CONVERSION OF GRASSLAND TO ARABLE DECREASES MICROBIAL DIVERSITY AND ALTERS COMMUNITY COMPOSITION

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Word count : 8,659

Keywords: agricultural intensification; metagenomics; soil management; soil pathogens; nitrogen-fixing bacteria; mycorrhizas

Abstract

The rapid global conversion of biodiverse landscapes to intensively managed arable fields may decrease microbial diversity and threaten the long-term fertility of native soils. Previous laboratory and experimental studies provide conflicting results: some have recorded declines in overall microbial diversity and certain beneficial microorganisms under intensified cultivation while others report no change (or even increased) diversity. However, few studies have been carried out in actual agricultural fields. We analysed the soil metagenomic communities of 36 current and former grasslands in Oxfordshire, England using Illumina sequencing of the bacterial 16S and fungal ITS1 regions to examine how agricultural intensification alters native microbial communities and whether arable reversion can reverse these processes. We demonstrate that land-use change did not affect bacterial diversity or specific beneficial taxa (nitrogen-fixing bacteria and mycorrhizas). However, fungal diversity declined and certain microbiota known to be plant pathogens (e.g. *Ophiostoma*) were significantly more abundant on agriculturally improved fields. On sites where arable reversion took place, microbial communities were similar to those from unimproved grasslands although overall vegetation diversity and fungal richness was lower. The conservation of species-rich grasslands and their associated microbial diversity could therefore be a key resource for sustainable agriculture in the future.

1. Introduction

Soil is the basis of agricultural productivity; and yet agricultural soils are heavily degraded worldwide. For example, many agricultural soils are thin and extremely low in organic matter (Jones et al. 2012; Matson et al. 1997). However, the biotic component of soil is equally important and often overlooked. Given that just one gram of soil contains over 50,000 bacteria and fungi, which play a key role in plant health, soil fertility, and wider ecosystem functioning (Chaparro et al. 2012; Garbeva et al. 2004; Salles et al. 2006; Singh et al. 2014; Zak et al. 2003), protecting and restoring soil fauna on agricultural lands will be key to supporting sustainable food systems in the future (Brussaard et al. 2007; Mendes et al. 2013).

Natural ecosystems are the main reservoir of soil microbial diversity (Lee and Lee 2013). For example, the microbiota from highly biodiverse ecosystems have been used in the development of new antibiotics, biofertilizers and even pigments (Balser et al. 2010; Celestino et al. 2014). However, natural ecosystems around the world are being replaced by agricultural systems threatening this diversity and the potential for future products (Chapin et al. 2000). Certain processes associated with intensification, such as the use of inorganic fertilizers, are already known to affect microbial diversity. For example, previous research suggests that microbial diversity generally and the diversity of highly beneficial arbuscular mycorrhizal fungi (which provide their plant hosts with phosphorus) specifically, decline with the application of nitrogen (N), phosphorus (P) and potassium (K) (Gosling et al. 2006; Kiers et al. 2002; Oehl et al. 2010; Verbruggen et al. 2013). Addition of nutrients to the soil may also reduce the effectiveness of microbiota associated with plant-defence and nitrogen-fixation (Salles et al. 2006; Fox et al. 2007). However, to better understand the changes in microbial communities that take place under intensification, more detailed studies of specific systems in the field are needed that reflect the changes taking place in real landscapes.

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60 In northern Europe agricultural intensification poses a particular threat to species-rich grasslands.
61 Intensification usually involves changes in land use and the application of inorganic fertilizers and
62 pesticides on fields. Species-rich ‘unimproved’ grasslands have developed over thousands of years of
63 human management, specifically through regular livestock grazing and cutting for hay. Unimproved
64 grasslands are known to contain exceptional levels of plant and animal diversity (Yeats et al. 1997).
65 Several studies have also reported that unimproved grasslands contain exceptional levels of microbial
66 diversity, although most only focus on mycorrhizal fungi and not entire microbial communities (Douds
67 and Millner 1999; Gosling et al. 2006; and van der Heijden et al. 2006). Since the 1950s, farmers have
68 increasingly ploughed up unimproved grasslands and replaced them with arable crops, or with
69 ‘improved’ grasslands – where a single species of grass is grown as an annual or bi-annual crop—
70 leading to a loss of over 97% of unimproved grasslands in lowland England alone (Blackstock et al.
71 1999). In certain conservation areas, arable fields have also been part of ‘reversion schemes,’ where
72 land owners and managers have attempted to restore species-rich grasslands on former arable land.
73 This system offers an ideal opportunity to compare the soil biota of different fields before and after
74 conversion to arable and to quantify the effectiveness of reversion on restoring the soil microbial
75 community to its pre-arable state.

