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Long-term fertilization and tillage regimes have limited effects on structuring bacterial and denitrifier communities in a sandy loam UK soil

Claire E. Moulton-Brown,[1] **Tianer Feng**,[2] **Shreiya Shivagni Kumar**,[2] **Luxi Xu**,[2] **Calvin Dytham**,[1] **Thorunn Helgason**,[1] **Julia M. Cooper**[2] **and James W. B. Moir**[1*]

[1]*Department of Biology, University of York, Heslington, York, UK.*

[2]*School of Natural and Environmental Sciences, Newcastle University, Newcastle Upon Tyne, UK.*

Summary

Denitrification causes loss of available nitrogen from soil systems, thereby reducing crop productivity and increasing reliance on agrochemicals. The dynamics of denitrification and denitrifying communities are thought to be altered by land management practices, which affect the physicochemical properties of the soil. In this study, we look at the effects of long-term tillage and fertilization regimes on arable soils following 16 years of treatment in a factorial field trial. By studying the bacterial community composition based on 16S rRNA amplicons, absolute bacterial abundance and diversity of denitrification functional genes (*nirK*, *nirS* and *nosZ*), under conditions of minimum/conventional tillage and organic/synthetic mineral fertilizer, we tested how specific land management histories affect the diversity and distribution of both bacteria and denitrification genes. Bacterial and denitrifier communities were largely unaffected by land management history and clustered predominantly by spatial location, indicating that the variability in bacterial community composition in these arable soils is governed by innate environmental differences and Euclidean distance rather than agricultural management intervention.

Introduction

Below-ground biodiversity plays a major role in the functioning of soil ecosystems and is considered a key measure of soil health and essential for the supply of ecosystem goods and services (Bardgett and van der Putten, 2014; Bender *et al*., 2016; Delgado-Baquerizo *et al*., 2016). Whilst farming practices are changing and efforts are being made to become more sustainable, in terms of meeting current food requirements whilst having minimal negative environmental effects (Rockström *et al*., 2017), it is important to consider what effects agricultural management practice will have on microbial communities and what the consequences of this will be for crop productivity and biogeochemical cycles.

The effect of soil microbial biodiversity on the nitrogen cycle is critical, as nitrogen is the primary limiting factor for productivity in agroecosystems (Rütting *et al*., 2018). To keep up with global crop demands, 110 Mt nitrogen fertilizer was applied in 2016 (FAO, 2018). Nitrogen fertilizer use remains a controversial issue, because of the need to balance high crop yield with the expense of fertilizers and the environmental impact associated with overuse, including eutrophication of waters, global warming and stratospheric ozone depletion (Ravishankara *et al*., 2009; IPCC, 2019; Stevens, 2019).

One option for balancing yield and environmental impact is through the use of organic fertilizers (OFs) which do not require fossil fuels for manufacture. Long-term OF amendments have been shown to increase microbial species richness relative to synthetic mineral fertilizers (SFs) (Hartmann *et al*., 2015; Francioli *et al*., 2016), which benefits ecosystem functioning. Additionally, farmers may seek to adopt different tillage practices to reduce their environmental impact. Reduced tillage, including no tillage (NT) and minimum tillage (MT), is thought to increase microbial diversity relative to conventional tillage (CT) by increasing soil organic carbon stocks (Cooper *et al*., 2016; Sun *et al*., 2016; Wang *et al*., 2016; Chen *et al*., 2020), with the added benefits of reduced soil erosion and improved water conservation (Derpsch *et al*., 2010). It should be noted, however, that there is often little consensus on the effects of tillage, with recent studies finding that reduced tillage does not increase soil carbon (Keel *et al*., 2019; Camarotto *et al*., 2020) or prokaryotic biodiversity (Degrune

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et al., 2016; Piazza *et al*., 2019). There is also little consensus on whether reduced tillage increases or decreases nitrogen losses through N_2O emissions (Six *et al*., 2004; Rochette, 2008; Krauss *et al*., 2017a; Krauss *et al*., 2017b).

Denitrification, the microbially mediated dissimilatory reduction of nitrate to nitrogen gases, accounts for 60% of global N_2O emissions (Kroeze *et al*., 1999) and contributes to decreased crop productivity through loss of available nitrogen from terrestrial ecosystems (Syakila and Kroeze, 2011). Although denitrification is considered beneficial for wastewater treatment and aquatic ecosystems, in terms of removing fixed nitrogen from the ecosystem and preventing undesirable consequences such as algal bloom (Lu *et al*., 2014), in agriculture the loss of nitrates from fertilizers is detrimental and costly. Understanding the impact of land management practices in structuring the denitrification community may enable the mitigation of these nitrogen losses.

