigants, the complexity of ARD may necessitate the combined use of multiple management strategies with the success of these measures expected to vary considerably between sites because of the varying importance of ARD causal agents among sites.

Poor establishment of apple trees on soils where the same, or a closely related species, has grown previously is a well-known problem for growers and tree nurseries worldwide (Agrios 1988;

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Auvil et al. 2011). This problem is commonly known as apple replant disease (ARD). Symptoms of ARD include uneven growth, stunting and shortened internodes above ground and discolored roots, root tip necrosis, and reduced root biomass below ground. The destruction of root tissues by a suite of cell wall degrading enzymes and cell-killing effector proteins, produced by both microbial and nematode components of ARD (Grabke et al. 2011; Isman 2000; Lobet et al. 2011; Mazzola and Gu 2000; Mazzola et al. 2008), impairs root function hampering plant responses to abiotic stresses such as drought, flooding, and nutrient deficiency (Mazzola and Manici 2012). Although the growth rates of surviving trees are similar to those of unaffected trees, they start bearing fruit some 2 to 3 years later with yields reduced by up to ~60% for the duration of the tree’s commercial life (Mazzola and Manici 2012; Mitchell 1978).

Current ARD management strategies are generally based on the principles of exclusion (crop rotation and tree placement within the orchard) or environmental alterations to modify the existing soil

microbial community (e.g., fumigation). Crop rotation exploits the natural decline in pathogen viability in the absence of a susceptible host and can be used effectively to reduce the severity of some diseases, especially in annual crops. However, it is a less practical option for perennial crops (Slykhuis 1990) and also economically unattractive to growers and nurseries specializing in fruit trees. Nonetheless, the exclusion of apple orchards from a site for 5 to 8 years is commonly recommended to reduce ARD severity (http://www.tfrec.wsu.edu/pages/cpg/Special_Programs). Since the 1950s, soil fumigation prior to planting has been the most common method to achieve effective control of ARD around the world (Tewoldemedhin et al. 2011a). Traditional soil fumigants (e.g., methyl bromide, metam sodium and chloropicrin) have a broad spectrum of activity on pathogenic and nonpathogenic microorganisms alike. However, over the last 30 years environmental and safety concerns have led to international legislation to prohibit, or severely restrict, their use. Thus, there is a need for alternative management strategies including more selective approaches for the modification of indigenous soil microbial communities, e.g., the addition of antagonistic and beneficial microorganisms (Tewoldemedhin et al. 2011a), incorporation of composted organic amendments (Mazzola et al. 2015) and the use of biofumigants (especially brassica seed meals) (Auvil et al. 2011; Wilcox 1990), and specific orchard ground cover management regimes (Utkhede and Smith 2000).

Several candidate organisms have been proposed as causal agents for ARD, such as *Pythium*, *Fusarium*, and *Cylindrocarpon* spp. (Mazzola and Manici 2012); these microbial genera contain well-known soilborne plant pathogenic species responsible for inducing a range of damping-off and root rotting symptoms in plants (Chaparro et al. 2014). However, the reports of these microbes causing replant disease on their own, or in combination, are inconsistent (Franke-Whittle et al. 2015; Mazzola 1998; Tewoldemedhin et al. 2011a, b); especially with respect to the role of root lesion nematodes such as *Pratylenchus penetrans* (Cobb) (Mai and Abawi 1978; Mazzola 1998). In addition to reports of the actions of deleterious microbes and nematodes, there are similarly variable accounts of increased growth and yield of apple trees grown in replant soils following the development of some specific fungal, pseudomonad, and other beneficial bacterial communities in the rhizosphere (Yao et al. 2006; Yim et al. 2015).

Next generation sequencing has greatly increased our understanding of soil microbial communities in many contexts, including that relating to crop health (Mazzola et al. 2015; Peruzzi et al. 2017; Yim et al. 2015). However, interpretation of the data produced can be difficult because often several hundreds of microbial groups (operational taxonomic units [OTUs]) differ in their relative abundance between contrasting samples. Also, the acquisition of a mechanistic understanding of soil microbial community composition is further hindered because most of the OTUs cannot be identified to genus level with confidence. We have previously used next generation sequencing to show that plant species is the principal determinant of the contrasting microbial communities which develop in the grass aisles and in the rows of trees in apple orchards (Deakin et al. 2018). Several hundreds of microbial groups differed in their relative abundance between the grass aisles and rows of trees and served to obscure what in all likelihood is a very small number of causal agents. Thus, identification of candidate organisms of ARD requires a sampling scheme in which the confounding effects of plant species and spatial variability are controlled.

In the present study we adopted a sampling strategy which has previously been successfully used by Wei et al. (2016) to identify

candidate causal organisms for strawberry yield decline. Specifically, we sampled rhizosphere soils from 15 pairs of neighboring trees (one apparently healthy and one showing ARD symptoms) selected at random. Next generation sequencing was then used to profile the communities of bacteria, fungi, oomycetes, and nematodes present in the rhizosphere of the apple trees and identified candidate causal organisms for ARD.

MATERIALS AND METHODS

Study site and soil sampling. The study site is an intensively managed orchard located on level ground near East Farleigh in Kent, U.K. (latitude: 51.24145, longitude: 0.46688, 105 m a.s.l.). Between 1988 and 2009, this orchard was cultivated with dessert apple trees (*Malus pumila* Miller) cultivar Cox on M.26 rootstock. In 2009, the orchard was replanted with *M. pumila* 'Gala' on M.26 rootstock. Symptoms attributed to ARD, i.e., greatly reduced growth and stunting of shoots were first noted in 2011, and the number of affected trees and the severity of symptoms have increased with time.

In order to reduce the influence of the inherent soil-related spatial variability in soil microbial community structure on detecting candidate ARD microbes, 15 pairs of neighboring trees (one apparently healthy and one showing ARD symptoms) were randomly selected for sampling of rhizosphere soils. Thus, the design was a randomized block design with 15 blocks (locations or pairs), each with two treatments (ARD and healthy). Sampling took place on 5 and 6 October 2016 to coincide with a phase of active root growth in apple trees and a period of enhanced soil microbial activity (Mazzola 1998). Fine roots (i.e., <2 mm diameter, nonwoody, feeder roots) from the zone where the propagules of soilborne plant pathogens are present at greatest density (i.e., ~10 to 20 cm depth and a distance of 50 cm from the trunk) were exposed and excised at three points around each tree (Horner and Wilcox 1996). Old and dying roots were not sampled to reduce the confounding incidence of secondary saprotrophic organisms (Tewoldemedhin et al. 2011b). Excised roots were shaken free of loosely attached soil and the remaining adhering soil was considered to be rhizosphere soil, which was removed with the aid of a sterile spatula (Bramwell et al. 1995) and collected in separate labeled, sealable polythene bags. Bags were placed on dry ice in the field and immediately transferred to a -80°C freezer when returned to the lab for storage prior to DNA extraction.