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77 To examine whether agricultural intensification in grasslands threatens native microbial diversity, we
78 conducted vegetation surveys, soil analysis, and metagenomic research at 36 current and former
79 grasslands in Oxfordshire, UK. All sites are part of active, working farms. We assessed: (1) how
80 agricultural intensification affects microbial diversity and community composition; and (2) whether
81 arable reversion can reverse any observed effects. Specifically, we compared five land-use types that
82 reflect real changes affecting the local landscape and environment within the study area. These land-
83 use types were: (1) unimproved grasslands which had never been ploughed or used for arable
84 cultivation; (2) improved grasslands which receive high levels of inorganic inputs and are sown with

commercial ryegrass (*Lolium perenne* L.) and clover-based seed mixes (*Trifolium pratense* L. and *T. repens* L.); (3) ‘conservation grasslands’ which are former arable fields which have undergone arable reversion; and two arable crops grown on former grassland sites: (4) oilseed rape (*Brassica napus* L.) and (5) poppy (*Papaver somniferum* L.). We selected these crop types because of concerns that growing non-mycorrhizal crops will negatively impact soil fungal diversity. At each site we determined the soil metagenomic profiles using Illumina sequencing of the bacterial 16S and fungal ITS1 regions and we compared differences in bacterial and fungal taxonomic diversity among the land-use types. We also looked for changes in two groups of microbiota with well-established functions, nitrogen-fixing bacteria and mycorrhizas, to determine whether agricultural intensification decreases the abundances of these agriculturally-important microbiota.

2. Materials and Methods

2.1 Study Area and Site Selection

The county of Oxfordshire is located in south-east England, UK. It has a maritime temperate climate with an annual rainfall of between 570-750mm (depending on elevation) (Killick et al. 1998). The daily average temperature in winter (November – February) ranges from 4.9 – 7.6 °C and the daily average temperature in summer (May – August) ranges from 12.5 – 17.6 °C. The three main soil types are alluvial, sandstone, and chalk. The primary crops are wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), oats (*Avena sativa* L.), and oilseed rape. Poppy is not a major crop but is increasingly grown due to demand for UK-derived codeine and morphine. Farmers also raise cattle and sheep for meat and dairy production.

Between June-July 2015, we conducted fieldwork at 36 sites in the region (**Supplementary materials Table 1 and Figure 1**). These sites fell under five major land-use categories (**Table 1, Figure 1**).

Unimproved grasslands are pastures and meadows managed by farmers (for fodder) or by conservation groups (to maintain rare plant species located there). All unimproved sites, as far as we know, have never been ploughed. Conservation grasslands are former intensively cultivated arable fields that have undergone arable reversion. This is achieved by (1) soil amendment (where soil is stripped to reduce soil nutrients) and (2) re-sowing former arable fields with plants characteristic of unimproved grasslands. Improved grasslands are leys sown with legume-based seed mixtures and treated with fertilizers (inorganic and organic). These fields are ploughed and re-sown every year or every other year depending on the yield of the first season and if a rotation system is used. The rapeseed and poppy fields were converted from grassland to arable within the past 30 years. All agriculturally improved arable fields receive inorganic N and P (and in one case, digestate from an anaerobic digester). None of these fields are tilled and all undergo rotation. In north Oxfordshire, farmers rotate rapeseed with wheat, barley, oats and peas (*Pisum sativum* L.). In south Oxfordshire, farmers rotate the rapeseed and poppy fields together along with wheat and barley.

Figure 1 Land-use types in the study region. Species-rich semi-natural grasslands are increasingly replaced with arable fields. A. Species-rich grassland (Wolvercote, Oxfordshire). B. Improved grasslands (Ipsden, Oxfordshire). C. Rapeseed field (Ipsden, Oxfordshire). D. Poppy field (Ipsden, Oxfordshire).

Table 1 Description of Land Use Categories. The study sites are divided into five land use categories: unimproved grasslands, conservation grasslands, improved grasslands, poppy fields, and rapeseed fields. Each category is characterized by type of land use (grassland or arable), function (agriculture or conservation), vegetation (unsown (i.e. natural) or sown), seed source (local or commercial), application of inorganic inputs (N, P, and K), use of rotation, and whether the field is grazed (G), mown (M), mown and grazed (MG), or cut.

Description	Unimproved grassland (n=14)	Conservation grassland (n=10)	Improved grassland (n=6)	Poppy field (n=3)	Rapeseed field (n=3)
Land Use	Grassland	Grassland	Grassland	Arable	Arable
Function	Agricultural	Conservation	Agricultural	Agricultural	Agricultural
Vegetation	Unsown	Sown	Sown	Sown	Sown
Seed source	Local	Local, commercial	Commercial	Commercial	Commercial
Application of inorganic N, P, K	No	No	Yes	Yes	Yes
Rotation	No	No	Yes	Yes	Yes
Grazed, Mown, or Cut	G, M, MG	G, MG	Cut	Cut	Cut

2.2 Soil Sample Collection and Vegetation Surveys

We conducted a systematic program of soil sampling at each site to ensure the soil samples reflected local grassland vegetation and soil diversity. We collected soil samples at each site at the same time of day (early morning), season (early summer), and weather (no rain) in order to ensure comparability between sampling units. This is particularly important because microbial abundance can decline during certain seasons (particularly winter) and increase under certain environmental conditions (e.g. rainfall) or management practices (e.g. fertilizer application) (Barni and Siniscalco 2000; Eriksson 2001; Schneider *et al.* 2010). At each site, we marked out a 10m by 10m area for sampling. These sample areas were not selected beforehand because the area sampled was based on the farmer's decision on the day of the site visit. The presence of grazing livestock, fertilizer application, and specific conservation regulations influenced farmer choice. We then walked in a 'w' pattern and took twenty evenly spaced cores. We used hand-held metal corers 5 cm x 13 cm in size. Contamination was avoided by using different corers for each field. We combined and manually homogenized the soil from each core and removed a small subsample (ca. 100 g). This subsample contained both roots and

soil to capture the variation in the rhizosphere communities at each site (Berg and Smalla 2009; Berendson et al. 2012; Chaparro et al. 2010). The subsamples were stored in a plant drying room for four weeks until completely dry.