Whilst the effects of tillage and fertilization regimes have received considerable attention, the potential impact on denitrifying bacteria remains relatively unclear. Previous studies have looked at the effect of NT compared to CT, with results suggesting that NT leads to an increase in denitrifier abundance and activity (Wang and Zou, 2020). This effect was shown to be fertilizer dependent (with vs. without N fertilizer) (Krauss *et al*., 2017a; Wang and Zou, 2020). Less is known about the effects of type of nitrogen fertilizer used, or the combined effects of fertilizer type and MT on total soil and denitrifier community structure.

The highly heterogeneous soil matrix contains many micro-scale soil habitats which affect microbial activity, diversity and abundance. Whilst many studies demonstrate the impact of long-term land management of microbial and denitrifier diversity, there is a conspicuous knowledge gap in how this is linked to natural processes in shaping the denitrifying community. Despite relatively weaker biogeographic patterns being observed in microbial taxa than in plants and animals (Meyer *et al*., 2018), it remains plausible that microbial and denitrifier community assembly is driven by local environmental conditions, dispersal limitations and selection (Dumbrell *et al*., 2010; Vos *et al*., 2013; Domeignoz-Horta *et al*., 2018; Jiang *et al*., 2020). However, little is known about how long-term agricultural practices affect these drivers.

The initial objective of this study was therefore to gain an understanding about the impact of fertilizer regime (mineral versus organic) and tillage (minimum versus conventional) on denitrifying functional guilds in arable soils in a long-term field trial. We hypothesized that (i) MT would be associated with increased denitrifier diversity due to reduced physical disturbance, reduced aeration and increased soil organic carbon which provides optimum conditions for full and partial denitrification; and (ii) SF would be associated with increased microbial abundance and denitrification gene diversity due to increased initial nitrate concentration relative to composted dairy manure. An additional objective of the study was to investigate the importance of inherent environmental variation within the field site, and intrinsic neutral processes, on microbial community composition. This study uses bacterial and select denitrifier gene diversity to address these specific objectives.

Results

Soil physicochemical properties

Soil physicochemical properties were measured to ascertain whether land management treatments affected soil properties, which could influence bacterial community composition. There was little chemical difference in the soil between treatments apart from extractable potassium content, which was significantly higher in soils treated with OF compared to SF (Table 1; Fertilizer: $F_{1,13} = 23.07$; $p = 0.0009$). All potassium concentrations had a K Index of 1, indicating a potential risk of potassium deficiency, whereas all soils were within range of P Index 2, the target index for arable crops (Alexander *et al*., 2008). Soil carbon was also slightly elevated in soils treated with OF, although this was not statistically significant ($p = 0.055$).

Treatment did not affect soil texture or pH. However, active microbial carbon biomass, measured through bacterial respiration, was greater in plots with MT compared to CT (Tillage: $F_{1,13} = 17.72$; $p = 0.002$). And, although not significant at the $p < 0.05$ level ($p = 0.054$), organic matter was also increased in sites that used MT.

The effect of tillage and fertilizer on bacterial taxa and abundance

The 16S ribosomal RNA gene sequence was used to investigate bacterial abundance and diversity. The relative abundance of each phylum remained relatively constant across plots (Fig. 1A), but clear differences were observed in the total abundance, with plots having between 1.45×10^9 and 3.41×10^{10} 16S rRNA genes per gram of soil (Fig. 1B). Although no significant difference was found between treatments due to high variance, MT appears to be associated with greater bacterial abundance. This is in accordance with the bacterial biomass result discussed above. When investigating the high sample variance further, it was clear that field-block had a significant effect on the 16S rRNA abundance in each soil sample (Block: $F_{3,12} = 5.77$; $p = 0.0111$), with Block 2 soils having significantly more 16S rRNA copies per gram of soil than any other block.

Table 1. Summary of a 16-year cropping systems management experiment at Nafferton Factorial Systems Comparison study (A) and soil physicochemical properties (B).