Soil characterization. Sixty-gram aliquots of soil from five neighboring healthy or ARD symptomatic trees were composited and homogenized by sieving to 2 mm in the field-moist state. Soil pH was measured in water (1:2.5, wt/vol), available K and Mg were quantified in 1 M ammonium nitrate extracts by atomic absorption spectroscopy, hot water extractable B, and cold water extractable SO_4^{2-} were quantified by ICP-OES and available P was quantified colorimetrically in 1 M bicarbonate extracts all according to MAFF/ADAS (1986). Soil organic matter content was determined by loss on ignition (550°C). Other extractable cations were quantified by atomic absorption spectroscopy. Ammonium acetate (1 M) was used to extract Ca, Na, Mn, and Fe and 0.05 M EDTA was used to extract Cu and Zn. Potassium sulfate extractable (0.5 M) $\text{NO}_3\text{-N}$ and $\text{NH}_4\text{-N}$ were determined colorimetrically according to Cataldo et al. (1975) and Anderson and Ingram (1993), respectively. Particle size distribution across three classes: sand (2.00 to 0.063 mm), silt (0.063 to 0.002 mm), and clay (<0.002 mm) were determined by laser diffraction, with subsequent texture attribution according to Avery (1973).

DNA extraction and sequencing. *DNA extraction.* Total genomic DNA was isolated from each of the three replicate 0.25 g

aliquots of soil collected from each tree using the PowerSoil DNA Isolation Kit (MoBio Laboratories) with minor modifications as described below. Before bead-beating, the samples were incubated in lysis solution at 65°C for 10 min. Samples were homogenized by two 20 s cycles at a power setting of 5.0 in a FastPrep instrument (FP120, Bio 101, Thermo Savant, Qbiogene), with 5 min on ice between the cycles. DNA was purified according to the kit protocol, quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific) and diluted to 2 ng μL^{-1} . The DNA from the three subsamples was then pooled, giving a total of 30 samples before PCR amplification (i.e., 15 biological replicates).

PCR. Nonoverlapping variable internal transcribed spacer (ITS) regions of ITS1 and ITS2 in the fungal genome were amplified using the primers EkITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') (Gardes and Bruns 1993) and Ek28R (5'-AT ATG CTT AAG TTC AGC GGG-3') ($\approx$ 3126T, Sequerra et al. 1997, and HC2 Navajas et al. 1994). This primer pair was chosen as we have shown previously that it gave better amplification when combined with the Illumina adapters (Xu et al. 2015). The V4 variable region of the 16S rRNA gene was amplified using the primers Bakt_341F (5'-CCT ACG GGN GGC WGC AG-3') and Bakt_805R (5'-GGA CTA CHV GGG TAT CTA ATC C-3') (Herlemann et al. 2011). The two primer sets were modified at the 5' end with adapters: TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG (forward adaptor) and GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA (reverse adaptor). ITS PCR conditions consisted of an initial denaturation step at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 60 s, elongation at 72°C for 60 s, and concluded with a final elongation step at 72°C for 7 min. PCR conditions for amplification of the 16S rRNA were as above, but the number of cycles was reduced to 25 and the annealing temperature was increased to 55°C.

The ITS1 region of the oomycete genome was amplified using primers ITS6 forward (5'-GAAGGTGAAGTCGTAACAAGG-3') (Cooke and Duncan 1997; White et al. 1990) and ITS7 reverse (5'-AGCGTTCTTCATTCGATGTGC-3') (White et al. 1990); PCR conditions were as above but with only 15 cycles. The 18S small subunit rRNA gene of the nematode genome was amplified using primers Nem18s forward (5'-CGCGAATRGCTCATTACAA CAGC-3') and Nem_18s reverse (5'-GGGCGGTATCT GATCGCC-3') (Floyd et al. 2005). PCR conditions consisted of an initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 57°C for 45 s, elongation at 72°C for 90s, followed by a final elongation step at 72°C for 7 min.

All PCR reactions were carried out in 1 $\times$ PCR buffer minus Mg (Invitrogen, Life Technologies), 2 mM MgCl_2 (Invitrogen, Life Technologies), 0.2 mM dNTP (Fisher Scientific), 0.2 mM forward and reverse primers each (Integrated DNA Technologies), 1 U of Platinum Taq DNA Polymerase (Invitrogen, Life Technologies), and 8 ng of template DNA; the reaction volume was complete to 25 μL with molecular biology reagent water (Sigma).

PCR clean up and next generation sequencing. PCR amplicon clean-up followed the manufacturer's instructions and used a bead based clean-up and size selection step (Agencourt XP Ampure beads). The fungal and bacterial amplicons originated from a single template and were pooled (according to individual soil samples) with the separate nematode and oomycete amplicons, before ligation to Illumina compatible adapters using a Nextera XT Index sample barcoding kit (Illumina). The quality of the final products was quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific) and a Qubit2.0 fluorometer (Life Technologies). The samples were diluted to 10 ng of DNA μL^{-1} and pooled in a 4 nM

library. The amplicon library was denatured using 1 mM NaOH and diluted to 17.5 pM as per the manufacturer's protocol. The diluted and denatured amplicon library was then combined with a denatured PhiX library at an equimolar concentration at a rate of 20% to increase heterogeneity of the samples, before sequencing using the 300 bp paired end protocol on an Illumina MiSeq running Miseq Reagent kit V3 (Illumina) (16 cycles indexing and 301 cycles, total 618 cycle run).

Quantification of 16S and ITS gene abundance. The absolute abundances of the target genes 16S rRNA and ITS, as proxy measures of microbial biomass for bacteria and fungi, respectively, were estimated by qPCR and the standard curve method using a CFX96 Touch Real-Time PCR Detection System with a C1000 Thermal Cycler (Bio-Rad). For fungi, ITS1-F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTT CTTCATCGATGC) were used as described previously by Badia-Fabregat et al. (2017), Op De Beeck et al. (2014), and Adams et al. (2013). The PCR thermocycling conditions were as follows: an initial denaturation at 95°C for 3 min; followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final extension phase at 72°C for 5 min, concluding with a melt curve (increasing from 65 to 95°C in increments of 0.5°C every 5 s). The reaction mixture contained 5 μL of qPCRBIO SyGreen mix Lo-ROX (PCR Biosystems), 0.3 μL of each primer (10 μM), 1.9 μL of nuclease-free water, and 2.5 μL of DNA, giving a total volume of 10 μL per well. Nuclease-free water was used in place of the DNA for nontemplate controls and standard curves were created using 10-fold serial dilutions of fungal (apple scab *Venturia inaequalis*) DNA from 10^0 to 10^{-4} with a starting quantity of 5 ng. Each sample was run in triplicate and the mean of the starting quantity used for further calculations.

The 16S rRNA target gene for the bacterial community was quantified following the method of Lansdown et al. (2016) using two sets of primers: Bakt_341F (CCTACGGGNGGCWGCAG) and Bakt_805R (GACTACHVGGGTATCTAATCC) for the 16S rRNA gene (Klindworth et al. 2013). The 16S rRNA abundances were quantified against a standard curve constructed from the purified PCR product using these primers with 10^1 to 10^6 copy numbers. The reaction volume was 20 μL and contained 400 nM of primers and 1 μL of DNA template. PCR conditions were 95°C for 2 min followed by 40 cycles at 95°C for 10 s then 60°C for 30 s. Each sample was run in duplicate and the mean copy number was used for further calculations. All raw sequencing data have been deposited in the GenBank SRA database under accessions SRR7281082 to SRR7281141.

Bioinformatic analysis of sequence reads. FASTQ reads were demultiplexed into bacterial (16S), fungal (ITS), oomycete (ITS), and nematode (18S) datasets based on their primer sequences. Reads with any ambiguous positions in the primer region, or nonmatching forward and reverse primers were discarded. All operational taxonomic unit (OTU) processing was carried out with the UPARSE 10.0 OTU clustering pipeline (Edgar 2013) unless specified otherwise.