We determined the nitrogen, phosphorus, potassium, and pH of the remaining soil from each core on the day of collection. Soil samples were dried in an oven and homogenized. All soil analyses were performed following the protocols in *Methods in Agricultural Chemical Analysis* (vol. 427). The pH of the soil was defined as the pH of the suspension obtained by shaking the soil with water under controlled conditions. The nitrogen in the soil was converted to ammonium-nitrogen by digestion with sulphuric acid and sodium sulphate with a copper-selenium catalyst. The ammonia liberated with sodium hydroxide was removed by steam distillation and determined titrimetrically. Phosphorus was extracted from the soil with a sodium bicarbonate solution of pH 8.5. The concentration of the blue complex produced by the reduction, with ascorbic acid, of the phosphomolybdate formed when acid ammonium molybdate reacts with phosphate is measured spectrophotometrically at 880 nm. Potassium was extracted from the soil with 1 M ammonium nitrate. The concentration of potassium in the extract was determined by flame photometry. Soil magnesium content was determined using 1 M ammonium nitrate to extract the magnesium in the soil. The concentration of magnesium in the diluted extract was determined by atomic absorption. Addition of a releasing agent eliminated interference by phosphate. All analyses were performed by Anglian Soil Analysis (Anglian Soil Analysis, Boston, Lincolnshire).

To determine the vegetation species richness at each site, we conducted vegetation surveys. We placed five 1m² quadrats randomly in each demarcated area where we took the soil cores. We recorded the species present in each quadrat. Abundance was determined as the number of quadrats in which each species occurred. Plants were identified using Fitter et al. (1984) and Rose (1989).

2.4 DNA Extraction and PCR

We extracted DNA from the soil samples using the protocols for the ZR Soil Microbe DNA Miniprep kit (Zymo Research). To identify both bacterial and fungal communities in the samples, we selected PCR primers targeting the V4 region of 16S rRNA for bacteria and the ITS1 region for fungi. The forward primer used for the 16S region was F515 5'-GTGCCAGCMGCCGCGGTAA-3' and the reverse primer was R806 5'-ATTAGAWACCCBDGTAGTCC-3' (Caporaso et al. 2011). Degenerate primers were used for the 16S region to account for the potential changes in base pairs (A and C) in bacterial sequences. The forward primer used for the fungal ITS1 region was ITS1F 5'-CTTGGTCATTTAGAGGAAGTAA-3' and the reverse primer was ITS2 5'-GCTGCGTTCTTCATCGATGC-3' (Buée et al. 2009; White 1999). We used barcoding to mark each sample so that samples could be combined after PCR and separated after sequencing.

We performed PCR twice (once for bacteria and once for fungi) using a Phusion High-Fidelity PCR kit (New England Biolabs). Each 20ml reaction mixture contained 4 µl Phusion, 0.4 dNTPs, 1 µl forward primer (10 pmol final concentration), 1 µl reverse primer (10 pmol final concentration), 1 µl template DNA, and 0.2 µl Phusion DNA polymerase according to standard protocols. Initial denaturation of the DNA was run for one cycle at 98° degrees for 3 minutes. Denaturation, annealing and extension were run for 30 cycles at 98° C (15 seconds), 55° C (15 seconds) and 72° C (30 seconds) respectively. The final extension was run for 7 minutes at 72° C.

To establish the quality of the DNA, we assessed 5µl of the PCR product from each sample by electrophoresis in 1.5% agarose gels. For samples where the quality of the DNA was low, we performed PCR a second time, ran an agarose gel to check DNA quality, and if the quality was higher than the first round of PCR, we used the second sample. We then combined 10 µl of PCR product for

each of the fungal and bacterial samples. To quantify the final PCR product before sending the combined sample for sequencing, Qubit™ fluorometric quantitation was performed using a Qubit™ Quantification Assay Kit (Thermo Fisher Scientific) following standard procedures. The final concentration of the DNA was 6.8 ng/μl in 200μl of product.