(A) Cropping system	MT OF	MT SF	CT OF	CT SF
Tillage	Shallow non-inversion	Shallow non-inversion	Deep inversion	Deep inversion
Fertility	Composted dairy manure	Ammonium nitrate	Composted dairy manure	Ammonium nitrate
Pest Control	None	None	None	None
Weed Control	None	None	None	None
(B) Soil chemical, physical and biological properties				
C (%)	2.24 (±0.09)	2.16 (±0.04)	2.21 (±0.05)	2.06 (±0.05)
N (%)	0.22 (±0.009)	0.21 (±0.003)	0.21 (±0.009)	0.21 (±0.005)
C:N	10.21 (±0.11)	10.35 (±0.08)	10.41 (±0.25)	10.08 (±0.21)
Extractable P (mg kg^{-1})	22.19 (±2.91)	17.14 (±3.24)	18.42 (±4.14)	18.42 (±3.92)
Extractable K (mg kg^{-1})	109.3 (±10.1)[a]	82.6 (±7.3)[b]	107.7 (±12.5)[a]	68.2 (±2.2)[b]
Moisture content (%)	23.0 (±0.92)	22.6 (±0.64)	22.4 (±1.26)	22.4 (±0.82)
pH	6.74 (±0.10)	6.51 (±0.13)	6.66 (±0.08)	6.69 (±0.16)
Clay (%)	16.50 (±0.65)	16.50 (±0.87)	16.50 (±0.29)	17.25 (±0.85)
Sand (%)	64.75 (±0.63)	64.25 (±1.80)	63.50 (±1.19)	62.50 (±1.55)
Silt (%)	18.75 (±0.25)	19.25 (±1.03)	20.00 (±1.00)	20.25 (±0.75)
Microbial biomass (μg C g^{-1} soil)	209.6 (±25.9)[a]	203.1 (±33.8)[a]	143.9 (±23.7)[b]	144.2 (±25.7)[b]
Organic matter (%)	6.19 (±0.19)	6.11 (±0.10)	5.99 (±0.13)	5.80 (±0.16)

All values are interaction means of four replicates with standard errors given in brackets.[1] MT = minimum tillage, CT = conventional tillage, OF = organic fertilizer & SF = synthetic mineral fertilizer.
[1]Means followed by the same letter in the same row are not significantly different. All soil properties measured were compared using Linear Mixed Effects model ($p < 0.05$).

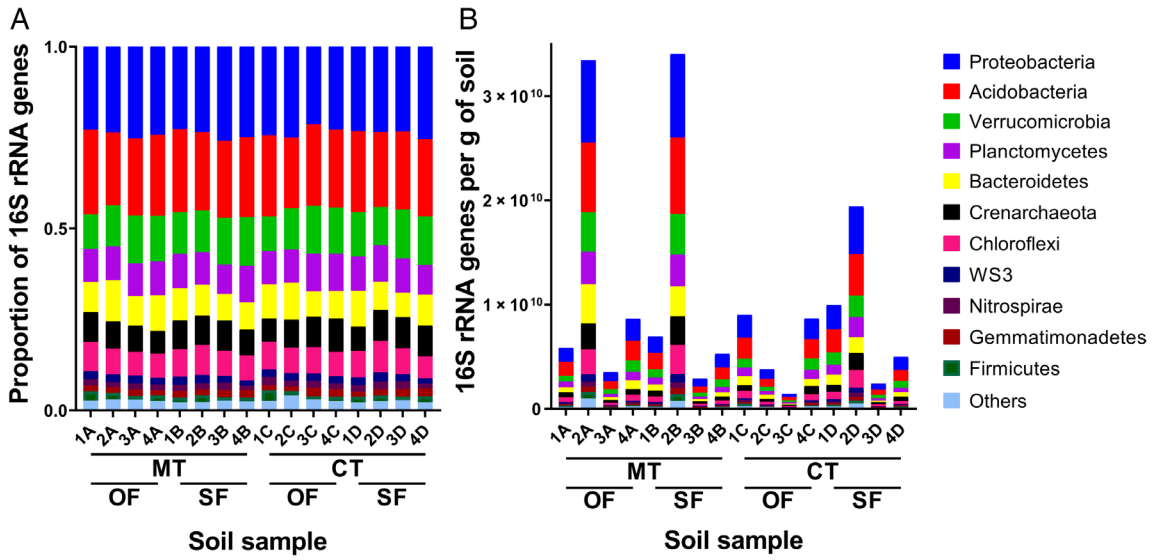

Fig. 1. Relative (A) and absolute (B) abundance of bacterial phyla in soils treated with different combinations of tillage and fertilizer. 'Others' refers to all phyla with an abundance of less than 1% across samples. Labels indicate field plot. MT and CT denote minimum and conventional tillage respectively. OF and SF denote organic and synthetic fertilizer respectively. [Color figure can be viewed at wileyonlinelibrary.com]

The dominant phyla across all samples were the Proteobacteria (with classes Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria dominating all samples) and Acidobacteria (Fig. 1). Indicator species analysis identified OTUs that were specifically associated with land management history. Although 3716 OTUs were detected in total, only 32 were associated with specific treatments, indicating that land management treatment did not affect the majority of taxa within the soil.