As recommended in the UPARSE pipeline, sequences were processed in two steps. First, sequences were filtered with very stringent criteria to generate OTUs and to obtain the representative sequence for each OTU. In the second step an OTU table (a sample-by-observation contingency table summarizing the frequency of each OTU in each sample) was generated, for which all sequences were filtered with far less stringent criteria and then aligned with the OTU representative sequences generated in the first step.

Generating OTUs and assignment of taxonomic rank. Two different approaches were used for (i) 16S bacterial and oomycete

ITS data, and (ii) fungal ITS and nematode 18S data as described below.

- (i) The 16S forward and reverse reads were merged with a maximum difference in overlap of 5% and the forward and reverse primers removed. Merged reads containing fragments of adapter sequences or fewer than 300 nucleotides were excluded from further analysis. Oomycete ITS reads were treated in the same way, but with the overlap difference set to 10% and the minimum length set to 150 nucleotides. Remaining merged reads were filtered for quality with a maximum expected error threshold of 0.5 per sequence (Edgar and Flyvbjerg 2015).
- (ii) The expected distance between fungal ITS and nematode 18S primers was larger than twice the MiSeq read length. Consequently, forward and reverse reads could not be merged, and thus only the forward reads were used and treated as unpaired reads. Unpaired reads that contained forward/reverse primers or the adapter sequence were excluded from analysis. An addition step was taken for nematodes, due to nonspecific amplification of prokaryote DNA, or unidentifiable sequences. These nonspecific sequences tended to have overlapping forward and reverse reads. Therefore, read pairs were merged (maximum 15% difference in overlap) and only nonmerging forward reads were retained. Reads shorter than 200 nucleotides for fungal and 150 nucleotides for nematode were discarded. Forward primers were removed, and then reads were quality filtered with an expected error threshold set to 1 per sequence.

Separately for each of the four datasets, sequences were dereplicated and unique sequences with fewer than two reads were discarded, then all unique sequence reads were sorted by their respective frequencies. These unique reads were clustered into OTUs at the level of 97% similarity and a representative sequence for each OTU was generated. The clustering algorithm also removed chimeras during clustering process. The UCLUST algorithm (http://drive5.com/usearch/manual/tax_conf.html) then assigned each OTU representative sequence to taxonomic ranks by alignment with the gene sequences in the reference databases “Unite V7” (fungal ITS) (Koljalg et al. 2013) and “RDP training set 15” (16S) (Cole et al. 2014). For 18S and oomycete ITS, the SINTAX algorithm (Edgar 2016) was used to assign taxonomy, using for both (separately) a subset of the SILVA_ssu database (Glöckner et al. 2017) covering Stramenopiles and Nematoda. Additionally the oomycete ITS database was combined with the Unite V7 fungal database.

OTU table generation. For 16S and oomycete ITS, the original sequence reads were merged with a maximum difference in the overlap set to 15%, and the merged pairs were aligned to the OTU representative sequences. For fungal ITS and 18S, the original unfiltered forward reads were aligned separately to the OTU representative sequences. In all clustering analyses the threshold similarity value was set to 97%.

Statistical analyses. All statistical analyses were carried out in R 3.4.0 (R Core Development Team 2008).

Library size normalization. Library size normalization was performed using two methods: (i) for all microbial communities using the median-of-ratios method implemented in DESeq2 (Anders and Huber 2010; Love et al. 2014) to produce size factors for each sample; and (ii) except for nematode, setting the size factors for each sample to the mean of the sample qPCR results. The abundance data for each sample was then divided by their respective size factor to produce two separate library size normalized datasets; raw (median-of-ratios normalized) data and qPCR data. All analyses were performed on both sets of normalized reads separately.

The qPCR adjustment (using the fungal qPCR results) was able to be applied to the oomycete sequencing data because the qPCR and oomycete sequencing samples were from the same source and the oomycete primers were not specific and also amplified fungi. Therefore, sequencing gave an abundance ratio of oomycete to fungi for the original sample, and the fungal portion can be adjusted by the qPCR values. To keep the proportions the same the oomycete abundance must also be adjusted by the same values.

Where appropriate, library size normalized reads were transformed using the DESeq2 variance stabilization transformation (VST). Principal component analysis (PCA) was applied to the VST-transformed OTU counts, to produce principal component (PC) scores per sample. Nonmetric multi-dimensional scaling (NMDS) of the Bray-Curtis distance used library size normalized reads due to the fact that VST may produce negative values, which cannot be used in NMDS. Supplementary Figure S1 is a schematic showing the how data were processed. All analyses were carried out separately for fungal, bacterial, nematode, and oomycete data.

Oomycete ITS OTUs which were not assigned by SINTAX to the stramenopile (heterokonts), alveolate, and Rhizaria (SAR) clade were excluded from all statistical analyses postsize normalization. Nematode OTUs that had a SINTAX confidence of less than 90% at the order level were excluded from all statistical analyses. OTUs with fewer than five reads across all samples were excluded from statistical analysis (except when calculating alpha diversity). Samples for which sequencing failed, or with very few total reads relative to other samples, together with the samples of their corresponding healthy or ARD symptomatic trees, were excluded as well.

Diversity indices. Alpha (α) diversity (Chao1, Shannon, and Simpson) indices were calculated using the R vegan 2.3-1 package (Dixon 2003). The rank of (α) diversity indices were subjected to analysis of variance (ANOVA) to assess the differences between healthy and ARD symptomatic trees via a permutation of significance. Beta (β) diversity was estimated from a distance matrix that was calculated from an appropriate OTU table with the USEARCH 10.0 calc_distmx function. Neighbor-joining trees were made from the distance matrices using the ape 3.4 package for R (Paradis et al. 2004). The R package Phyloseq 1.12.2 (McMurdie and Holmes 2013) was used to calculate weighted (by OTU count) UniFrac distances between samples. Permutation multivariate ANOVA (PERMANOVA) as implemented in R with vegan 2.3-1 was carried out to assess the differences between healthy and ARD symptomatic trees.

Differential OTU abundance. In addition to the overall comparison among samples in relation to tree health status through PCA of the VST-transformed OTU counts and NMDS of the Bray-Curtis dissimilarity matrix, DESeq2 was used to detect OTUs with differential relative abundances between healthy and ARD symptomatic trees. Both raw and qPCR-adjusted OTU count data were analyzed without rarefaction (McMurdie and Holmes 2013) using DESeq2. In addition, DESeq2 also implements an algorithm for the automatic filtering of OTUs before differential abundance analysis. Several criteria are used for automatic filtering, including variance in abundance across samples and overall abundance level. To correct for the false discovery rate associated with multiple testing, the Benjamini-Hochberg (BH) adjustment was used with DESeq2 (Benjamin and Aikman 1995). Statistical significance was determined at the 5% level (BH adjusted).

BLASTn searches against the GenBank nonredundant database was then used to further characterize significant OTUs. For fungal OTUs this included an additional step to include sequence covering the ITS2 region. Forward reads aligning to each OTU and their reverse pairs were extracted. Reverse reads were truncated to 250

bases to remove poor quality ends and the pairs were then joined using USEARCH fastq_join with default parameters. The joined pairs were quality filtered with a maximum expected error threshold of three per sequence (Edgar and Flyvbjerg 2015), and clustered separately for each OTU at 97% similarity. Clusters comprising the largest proportion of reads (where more than one) were taken as the representative sequence for the BLASTn search.