2.5 Sequencing and Analysis of Microbial Data

The PCR product was sent to MR DNA in Texas for Illumina MiSeq v3 chemistry sequencing. Once the sequences were received, FLASH (**F**ast **L**ength **A**ddress of **S**hort reads) was used to join pair-ended reads and to remove low-quality sequences with the settings as follows: -O, -m 50 -M 500 -x 0.1 (allow for “outies”, minimum overlap of 50bp, maximum overlap of 500bp and to discard reads with 10% or more of mismatches in their overlap region) (Magoč and Salzberg 2011). Sequences with a phred score below 33 were cut to improve the overall accuracy of the analysis (Walshaw et al. 2011). Reads were processed using FASTX (http://hannonlab.cshl.edu/fastx_toolkit/) bash scripts and custom Python scripts. After separation of amplicons based on a double barcode system as used previously (Fadrosh et al. 2014) we obtained 744389 and 241090 usable reads for bacteria and fungi, respectively. The barcoded samples were then separated using Bash, resulting in one bacterial and one fungal sample representative of the microbial community for each of the 36 sites. To obtain the taxonomic profile for each sample, we annotated the metagenomic data using the SILVA small-subunit rRNA (SSU) database (version 126) (Quast et al. 2013) for bacteria and the NCBI Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast>) for fungi. In SILVA, the maximum e-value cut off was set to 10^{-5} , the minimum alignment identity was set to 100%, and the minimum alignment length cut-off was set to 15bp in order to achieve a high rate of certainty in taxonomic identifications. In the BLAST search, we optimized the nucleotide database (nr/nt) query for highly similar sequences (megablast). We used MEGAN 5 (MEtaGenome ANalyzer, Center for Bioinformatics, Tübingen, Germany, <http://ab.inf.uni-tuebingen.de/software/megan5/>) to determine the taxonomic profile for

each sample (Huson et al. 2011; Oulas et al. 2015). We determined the bacterial and fungal taxonomic richness based upon the number taxa for each sample. To calculate evenness, we used the Shannon Weaver Index.

2.6 Statistical Analysis

We used one-way analysis of variance (ANOVA) to determine whether land use had a significant effect on vegetation diversity, soil fertility, microbial diversity, and specific microbial taxa. The data were log-transformed where necessary to meet assumptions of normality and homoscedasticity. Differences among land-use types were assessed by reference to the standard *F* tests. To further investigate the nature of the differences among land-use types, we performed a limited number of contrasts (which are allowed within the analysis) and mean that further adjustments for multiple testing are unnecessary. In our case, we were particularly interested in whether other types of land use differed significantly from the ‘unimproved grassland’ – hence we examined contrasts between unimproved grassland and each of the remaining land-use types. The significance of these contrasts was assessed using *t*-tests. To determine the effect of soil fertility on microbial communities, we used Redundancy Analysis (RDA). We created Generalized Additive Models (GAMs) based on this data to determine the effect of soil N, P, K and Mg on bacterial and fungal taxonomic diversity (Yee and Mitchell 1991). We used Pearson Correlation analysis to determine whether there was a correlation between vegetation, bacterial and fungal taxonomic richness. We performed ANOVA and correlation analysis in R (version 3.2.2, "Fire Safety") and RDA in Canoco (version 4.5, Lepš and Šmilauer 2003).

3. Results

3.1 Microbial Diversity and Community Composition

Metagenomic analysis of the bacteria data generated a total of 158,781 reads, 112,280 of which were assigned to 626 taxa in MEGAN. Bacterial taxonomic richness did not vary among land-use types ($F_{4,31} = 0.44$, $P = 0.779$) and was highest in the samples from the rapeseed fields (161 ± 18.38 taxa) and lowest in samples from the poppy fields (137.7 ± 18.32 taxa). There was no difference in the evenness of the bacteria among land-use types ($F_{4,31} = 2.114$, $P = 0.103$). 28% of bacteria taxa were site specific (found only at one site; **Supplementary Tables 2 and 3**). The dominant bacterial taxa were Actinobacteria, Cyanobacteria, *Gaiella*, Rubrobacteriadae, *Spartobacteria*, Acidobacteria, Solirubrobacteria, Rhizobiales, Chloroflexi and *Bacillus*. The total taxonomic profile for all sites is shown in **Supplementary Materials Figure 2**. There was significant variation in the abundance of *Spartobacteria* ($F_{4,31} = 3.494$, $P = 0.018$), Acidobacteria ($F_{4,31} = 3.408$, $P = 0.02$), Solirubacterales ($F_{4,31} = 2.986$, $P = 0.034$) among land-use types, with the highest numbers in unimproved grasslands (**Figure 2**). There was also significant variation in the abundance of Actinobacteria ($F_{4,31} = 3.671$, $P = 0.015$), Cyanobacteria ($F_{4,31} = 7.39$, $P < 0.001$), and *Bacillus* ($F_{4,31} = 4.567$, $P = 0.005$) all of which were significantly more abundant on the agriculturally improved fields compared to the unimproved grasslands. There was no significant variation in *Gaiella* ($F_{4,31} = 1.222$, $P = 0.322$), Rhizobiales ($F_{4,31} = 1.697$, $P = 0.176$), and Chloroflexi ($F_{4,31} = 1.743$, $P = 0.167$) among the five land-use categories.