OTUs with differential expression have been deposited in the GenBank nucleotide database under accessions MK184957 to MK184981 (bacteria), MK192880 to MK192896 and MK192861 to MK192877 (fungi), MK192833 to MK192860 (oomycetes), and MK184983 (nematodes).

RESULTS

Soil physico-chemical properties. The soil at the site was a profundic chromic Luvisol with clay loam texture (42% sand, 32% silt, and 26% clay). Reflecting the small sampling scale used, there were no statistically significant differences in the physico-chemical properties of the rhizosphere soil associated with healthy and ARD symptomatic trees (Table 1).

Data filtering and OTU information. Primary filtering of the data to remove sample pairs containing an individual sample with a very low number of mapped sequences (<1,000 total sequences,

Supplementary Fig. S2) reduced the number of sample pairs to 14 for bacteria, fungi, and oomycetes and to 11 for nematodes. Therefore, all discussed results are based on 11 paired samples for nematodes and 14 paired samples for the other three microbial communities. OTUs representing the SAR clade were also removed from the oomycete data. Finally, all OTUs with very low total counts were excluded from further statistical analysis, except for calculation of alpha diversity indices (Table 2). The overall summary of sequence and OTU processing is given in Supplementary Table S1.

Rarefaction analysis showed that the present sequencing depth was sufficient for all of the samples retained after primary filtering (Fig. 1, Supplementary Fig. S3). Although there are large numbers of OTUs within all samples, a small proportion of these OTUs accounted for the majority of mapped reads. For instance, out of the total 1,993 fungal OTUs, the top-most 225 OTUs accounted for more than 90% of mapped reads (Fig. 1).

The statistical confidence with which fungal OTUs could be mapped onto known taxonomic units was much less than for bacteria. For example, 49.1% of fungal OTU representative ITS sequences could not be mapped to a rank lower than kingdom fungi with >90% confidence, but 92.6% of bacterial OTU representative 16S sequences could be mapped to a taxonomic rank lower than kingdom bacteria (Supplementary Table S2). The BLASTn search results of randomly selected sequences from these “unknown” OTUs

TABLE 1
Selected physico-chemical properties of soil associated with healthy trees without obvious symptoms of apple replant disease (ARD) and trees with symptoms attributed to ARD^a

Physico-chemical property	Healthy	ARD symptomatic	P value
pH	6.5 (0.12)	6.6 (0.03)	0.802
Organic matter (g kg ⁻¹)	55.0 (3.60)	59.3 (2.91)	0.402
Nitrate (mg kg ⁻¹)	32.6 (1.82)	29.1 (1.92)	0.259
Ammonium (mg kg ⁻¹)	8.1 (0.44)	8.9 (0.48)	0.296
Phosphorus (mg kg ⁻¹)	66.2 (3.53)	69.8 (1.33)	0.395
Sulfate (mg kg ⁻¹)	43.7 (3.89)	46.0 (2.50)	0.643
Boron (mg kg ⁻¹)	2.3 (0.26)	2.4 (0.13)	0.724
Calcium (mg kg ⁻¹)	2,687 (134.5)	2,711 (167.1)	0.919
Copper (mg kg ⁻¹)	25.7 (2.43)	26.2 (2.00)	0.891
Iron (mg kg ⁻¹)	223 (34.2)	236 (11.8)	0.739
Magnesium (mg kg ⁻¹)	143 (2.2)	140 (1.0)	0.238
Manganese (mg kg ⁻¹)	19.6 (2.27)	21.1 (2.42)	0.672
Potassium (mg kg ⁻¹)	429 (20.2)	426 (13.2)	0.907
Sodium (mg kg ⁻¹)	28.2 (0.21)	26.7 (1.84)	0.459
Zinc (mg kg ⁻¹)	24.8 (3.27)	26.0 (1.64)	0.769

^a Nutrient values are extractable and all values are the mean of three replicates with the standard error of mean given in parentheses.

TABLE 2
Summary of the number of operational taxonomic units (OTUs) before and after filtering, and with significant differential relative abundance in rhizosphere samples from healthy and apple replant disease symptomatic apple trees via DESeq2 analysis

Number of OTUs	Bacteria		Fungi		Oomycetes		Nematodes
	Raw data	qPCR-adjusted	Raw data	qPCR-adjusted	Raw data	qPCR-adjusted	
Unfiltered	3,094	3,094	1,983	1,983	100	100	61
Low counts filtered	3,067	3,088	1,903	1,968	99	99	54
After DESeq2 filtering	927	1,053	1,903	671	68	80	54
With Benjamini-Hochberg adjusted $P < 0.05$	11	22	5	16	22	31	1

confirmed the uncertainty in assigning taxonomy to these OTUs: (i) some OTUs had 99 to 100% similarity with sequences in the NCBI, but these were all from unidentified bacterial or fungal strains, (ii) some OTUs had 97% similarity with sequences from more than one distinct taxonomic unit (one or more could be uncultured/unidentified strains), and (iii) some OTUs did not have 97% similarity with any sequences in the NCBI database.

Based on the DESeq2 normalized sequence data, the three most abundant bacterial phyla were *Proteobacteria* (40.3%), *Acidobacteria* (20.2%), and *Bacteroidetes* (12.7%) (Fig. 2); and for fungi the three most abundant phyla were Ascomycota (57.3%), Basidiomycota (22.6%), and Zygomycota (2.5%) (Fig. 2). Adjustment of the raw sequence data according to qPCR data of individual samples had little impact on the overall profiles of both bacterial and fungal communities (Fig. 2).

For the oomycete data, only 100 of 317 OTUs were assigned to the SAR clade, with 7 assigned to kingdom plantae and 210 to kingdom fungi. BLASTn searches for a random selection of the “plant” and “fungal” OTUs confirmed their likely identity. For 86 of the 100 oomycete OTUs, taxonomy predictions made by the SINTAX algorithm had confidence estimates >0.75 at the kingdom taxonomic rank. BLASTn searches for all the remaining 14 OTUs confirmed them to be (probable) members of the SAR clade. More than 80% of oomycete reads were classified to Pythiaceae (Supplementary Fig. S4). The SINTAX taxonomy predictions for the 61 nematode OTUs had confidence estimates ≥ 0.9 at the order level and the likely identity of a random selection was confirmed by BLASTn searches. However, for a random selection of OTUs below this cut-off value, BLASTn was either unable to identify homologs with certainty, or identified the sequences as being of bacterial origin.

Fig. 1. Cumulative number of mapped internal transcribed spacer sequences combined for all samples over the total 1,993 fungal operational taxonomic units (OTUs) arranged on a descending order by number of sequences per OTU. The vertical lines indicate the proportion of the total sequence count contained within the number of OTUs on the x-axis, e.g., 90% of all sequences are contained within approximately the 225 most abundant OTUs.

In all cases, the uncertainty in assigning OTUs to specific taxonomic ranks was not associated with the sample tree status (ARD or healthy).

Rhizosphere microbial community diversity. The ratio of bacterial quantification with fungal quantification per sample was tested for significant difference between healthy and ARD symptomatic samples using ANOVA with pairs of trees and tree healthy status as two factors, but no statistically significant difference was found ($F_{1,14} = 0.015$, $P > 0.90$).

Although alpha diversity was very variable between samples, for library size normalized data (i.e., non-qPCR-adjusted data), there was a statistically significant difference between the fungal diversity (Chao1) in the rhizospheres of healthy and ARD symptomatic trees ($P < 0.05$): Chao1 = 561.60 ± 18.92 for ARD symptomatic trees compared with 648.86 ± 24.93 for the healthy trees (Fig. 3). For alpha diversity calculated using qPCR-adjusted data there was no comparable significant difference between healthy and ARD symptomatic trees (Supplementary Fig. S5). For bacteria, irrespective of whether the reads were normalized by qPCR data or not, there were no significant differences between healthy and ARD symptomatic trees for any of the alpha indices (Supplementary Figs. S6 and S7). Similarly, for oomycetes and nematode, none of the alpha indices differed significantly between samples from healthy and ARD symptomatic trees (Supplementary Figs. S8, S9, and S10).

NMDS ordination of dissimilarity for Curtis-Bray beta diversity indices for bacterial community composition showed that the differences between healthy and ARD symptomatic tree samples were very small (Supplementary Fig. S11). Permutation MANOVA analysis of weighted UniFrac distances indicated that although

Fig. 2. Proportion of DESeq2 normalized (black) or qPCR-adjusted (orange) sequence reads assigned to different phyla at ≥ 90 confidence for **A**, bacteria and **B**, fungi. For both kingdoms, phyla accounting for less than 1% of reads were summed into the “others” group and operational taxonomic unit that could not be reliably assigned to a phylum were grouped together under the respective kingdom.

there was a statistically significant difference between ARD symptomatic and healthy trees ($P < 0.05$), the difference only explained ca. 5% of total variability. In contrast, the differences between pairs of trees, i.e., their spatial location within the orchard, accounted for ca. 65% of the variation in the dataset. Similar results were obtained when the UniFrac distance was weighted by qPCR data. For PCA of raw read data, only differences in PCA3 were significant ($P < 0.01$) between healthy and ARD symptomatic samples (Fig. 4A). The influence of spatial location on community structure was also emphasized by PCA and was far greater than the variation due to health status (Supplementary Table S3). For bacteria the differences between pairs of trees were significant for both PCA1 and PCA3 ($P < 0.01$), and were close to significance for PCA2 ($P = 0.056$). For reads adjusted by qPCR data, none of three PCAs differed significantly between healthy and ARD symptomatic samples (Supplementary Table S4) and between pairs of trees were significant for PCA2 only ($P < 0.01$).

NMDS analysis and PCA showed that the overall differences in the fungal community between healthy and ARD symptomatic tree samples were also very small. For both raw and qPCR-adjusted fungal data, differences in the first three PCAs between healthy and ARD symptomatic tree samples were not significant. In contrast, differences between pairs of trees (spatial location) were significant ($P < 0.01$) for all PCAs (Fig. 4B), except for PCA2 of the raw counts data. The effect of spatial location accounted for ca. 97, 86, and 78% of the total variability in PCA1, PCA2, and PCA3, respectively. Permutation MANOVA analysis of weighted UniFrac distances also indicated the difference between ARD symptomatic and healthy trees was not significant, but differences among tree pairs were highly significant. Similar results were obtained when the UniFrac distance was weighted by qPCR data.

NMDS analysis and PCA showed that there was not much overall difference in the nematode community between healthy and ARD symptomatic tree samples. Differences between ARD symptomatic and healthy tree samples were only significant ($P < 0.05$) for PCA3. UniFrac distance did not differ between the ARD symptomatic and healthy tree samples. Similar results were obtained for oomycetes, with the difference between tree pairs accounting for greater variability in the oomycete community than in the nematode community and difference in the health status was distinguishable in PC2 for qPCR-adjusted data (Supplementary Fig. S12).

Differential abundance of individual OTUs. Many OTUs were filtered out by the DESeq2 default criteria, particularly in the bacterial dataset where approximately two thirds of OTUs were discarded. Up to 24 OTUs had significant differences in their relative abundance between ARD symptomatic and healthy tree samples for each type of microbe (Table 2). Tables 3, 4, and 5 give the summary of those OTUs with differential abundance, together with their possible roles summarized from published literature. Supplementary Tables S5, S6, S7, and S8 present frequencies of top 200 bacterial, fungal, oomycetes, and nematode OTUs in each sample.

DESeq2 analysis of retained bacterial OTUs as raw and qPCR-adjusted sequence data identified a total of 25 unique OTUs with significant (BH adjusted $P < 0.05$) differential abundance between ARD symptomatic and healthy tree samples (Table 3). The abundance of all 25 OTUs showed a modest reduction in the rhizosphere of ARD symptomatic trees relative to the rhizosphere of healthy trees with the log₂-fold change ranging between -0.72 and -3.2 (Table 3). None of these OTUs can be reliably classified to the genus rank, but eight OTUs were from phylum *Acidobacteria* and five were from phylum *Verrucomicrobia* and the responsive taxa were predominantly rhizosphere competent free-living heterotrophs and root endophytes.

The relative abundances of three fungal OTUs were greatly reduced (i.e., virtually absent) in rhizosphere soil from ARD

symptomatic trees, with the log₂-fold change for these OTUs ranging between -10.2 and -27.1 (Table 4). These OTUs were attributed to uncultured Agaricomycetes and uncultured Glomeromycota, taxa which contain root symbionts and wood decay fungi able to decompose selectively either the hemicellulose and cellulose, or lignin fractions of dead wood. The log₂-fold change of the remaining 13 OTUs ranged between 9.9 and 21.1 (Table 4), which indicates that the rhizosphere fungal community of healthy trees is depleted with respect to saprotrophs and pathogens.

Eleven oomycete OTUs had a substantially lower relative abundance in rhizosphere soil associated with ARD symptomatic trees with the log₂-fold change ranging from -5.7 to -30.0 (Table 5). Four OTUs were *Pythium* spp. (potentially either saprotrophs or pathogens); they also included an OTU (*Lagenidium* sp.) as a known parasite of *Pythium* sp. and nematodes, and several OTUs as pathogens of nonapple hosts. For the other 17 OTUs, the log₂-fold change ranged from 5.1 to 29.9 (Table 5), indicating that these OTUs were virtually absent in the rhizosphere of healthy trees. Many of the OTUs were associated with pathogens of brassicas, including the ruderal weed species prevalent in the tree rows but *Pythium intermedium* is a known root pathogen of replanted apple trees.

Only 1 of the 61 nematode OTUs had differential relative abundance between ARD symptomatic and healthy tree samples (Table 5). *Paratylenchus projectus* is more abundant (log₂-fold change = 12.3) in the rhizosphere of ARD trees than in healthy trees.