Metagenomic analysis of the fungal data produced a total of 19,717 reads, 19,085 of which were assigned to 882 taxa in MEGAN. Fungal taxonomic richness varied significantly among the five land-use types ($F_{4,31} = 9.003$, $P < 0.001$) and was highest in unimproved grasslands (84.07 ± 3.192 taxa) and lowest in the rapeseed (57.33 ± 7.6 taxa) and poppy fields (57.33 ± 7.6 taxa). Fungal taxonomic richness was significantly lower (on average by 21.37 ± 4.946 taxa) on conservation grasslands

compared with unimproved grasslands ($t = -4.321$, $P < 0.001$). There was no difference in the evenness
 of the fungi among land-use types ($F_{4,31} = 0.867$, $P = 0.495$). In addition, there was no correlation
 between bacterial and fungal taxonomic diversity ($r = -0.073$, $t = -0.424$, $df = 34$, $P = 0.671$). 40% of
 fungal taxa were site specific (**Supplementary Tables 2 and 3**). The dominant fungal taxa were
 Ascomycota, Mortierellamycotina, *Mortierella*, Agaricales, Cladosporium, Basidiomycota,
 Tremellomycetes, Leotiomyces, *Fusarium* and Pleosporales (**Figure 2; Supplementary Materials**
Figure 2). There was significant variation in the abundance of Tremellomycetes ($F_{4,31} = 3.475$, $P =$
 0.017) and Pleosporales ($F_{4,31} = 2.51$, $P = 0.061$) among the land-use types, with the highest numbers
 in the poppy fields. The abundance of Cladosporium varied significantly among the land-use types ($F_{4,31} = 3.222$, $P = 0.025$) with the highest abundances found in conservation grasslands. There was no
 significant variation in Ascomycota ($F_{4,31} = 1.324$, $P = 0.283$), Mortierellamycotina ($F_{4,31} = 0.8$, $P =$
 0.534), *Mortierella* ($F_{4,31} = 0.426$, $P = 0.788$), Agaricales ($F_{4,31} = 1.077$, $P = 0.409$), Basidiomycota
 ($F_{4,31} = 10.752$, $P = 0.477$), *Fusarium* ($F_{4,31} = 1.848$, $P = 0.013$), and Leotiomyces ($F_{4,31} = 0.619$, P
 $= 0.652$) among the five land-use categories.

Figure 2 Management-sensitive microbiota. Spartobacteria, Acidobacteria, and Solirubacterales were more abundant on species-rich grasslands while Actinobacteria, Cyanobacteria and *Bacillus* were more abundant on agriculturally improved fields. Trymellicetes and Pleosporales were more abundant on agriculturally improved fields while *Cladosporium* was most abundant on grasslands under arable reversion. U = Unimproved grassland; C = Conservation grassland; I = Improved grassland; P = Poppy field; R = Rapeseed field. Plots show means $\pm$ 1 standard error. The y-axis represents number of taxa.

3.2 Effect of Vegetation Species-Richness and Soil Fertility on Microbial Diversity

There was no correlation between vegetation species richness and bacterial diversity ($r = 0.135$, $P = 0.432$). Fungal diversity was positively correlated with vegetation species richness ($r = 0.552$, $P < 0.001$). Vegetation species-richness varied significantly among the land-use types ($F_{4,31} = 16.61$, $P < 0.001$). Unimproved grasslands had the highest species-richness (16.42 ± 1.1 species) and were dominated by a wide variety of grasses (e.g. *Festuca gigantea* (L.) Vill., *Festuca pratensis* Huds., *Dactylis glomerata* L., *Glyceria fluitans* (L.) R. Br.) and legumes (e.g. *Lathyrus pratensis* L., *Vicia cracca* L.). Although the botanical composition of conservation grasslands closely mirrored that of unimproved grasslands, the species richness of conservation grasslands was on average lower by 5.23 ± 1.716 species ($t = -3.047$, $P = 0.005$). Species richness was lowest on the rapeseed and poppy fields.

The soil N, P, K, Mg and pH for each site can be found in **Supplementary Materials Table 1**. Redundancy analysis (RDA) shows that agriculturally improved sites were associated with soils high in P and K and species-rich grasslands were associated with soils high in N and Mg (**Supplementary Materials Figure 3**). Analysis of the bacteria samples and soil variable explains 29 % of the variation in the microbial data and 96 % of bacterial-environment correlations ($F_{2,31} = 2.601$, $P = 0.002$) and 18.5 % of the variation among fungal taxa and 87.6 % of fungal-environment correlations of the fungal samples ($F_{2,31} = 1.6$, $P = 0.002$). The Generalized Additive Models (GAMs) in **Figure 1** show the sensitivity of bacterial and fungal diversity to increased soil fertility. Bacterial diversity did not decrease with increased soil N ($F_{2,31} = 2.69$, $P = 0.069$) or P ($F_{2,31} = 1$, $P = 0.378$) but declined under

increased K ($F_{2,31} = 6.19$, $P = 0.005$). Fungal diversity was not affected by increased N ($F_{2,31} = 0.968$, $P = 0.39$) or K ($F_{2,31} = 1.06$, $P = 0.17$) but declined under increased P ($F_{2,31} = 6.29$, $P = 0.004$). Increased soil pH did not affect bacterial diversity ($F_{2,31} = 0.96$, $P = 0.39$) or fungal diversity ($F_{2,31} = 0.631$, $P = 0.461$).

Figure 3 Redundancy Analysis (RDA) showing the impact of soil N, P, K, Mg and pH on bacterial and fungal community composition and diversity. A. and B. Agriculturally improved fields were high in P and K while unimproved grasslands and most grasslands under arable reversion were high in Mg and N. C. Bacterial diversity declined under increased K. D. Increased levels of soil P caused a sharp decline in fungal diversity.