DISCUSSION

The etiology of ARD has been difficult to elucidate for several reasons. Firstly, unlike many other soilborne diseases no single causal agent has been consistently identified across affected sites. Not only have different types of microorganisms and nematode

Fig. 3. Chao1, Shannon, and Simpson alpha (α) diversity measures for fungal raw data from healthy (H) and apple replant disease symptomatic (S) trees. The Shannon measure was calculated using the base of the natural logarithm. The Chao1 metric includes estimates of unseen species and therefore error bars are given to indicate standard error.

worms been proposed as causal agents, their relative importance may vary considerably among sites (Mazzola and Manici 2012). Secondly, until the development of culture-independent molecular tools that could profile soil microbiota without apparent biases, the identification of microbial causal agents was reliant on traditional culture techniques to which only a small proportion of microorganisms are amenable (Bakken 1997). Thirdly, the search for microbial causal agents has been limited primarily to comparing bulk and/or rhizosphere soils from trees of contrasting health status following the application of various soil treatments, or comparisons of healthy and ARD symptomatic trees from different orchards/plots, or based on assumptions of ARD risk associated with different locations within orchards, e.g., comparisons of the soil microbial community in the aisles (low ARD risk) with that in the tree rows (high ARD risk). These strategies generally lead to the discovery of a large number of microbial groups with differential abundance between the variously defined “ARD” and “non-ARD” samples because of the effects of soil amendment on soil microbial communities (Peruzzi et al. 2017; Yim et al. 2015), the large variability in soil microbiota even at very small spatial scales (Buckley and Schmidt 2001b; Naveed et al. 2016), and the inherent microbial association with plant species (Burns et al. 2015) and hence vegetation communities (Deakin et al. 2018).

We used a sampling scheme that controlled small-scale spatial variability to identify candidate causal organisms of ARD. Sampling paired trees reduced the influence of spatial variation in microbial community structure as much as practically possible because samples with close physical proximity had greater similarity to each other than with other sample pairs (Fig. 4). Thus, we have more confidence that OTUs with differential abundance between two neighboring (ARD and healthy) trees are more likely to be associated with the tree healthy status than due to spatial variability in soil microbial

communities. This expectation is supported by the present results: the number of OTUs with differential abundance between ARD symptomatic and healthy trees was greatly reduced. Thus, only 1 and 1.5% of bacterial and fungal OTUs, respectively, from raw sequence reads data showed significant differential abundance between ARD symptomatic and healthy trees. In contrast, our previous study indicated that about 35% of fungal and bacterial OTUs differed in their abundance between the grass aisle and original tree stations (Deakin et al. 2018) and, as in this study, the distance between neighboring samples (ARD symptomatic versus healthy, and grass aisle versus tree station) was similar at ca. 1.5 to 2.0 m.

For fungi and bacteria, analysis of the absolute abundance of individual OTUs (estimated by qPCR) identified more OTUs with differential abundance between healthy and ARD symptomatic samples than analysis of the raw median-of-ratio normalized sequence data. For bacteria, eight out of eleven OTUs with differential abundance identified by the analysis of raw data were also identified by the qPCR-adjusted data. For fungi, all five fungal OTUs with differential abundance identified in raw data were identified by the qPCR-adjusted data. It should be noted that qPCR can only provide an estimate of gene abundance associated with individual OTUs in a given sample. Bacteria can have between one and 15 copies of the 16S rRNA gene (Klappenbach et al. 2001; Větrovský and Baldrian 2013) and similarly there is a high level of variation in the ITS copy numbers (per nanogram of DNA) among fungal taxa (Baldrian et al. 2013; Song et al. 2014). Thus, the abundances of 16S and ITS genes are a function of genomic copy number and the number of organisms, and without taxa specific conversion factors it is difficult to obtain true estimates of microbial biomass for different taxa.

Soil microbial communities and soil biodiversity are intimately associated with many ecosystem properties and soil functions above- and below-ground, including plant diversity, decomposition, nutrient retention, nutrient cycling, and the suppression of soilborne plant diseases (Barrios 2007). Out of the four sectors of the soil biological community investigated only fungal diversity was greater in the rhizosphere of healthy trees, relative to ARD symptomatic trees. For the other groups (bacteria, oomycetes, and nematodes) there were no significant differences between healthy and ARD symptomatic trees and these results support the conclusion of Mazzola and Strauss (2014) that ARD is not ameliorated by enhanced rhizosphere biodiversity per se. Thus, it is likely the abundance of specific microbes or microbial functions that has a greater influence on the development of ARD. Both significant positive and negative associations of many bacterial OTUs with plant growth parameters have been demonstrated previously (Franke-Whittle et al. 2015; Yang et al. 2012). The abundance of all bacterial OTUs showing differential abundance between ARD symptomatic and healthy tree samples was reduced in the rhizosphere of ARD symptomatic trees. Although it was not possible to classify all of the OTUs to the rank of genus or species and hence to infer their potential roles in the development of ARD, the abundance of some genera with potentially beneficial activities, i.e., potential degraders of phenolic compounds (Yim et al. 2015) and PGPR (St. Laurent et al. 2010; Yao et al. 2006), was greater in the rhizosphere of healthy trees. Interestingly, unpublished data from a few samples of rhizosphere soil of ARD symptomatic and healthy trees in a Dutch orchard showed the same trend: 42 out of 45 bacterial OTUs with differential abundance had much greater abundance in healthy tree samples than in ARD symptomatic samples, including four *Bacillus* spp. (G. Deakin, T. Passey, J. Bennett, and X. Xu, *unpublished data*).

In contrast with bacteria, 13 out of the 16 fungal OTUs with differential abundance had much greater representation in ARD symptomatic samples than in healthy tree samples. Furthermore,

Fig. 4. Principal component plots of bacterial and fungal data. **A**, Principal component plot (PC1 versus PC3) for raw bacterial data. **B**, Principal component plot (PC1 versus PC2) for raw fungal data. Color indicates samples taken from either healthy (black) or apple replant disease symptomatic (orange) trees. Dotted lines connecting points indicate paired sample.

the magnitude of the difference in the abundance between healthy and ARD tree samples was much greater for fungi than for bacteria, suggesting that these OTUs are almost absent either in the healthy tree samples (13 OTUs) or in the ARD symptomatic

samples (3 OTUs). However, BLAST searches showed that none of those 13 fungal OTUs is directly related to known apple pathogens. Previous studies (either through isolation or sequencing) have suggested several fungal species as candidate causal agents for

TABLE 3
Bacterial operational taxonomic units (OTUs) with significant differential abundance in the rhizospheres of healthy versus apple replant disease symptomatic apple trees and the putative lifestyle and ecology associated with each taxa

Taxonomy ^a	Log ₂ -fold change ^b	Putative lifestyle and ecology
<i>Acidobacteria</i> _Gp1	−1.4	Free-living heterotrophs. Common soil bacteria, but not commonly associated with plants (Chelius and Triplett 2001). They are oligotrophic bacteria and less abundant in organic matter-rich soils (Fierer et al. 2007).
<i>Acidobacteria</i> _Gp3	−2.3	
<i>Acidobacteria</i> _Gp4	−2.0 (−1.6)	
<i>Acidobacteria</i> _Gp4 (c)	(−0.8)	
<i>Acidobacteria</i> _Gp5 (c)	(−0.9)	
<i>Acidobacteria</i> _Gp6	−1.3 (−0.9)	
<i>Acidobacteria</i> _Gp6	−2.5	Endophyte and copiotrophic free-living heterotroph (Fierer et al. 2007; Janssen 2006). Dominant root endophyte of apple (Bulgari et al. 2012).
<i>Acidobacteria</i> _Gp6	−2.2	
<i>Bacteroidetes</i> <i>incertae sedis</i> (c)	−2.2	
<i>Betaproteobacteria</i>	−1.6 (−1.3)	
<i>Chitinophagaceae</i>	−1.6 (−1.3)	
<i>Chitinophagaceae</i>	−1.3	
<i>Chlamydia</i> sp.	−2.2	Pathogen of vertebrates and symbionts of free-living amoebae. <i>Chlamydia</i> strains are widely present in the environment, but little is known about their ecology (Collingro et al. 2005).
<i>Dyadobacter</i> sp.	−2.1	Free-living heterotroph. Abundant in the rhizosphere of lettuce (Schreiter et al. 2014), and wheat and oilseed rape (Haichar et al. 2008).
<i>Latescibacteria</i>	−2.6 (−2.1)	Probable saprotroph and possibly tolerant of seasonal water-logging (Farag et al. 2017).
<i>Pseudoxanthomonas</i> sp.	−3.1 (−2.6)	Saprotroph. Able to degrade cellulose (Hou et al. 2015) and benzene, toluene, ethylbenzene, and o-, m-, and p-xylene (Kim et al. 2008).
<i>Sinobacteraceae</i>	−1.7	Endophytes. More abundant in the apple roots not infected by ' <i>Candidatus</i> Phytoplasma mali' (Bulgari et al. 2012).
<i>Spartobacteria</i> genera <i>incertae sedis</i> (g)	−1.1 (0.7)	Free-living heterotrophs and symbionts of nematodes. Common soil bacteria, and are the major group of <i>Verrucomicrobia</i> found in soil (Janssen 2006).
<i>Spartobacteria</i>	−1.9	
<i>Spartobacteria</i>	−2.0	
<i>Spartobacteria</i> (c)	−1.8 (−1.4)	
<i>Verrucomicrobia</i> subdivision 3	−1.9	Free-living heterotrophs. Abundant in grasslands and in subsurface soil horizons. Abundance is affected by soil moisture (Buckley and Schmidt 2001a) and reduced in apple replant soil (Sun et al. 2014).
<i>Verrucomicrobia</i> subdivision 3	(−0.7)	
Uncultured bacterium	−1.9	
Uncultured bacterium	−1.5	

^a Taxonomic identities were ascribed by interrogation of the Ribosomal Database Project and NCBI (BLASTn) databases. Differential abundances are statistically significant at $P < 0.05$ (Benjamini-Hochberg corrected). The representative sequences of these OTUs were deposited in GenBank nucleotide database under accessions MK184957 to MK184981.

^b A negative log₂-fold change indicates greater abundance in the rhizosphere of healthy trees. The log₂-fold change is from the qPCR data, bracketed values are from raw data.

ARD, including *Cylindrocarpon*, *Rhizoctonia*, and *Fusarium* spp. (Braun 1995; Manici et al. 2013; Mazzola and Manici 2012; Tewoldemedhin et al. 2011a). In two orchards in the U.K., a *Cylindrocarpon* OTU was much more abundant in apple tree stations than in the corresponding aisles (Deakin et al. 2018). Although replanting in the interrows can minimize replant disease, it should be noted that potential fungal pathogens (e.g., *Fusarium solani* and *F. oxysporum*, *Cylindrocarpon* spp., and *Rhizoctonia* sp.) are endemic in soil and their distribution may not differ between row and interrow locations (Kelderer et al. 2012). For the three OTUs with greater abundance in healthy tree samples, two are probably wood decay fungi rather than ectomycorrhizal fungi because apple trees do not readily form ectomycorrhizal associations (Miller et al. 1985), and the remaining one is likely to be an arbuscular mycorrhizal fungi (AMF). Recent work has shown that

AMF are able to provide disease resistance benefits to apple (Berdeni 2016).

Several oomycete OTUs also had increased abundance in healthy tree samples, with others having increased abundance in samples from ARD symptomatic trees. Most of the OTUs either cannot be reliably identified to the species level, or are not known to be pathogens of apple. Only one OTU, *Pythium intermedium*, is known to cause root rots in Rosa (Metzger et al. 2007) and was more abundant in ARD symptomatic samples. This species has previously been reported as a causal agent of apple in a study of at six orchard sites in Washington State (Mazzola et al. 2002). Isolation and cultivation based studies on ARD indicate that *Pythium* sp. are frequently isolated from apple roots in combination with fungal pathogens (Braun 1991). Dual inoculation of both *Pythium irregulare* and *C. lucidum* results in greater root damage and

TABLE 4
Fungal operational taxonomic units (OTUs) with significant differential abundance in the rhizospheres of healthy versus apple replant disease symptomatic apple trees and the putative lifestyle and ecology associated with each taxa

Taxonomy ^a	Log ₂ -fold change ^b	Putative lifestyle and ecology
Uncultured Agaricomycetes	-24.4	Predominantly saprotrophs, pathogens, and mutualists, including the major concentration of ectomycorrhizal taxa, and white-rot and brown-rot wood decay fungi (Kirk et al. 2008).
Agaricomycetes	19.1	
Uncultured Agaricales	-27.1 (-26.6)	The gilled mushrooms, an order in Agaricomycetes.
Uncultured Glomeromycota	-10.2	Mutualists-arbuscular mycorrhizas, symbionts of nonvascular plants and endocytosymbionts of cyanobacteria (Schüßler et al. 2001).
Basidiomycota	10.4	Predominantly saprotrophs, but also pathogens and mutualists-ectomycorrhizas. The most abundant fungal taxon in soil (Fierer, 2017). Abundant in soil under apple trees (Wang et al. 2016) and increased by the presence of ryegrass in alleys (Qin et al. 2016). Abundance is reduced by brassica seed meal treatment (Mazzola et al. 2015) and increased by ryegrass in alleys (Qin et al. 2016).
<i>Cryptococcus</i> sp.	19.7 (20.5)	Soil yeast. Abundant in the rhizosphere and in bulk soils (Mestre et al. 2011). Known to influence soil aggregation, contribute to nutrient cycles, and interact with vegetation (Botha 2011; Cloete et al. 2009).
Dothideomycetes (possibly <i>Leptospora gali</i>)	19.3	Saprotrophs and endophytes. Rhizosphere competent with grass (Mouhamadou et al. 2011) and apple (Mazzola et al. 2015). Populations are reduced by brassica seed meal treatment (Mazzola et al. 2015), but increased after the addition of leaf litter (Winder et al. 2013).
Dothideomycetes	10.7	
Pleosporales	19.9 (20.7)	An order in Dothideomycetes; possibly <i>Neoconiothyrium rosae</i> or <i>Rousoella euonymi</i> .
Uncultured Dipodascaceae	9.9	Probable saprotrophs. <i>Dipodascus</i> spp. isolated from rotting cauliflower and soil were able to produce phytase (Ranjan and Sahay 2013); possibly <i>Galactomyces</i> or <i>Geotrichum</i> .
Uncultured fungus	18.6	
Uncultured fungus	18.7	
Uncultured fungus	18.8	
Uncultured fungus	19.0	
<i>Achaeta</i> sp.	19.4 (20.7)	A genus of annelid worms.
Unknown fungus	21.1 (22.0)	

^a Taxonomic identities were ascribed by interrogation of the Ribosomal Database Project and NCBI (BLASTn) databases. Differential abundances are statistically significant at $P < 0.05$ (Benjamini-Hochberg corrected). The representative sequences of these OTUs were deposited in the GenBank nucleotide database under accessions MK192880 to MK192896 (forward reads) and MK192861 to MK192877 (reverse reads).