Figure 4 Redundancy Analysis (RDA) showing the impact of soil N, P, K, Mg and pH on bacterial and fungal community composition and diversity. A. and B. Agriculturally improved fields were high in P and K while unimproved grasslands and most grasslands under arable reversion were high in Mg and N. C. Bacterial diversity declined under increased K. D. Increased levels of soil P caused a sharp decline in fungal diversity.

3.3 Management Sensitive Taxa

A number of less dominant taxa showed a strong sensitivity to particular land uses and were more abundant on either species-rich grasslands or agriculturally improved fields. The abundance of Planctomycetes varied significantly among the land-use types ($F_{4,31} = 4.22$, $P = 0.008$) and was most abundant on unimproved grasslands (144.1 ± 20.48) and lowest on the poppy fields (9.33 ± 48.76).

There was significant variation in the abundance of Rickettsiales among the five-land use types ($F_{4,31} = 6.831$, $P = 0.005$), with the highest levels found in the poppy fields (388 ± 78.46) and the lowest found in conservation grasslands (9.1 ± 51.062). The abundance of *Pseudomonas* ($F_{4,31} = 13.14$, $P < 0.001$), *Massilia* ($F_{4,31} = 9.218$, $P < 0.001$), *Olpidium* ($F_{4,31} = 11.08$, $P < 0.001$), *Pyrenochaeta* ($F_{4,31} = 13.31$, $P < 0.001$), *Cadophora* ($F_{4,31} = 11.49$, $P < 0.001$), and *Verticillium* ($F_{4,31} = 3.466$, $P = 0.018$) varied significantly among the land-use types and were up to ten times more abundant on the poppy and/or rapeseed fields. Other microbiota only found on species-rich grasslands (unimproved and conservation grasslands) and those only found on agriculturally improved fields (improved grasslands, poppy fields, and rapeseed fields) are listed in **Supplementary Materials Tables 2 and 3**.

Specific microbiota known to have a beneficial effect on crops also varied significantly among land-use categories. The abundance of one genus known to fix nitrogen, *Rhizobium* spp., varied significantly among the different land-use types ($F_{4,31} = 11.78$, $P < 0.001$) and, surprisingly, was most abundant on the poppy and rapeseed fields. The types of nitrogen-fixing bacteria varied greatly among land-use types: unimproved and conservation grasslands were dominated by *Bradyrhizobium* spp., *Sinorhizobium* spp., *Frankia* spp., *Heliobacter* spp., *Nitrosomonas* spp., and *Paenibacillus polymyxa* while poppy and rapeseed fields were dominated by *Rhizobium* spp., *Cyanobacteria* sp., and *Azotobacter* spp. (**Figure 4**). Although the abundance of one common mycorrhizal genera, *Glomus* sp., did not vary significantly among the land-use types ($F_{4,31} = 1.327$, $P = 0.282$), specific genera (*Paraglomus* sp. and *Acaulospora* sp.) were found only in samples from unimproved and conservation grasslands.

Figure 5 RDA showing differences in abundances of N-fixing bacteria and mycorrhizas among sites. *Bradyrhizobium* spp. was associated with unimproved grasslands and fields under arable reversion while *Rhizobium* spp. and *Paenibacillus* spp. were associated with agriculturally improved fields. *Nitrosomonas* spp. were only found on unimproved grasslands. *Glomus* spp. were found across the study sites and while *Paraglomus* sp. was found only on unimproved grasslands and grasslands under arable reversion. In the figures, samples are represented by the abundance of each N-fixing bacteria or mycorrhiza (absence is symbolized by a '+') and are color-coded by land-use type. The figures are based on the RDAs in Figure 1.

4. Discussion

Our analysis presents the first multi-site comparison of the effect of agricultural intensification and land-use change on microbial diversity within a real agricultural setting. We found that agricultural intensification and land-use change do not decrease overall bacterial taxonomic diversity but do cause fungal taxonomic diversity to decline. We also found that specific taxa were either more abundant or only associated with specific land uses. Here, we discuss the resilience of microbial diversity to agricultural intensification, the ecological and agricultural ramifications of management on specific taxa, and the importance of sustainably managing, protecting and harnessing the microbial diversity of

unimproved grasslands to improve the success of arable reversion initiatives and to naturally boost the soil fertility of arable fields.

4.1 Resilience of Microbial Diversity

Bacterial communities were resilient to increases in soil fertility and changes in land use in our study area yet fungal communities showed a strong negative response to these changes. The rapid evolution of bacterial communities may explain their resilience to changes in land use and increased soil fertility. The lifecycles of bacteria are short and 100-300 generations can occur each year, leading to high rates of competition between bacteria, therefore new communities adapted to new environmental constraints might emerge quickly (Pennisi 2013; Wiser et al. 2013). In contrast, the sensitivity of fungi to changes in agricultural practices may be linked to their slower reproductive cycle and reliance on specific plant hosts to survive. We also found that fungi were more sensitive to increased soil P and K, which is supported by other field-based and experimental studies (Hansen et al. 2000; Raun and Johnson 1999).