^b A negative log₂-fold change indicates greater abundance in the rhizosphere of healthy trees. The log₂-fold change is from the qPCR data, bracketed values are from raw data.

TABLE 5
Oomycete and nematode operational taxonomic units (OTUs) with significant differential abundance in the rhizospheres of healthy versus apple replant disease (ARD) symptomatic apple trees and the putative lifestyle and ecology associated with each taxa

Taxonomy ^a	Log ₂ -fold change ^b	Putative lifestyle and ecology
<i>Pythium</i> sp.	21.1 (22.5)	Many are soil saprotrophs, but a large number are pathogens. Pathogenic species typically have a large host range, but some are restricted to specific plant hosts. Wet, cool conditions frequently favor the development of disease. <i>Pythium</i> spp. have been implicated in ARD (Mazzola 1998; Mazzola et al. 2002).
<i>Pythium</i> sp.	(19.8)	
<i>Pythium</i> sp.	10.4 (11.1)	
<i>Pythium</i> sp.	(5.1)	
<i>Pythium</i> sp.	−6.3 (−5.7)	
<i>Pythium</i> sp.	(−6.8)	
<i>Pythium</i> sp.	−10.4 (−9.8)	
<i>Pythium</i> sp.	−11.7 (−10.3)	
<i>Pythium aquatile</i>	(21.3)	Pathogen with moderate pathogenicity. Has a broad host range including vegetables, ornamentals and <i>Eucalyptus</i> sp. (Kumar 2016; Robertson 1980).
<i>Pythium intermedium</i>	(19.7)	Pathogen. A dominant <i>Pythium</i> sp. isolated from apple roots, and known to cause stunting of apple seedlings and a decrease in plant biomass (Mazzola et al. 2002).
<i>Pythium intermedium</i>	11.2 (9.8)	
<i>Pythium polymastum</i>	9.2	Pathogen causing root rot of Brassicas (Botha and Coetzer 1996).
<i>Pythium volutum</i>	−4.7	Pathogen causing root rot in grass species.
<i>Pythium tracheiphilum</i>	−10.0 (−9.9)	Pathogen causing wilting and stem and root rot of lettuces (Matta 1965) and other vegetables, but also reported from turf grasses (Abad et al. 1994).
<i>Peronospora</i> sp.	(13.9)	Pathogens causing downy mildews, especially on Amaranthaceae and Brassicaceae (Smith et al. 1988). Both plant families contain many common weeds of orchards.
<i>Hyaloperonospora</i> sp.	29.9 (28.1)	
<i>Myzocytiopsis vermicola</i>	18.6 (19.2)	Parasite of nematodes (Glockling and Beakes 2000).
<i>Pustula obtusata</i>	(19.3)	Pathogen causing white rust of <i>Tragopogon</i> sp. (Smith et al. 1988), a locally common Asteraceae weed of apple orchards.
<i>Aphanomyces euteiches</i>	15.9 (16.2)	Pathogen causing root rot of legumes (Papavizas and Ayers 1974).
<i>Aphanomyces</i> sp.	−27.2 (−23.7)	
<i>Apodachlya brachynema</i>	−7.7	Reported from the rhizosphere of <i>Phragmites</i> sp. (Nelson and Karp 2013).
Uncultured oomycete	11.2 (11.1)	
Uncultured oomycete	(10.2)	
Uncultured oomycete	(20.2)	
<i>Lagenidium</i> sp.	−30.0 (−29.1)	Mycoparasite of <i>Pythium</i> spp. (Whiffen 1946), and a parasite of nematodes (Barron 1976; Shaffer and Jaffee 1987).
<i>Lagenidium</i> sp.	21.3 (21.0)	
<i>Saprolegnia</i> sp.	−29.9	Saprotrophs. Common in soil, seasonally abundant in the surface and litter layers of soil during the spring and autumn (Dick and Newby 1961). Some are notorious pathogens of aquatic animals.
<i>Albugo candida</i>	(−24.7)	Pathogen causing white blister rust of Brassicas, often in close association with <i>Peronospora</i> (Smith et al. 1988).
Nematode		Ectoparasite of roots (Yeates et al. 1993), plant hosts include brassicas, legumes, and grasses (Goodey et al. 1965; Townshend and Davidson 1989; Viketoff and Sohlenius 2011).
<i>Paratylenchus projectus</i>	12.3	

^a Taxonomic identities were ascribed by interrogation of the Ribosomal Database Project and NCBI (BLASTn) databases. Differential abundances are statistically significant at $P < 0.05$ (Benjamini-Hochberg corrected). The representative sequences of these OTUs were deposited in the GenBank nucleotide database under accessions MK192833 to MK192860 (oomycetes) and MK184983 (nematodes).

^b A negative log₂-fold change indicates greater abundance in the rhizosphere of healthy trees. The log₂-fold change is from the qPCR data, bracketed values are from raw data.

reduction in shoot growth than inoculation of either pathogen alone (Braun 1995; Tewoldemedhin et al. 2011a). As with many of the putative causal agents of ARD, the involvement of oomycetes appears to be site-specific (Manici et al. 2013). Interestingly, ARD tree samples had much lower abundance of two OTUs (*Lagenidium* sp.), known parasite of *Pythium* spp. (Whiffen 1946) and nematodes (Barron 1976; Shaffer and Jaffee 1987).

Root lesions caused by nematodes, such as *Pratylenchus penetrans*, have been implicated in exacerbating ARD by facilitating pathogen entry into the host's root tissues (Galkovskiy et al. 2012; Mai and Abawi 1978; Tewoldemedhin et al. 2011a). However, at our study site the only nematode OTU (*Paratylenchus projectus*) for which abundance was much greater in samples from ARD symptomatic trees than in samples from healthy trees was an ectoparasite of a wide range of plant hosts, including brassicas, legume, and grasses (Goodey et al. 1965; Townshend and Davidson 1989; Viketoft and Sohlenius 2011). However, it is not clear whether *Paratylenchus projectus* could also parasitize apple.

Conclusion. The present results are consistent with the current consensus that ARD is a complicated disease, for which a variety of organisms are responsible (Franke-Whittle et al. 2015; Mazzola 1998; Mazzola and Manici 2012; Tewoldemedhin et al. 2011a), with the relative importance of these factors varying from site to site. The structural differences of microbial communities in the rhizospheres of ARD symptomatic and healthy trees are more subtle than has previously been suggested by studies in which the microbial communities of replant sites with closely situated control fallow soils, or in replant soils subjected to different treatments have been compared. Using a spatially explicit sampling strategy, we have shown that for this particular study site, ARD is associated with reduced abundance of a small number of bacterial OTUs and one AMF OTU, and increased abundance of at least one pathogenic *Pythium* OTU, one nematode OTU, and a small number of specific fungal OTUs. In the absence of broad spectrum soil fumigants, manipulation of specific microbial groups may thus be a feasible approach to managing ARD. Furthermore, given the ARD complexity, combined use of alternative management strategies, including soil amendment (Mazzola and Strauss 2014), use of specific beneficial organisms (Forge et al. 2001), and deployment of different rootstock genotypes (Zhu et al. 2016), is another approach to take. It should be stressed that the success of ARD control measures can be expected to vary considerably with sites because of the varying importance of ARD causal agents among sites.

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