Specific ‘beneficial’ microbiota were also unaffected by increased inputs. Specifically, bacteria with nitrogen-fixing capabilities (e.g. *Cyanobacteria*) were actually more abundant on the agriculturally improved fields. Similarly, arbuscular mycorrhizas were found across all sites (in very low amounts). Saprotrophic fungi associated with vegetation decomposition (e.g. *Acomycota*, *Basidiomycota*, *Mortierella*) were also ubiquitous (Boddy et al. 2008; Štursová et al. 2012). This agrees with recent experimental and field-based research demonstrating that nitrogen-fixing bacteria and mycorrhizas persist under increased soil fertility and even pesticide use (Ahern et al. 2002; Saia et al. 2015; Shafiani and Malik 2003).

The use of crop rotation, no-till agriculture, and arable margins may explain the high abundance of beneficial microbiota despite increased inputs. For example, rotation followed by no-tillage can

promote the resilience of mycorrhizas in the soil year-to-year despite a lack of mycorrhizal hosts (Barni and Siniscalco 2000). Roots infected from previous years may also auto-inoculate the soil the following year. In addition, no-till regimes can protect mycorrhizal hyphal networks and thus mycorrhizal abundance (compared to tilled fields) (Lupwayi et al. 1998). The use of arable field margins around fields may also serve as reserves for ‘beneficial’ fungi and bacteria. In Oxfordshire, field margins were composed of a variety of plants usually associated with grasslands and may have microbial communities similar to unimproved grasslands. Kivlin et al. (2011) have shown that wind, water, and animals can move the spores of these communities up to 10 m from their origin. This suggests that field margins could potentially serve to ‘restore’ the microbial communities within the arable fields that they border. The effect of agricultural intensification on microbial diversity, and on a limited number of beneficial microbiota, is thus complicated and may vary among sites and among regions due to a variety of local biotic and anthropogenic factors.

4.2 Agricultural Intensification and Soil Pathogens

Our analysis showed that species-rich grasslands and agriculturally improved fields contained unique taxa. This indicates that changes in land management may significantly alter microbial communities. This was most striking in relation to *Pseudomonas*, *Massilia*, *Olpidium*, *Pyrenochaeta*, *Cadophora*, and *Verticillium* (**Figure 5**). As the genus *Pseudomonas* contains both beneficial (e.g. *Pseudomonas fluorescens*) and pathogenic (e.g. *Pseudomonas viridiflava*) species (Zeilinger et al. 2015), caution should be taken when interpreting these results. Nevertheless, in our analysis species assigned to these genera included *Pseudomonas cichorii*, *Pseudomonas viridiflava*, *Olpidium brassicae*, *Verticillium dahlia*, *Pyrenochaeta lycopersici*, all of which have shown pathogenic activity in plants and/or humans and livestock in other studies (D’Amico et al. 2008; Garibaldi 2013; Goodner et al. 2001; Mansfield et al. 2012; Sarris et al. 2012; Waipara et al. 1996). In addition, some taxa affecting humans and livestock health (e.g. *Chaetomium* spp., *Listeria monocytogenes*, *Stenotrophomonas maltophilia*,

Yersinia seeligeri) (Barron et al. 2003; Berg et al. 2005; Correia et al. 2014; Mendes et al. 2013; Kozak et al. 1996; Strydom and Witthuhn 2015), were only found on the agriculturally improved fields, and specifically, one of the rapeseed fields fertilized with digestate from a local anaerobic digester

Figure 6 Abundance of potentially pathogenic taxa on agriculturally improved fields. *Pyrenochaeta*, *Cadophora*, *Verticillium*, and *Olpidium* were all more abundant on the rapeseed fields. *Pseudomonas* and *Massilia*, and were most abundant on the poppy fields. Aphid infestation of poppy flower and pod damage (© K. French) (bottom right) and *Alternaria* affecting rapeseed pods (©Bayer Crop Science) (top right). U = Unimproved grassland; C = Conservation grassland; I = Improved grassland; P = Poppy field; R = Rapeseed field. Plots show means $\pm$ 1 standard error.

Although we did not specifically set out to identify soil pathogens in our analysis and we did not test for pathogenicity (e.g. by immunofluorescence assay, etc.; Cahill 1999), these results are striking and agree with visual observation of the arable fields involved in the study and information provided by farmers. For example, during the time of sampling, the poppy fields suffered severe infestations of aphids (*Aphis fabae*) and an unidentified fungal disease (which caused damage to poppy roots, stems and pods) and the rapeseed field were infected with several fungal pathogens, including *Alternaria raphani* (which leaves black spots on the pods and causes early dehiscence) (R. Fanshaw, pers.

comm.). These fields contained the aforementioned taxa, which have been shown to reduce crop productivity in other field studies and laboratory experiments. For example, several studies have highlighted how increased abundances of *Massilia* can reduce crop productivity while increases in *Pseudomonas viridiflava* and *Verticillium dahlia* cause stem and root lesions (Berg and Smalla 2009; Carrieri et al. 2014). Decreased vegetation diversity may partially contribute to increased levels of potential soil pathogens on these arable fields, although further research would be required to show this. Other studies have shown that host-specific pathogens can rapidly multiply on arable fields sown with monocultures (Dick 1992; Knops et al. 1999; Mitchell et al. 2002; Stuckenbrock and McDonald 2008) while increased application of inorganic inputs can increase fungal pathogens (Perez-Brandan et al. 2014). Application of digestate from anaerobic digesters as a fertilizer on arable fields may also have contributed to the unique community of microbiota in one of the rapeseed fields. Other studies have reported that application of digestate to arable fields can increase the abundance and diversity of soil pathogens, in part, because spores from pathogens like *Salmonella* and *Listeria* can survive the pasteurization process (Bonetta et al. 2014; Nkoa 2014; Sahlström 2003).

Our results strongly suggest that future metagenomic analysis of soil microbial communities from arable fields should be combined with metatranscriptomics and metaproteomics to determine more fully the potential effect of agricultural intensification on soil pathogens. Although several studies have proposed that metagenomic analysis can be a way to quickly identify pathogens in soil and water (Cahill 1999; Delmont et al. 2011; Deng et al. 2015; Pettengill et al. 2012; Pongor et al. 2014, Zhu et al. 2015), metagenomic data alone cannot differentiate between expressed and non-expressed genes, and thus, whether a ‘potential’ pathogen is ‘active.’ Complementing metagenomic analysis with metatranscriptomics could highlight differences in expressed genes among sites while metaproteomics could be used to assess the catalytic potential of the different microbial communities at each field (Bastida et al. 2008; Gilbert and Hughes 2011; Simon and Daniel 2010; Wilmes and Bond 2010). Although perhaps more expensive and time consuming, pursuing these additional analyses can identify

the relationship between specific soil pathogens and particular land uses, crops, or fertilizers. Further analysis of fields fertilized with digestate is also particularly important as current bioenergy initiative increasingly promote digestate as a nutrient-rich fertilizer to local farmers.

4.3 The Conservation and Agricultural Importance of Grassland Microbial Diversity

Our analysis shows that arable reversion was only partially successful in restoring microbial diversity. For example, fungal diversity was significantly lower on fields under arable reversion. Previous work (Plassart et al 2008; Smith et al 2003) had shown that arable reversion could restore grassland vegetation diversity and increase soil microbial biomass. However, these studies only measured overall microbial diversity, rather than microbial community composition and specific taxa. Although arable reversion can increase microbial biomass and C sequestration it cannot ‘restore’ native soil communities (Jangid et al.2003). Species-rich grasslands thus harbour unique microbial communities which cannot be replaced. Inoculum could be created from these grasslands and applied to fields under arable reversion to aid bioremediation efforts. Previous studies have found that using inoculum created from native grasslands boosted the success of grassland restoration by increasing establishment of seeded plots and vegetation diversity on degraded land compared to treatments using commercial inoculum or no inoculum (Emam 2016; Richter and Stutz 2002; Torez et al. 2016). In addition, inoculating intensively cultivated arable fields with soils from species-rich grasslands could potentially boost soil fertility sustainably and decrease reliance on chemical fertilizers. Although inoculum for arable fields is available commercially, it usually contains a very limited number of taxa (e.g. *Bacillus* spp. or *Glomus* spp.) (Garg and Chandel 2010). In contrast, the heterogeneity of microbiota in inoculum from native grasslands may provide a greater range of ‘beneficial’ microbiota to the soil. As such, the microbial diversity found on species-rich grasslands potentially has a strong economic and agricultural value, lending further support to calls to conserve these landscapes.

5. Conclusion

Based on field research in Oxfordshire, we show that agricultural intensification has the power to significantly alter soil microbial communities. While specific microbiota with established ‘beneficial’ activities, such as mycorrhizas and nitrogen-fixing bacteria, were found across the study sites, fungal diversity significantly declined under increased soil inputs and reduced vegetation diversity. Although our study did not aim to specifically identify pathogens in the soil, our analysis found that specific microbiota found to be pathogenic in other situations increased dramatically on agriculturally improved fields. Over the long term, continued conversion of species-rich grasslands to arable fields could lead to the loss of the microbial genetic resources specific to unimproved grasslands and an overall decline in soil fertility. Future research should look more specifically at how certain land management activities, such as crop rotation, the use of arable margins, and even inoculating fields with soil from species-rich grasslands, may help mitigate these effects and how and why agricultural intensification may potentially increase soil pathogens. Our results thus provide strong support for the conservation of species-rich grasslands and highlight the potential importance these landscapes may play in creating more sustainable agricultural systems.

Data accessibility:

We have deposited the raw sequence data and the metadata associated with each sample with the European Nucleotide Archive (ENA). It can be accessed at <http://www.ebi.ac.uk/ena/data/view/PRJEB13388>.

Funding

This research was part of K.F.'s doctoral research and was funded by the British Marshall Scholarship and Trinity College, Oxford. Fieldwork expenses were partially covered by a grant from the Biosocial Society.

Conflict of interest. None declared.

Acknowledgements

K.F. wishes to thank Natural England, Earth Trust, and the landowners involved in the study for providing access to their fields and information about land use practices and Emily from Anglian Soil Analysis for explaining soil testing procedures.

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Author contributions:

KF designed the experiment and carried out the fieldwork. KF and AT conducted the lab work. AT and KF performed the sequence analyses and taxonomic assignment. KF and LT performed the

726 statistical analysis of the data. KF wrote the initial draft of the manuscript which was then revised with
727 input from all authors.