Five indicator species were specifically associated with plots treated with SF. These were the ammonia oxidizing *Nitrosospira multiformis*, a Chloroflexi in the Kouleothrixaceae family, a *Spartobacterium* thought to be an obligate symbiont of plant pathogenic nematodes of the *Xiphinema americanum* group (Vandekerckhove *et al*., 2000) and two Phycisphaerae [a bacterial class of mainly unknown ecophysiologies often found in ANAMMOX communities (Costa *et al*., 2014)]. Two OTUs were specifically associated with MT treatments.

One is a member of the DA101 genus of the Chthoniobacteraceae family that is known to be common in grassland soils (Felske and Akkermans, 1998), the other is a Gammaproteobacteria of the Sinobacteraceae family.

The effect of tillage and fertilizer on bacterial rRNA and denitrification gene alpha diversity

To compare the effects of land management on within-plot biodiversity, three measures of alpha diversity were used. The number of observed variants did not differ significantly in the 16S rRNA, *nirK* or *nosZ* amplicons, but there were approximately 32% more observed *nirS* variants in the MT-treated plots than the CT (Fig. 2A; Tillage: $F_{1,9} = 13.7$; $p = 0.0048$). Likewise, *nirS* had significantly greater Shannon diversity under MT compared to CT (Fig. 2B; Tillage: $F_{1,9} = 12.08$; $p = 0.0069$). Notably, land management treatment was not associated with any differences in variant evenness for any of the amplicons (Fig. 2C). Overall, there is very little impact of variation in tillage or fertilizer regime on below-ground biodiversity judged on the basis of 16S, *nirK*, *nirS* or *nosZ* alpha diversity.

All genes had significantly different levels of evenness, with 16S rRNA variants being more evenly distributed within plots compared to the other genes which had more dominant variants, particularly *nirS* (Fig. 2C; Evenness by gene: $F_{3,60} = 106.4$; $p = 2e^{-16}$).

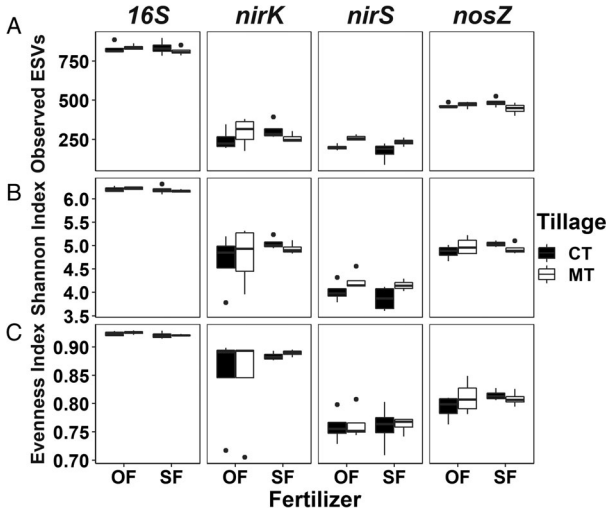

Fig. 2. Boxplots of alpha diversity measures of each sample treatment.
A. Observed ESVs by sample treatment.
B. Shannon diversity index.
C. Pielou's evenness index. Conventional tillage is shown in black, with minimum tillage shown in white. OF and SF denote organic and synthetic fertilizer respectively.

The effect of tillage, fertilizer and sample location on bacterial community composition

To explore the differences in the composition of bacterial communities between land management treatments, a non-metric multidimensional scaling (NMDS) ordination of whole bacterial community 16S rRNA gene amplicons was constructed (Fig. 3A). To confirm if the differences between community compositions can be explained by land-management factors, a PERMANOVA test was used. Neither tillage method nor fertility treatment nor a combination of the two were found to have significant effects on community composition (Tillage: $F_{1,15} = 0.73$; $p = 0.81$; Fertilizer: $F_{1,15} = 1.2$; $p = 0.18$; Tillage $\times$ Fertilizer: $F_{1,15} = 0.59$; $p = 0.98$), despite block effects being included as a random factor. The variable accounting for most variance among samples was blocked, i.e. the location of the plots (Fig. 3; Block: $F_{3,15} = 5.7$; $p = 0.001$), suggesting that field location had a greater effect on soil bacterial community than either tillage or fertilizer treatments.

Amplicon analyses for the denitrification genes *nirK*, *nirS* and *nosZ* (clade I) were then used to ascertain whether functional diversity followed the same patterns as taxonomic diversity in the treated soils. Like bacterial taxa, the variants of the nitrite reductase gene *nirK* were not significantly associated with tillage or fertilizer treatment (Tillage: $F_{1,15} = 0.92$; $p = 0.53$; Fertilizer: $F_{1,15} = 1.02$; $p = 0.35$; Tillage $\times$ Fertilizer: $F_{1,15} = 0.70$; $p = 0.96$), but were strongly associated with field block (Fig. 3B; Block: $F_{3,12} = 2.41$; $p = 0.001$). This was also true for *nirS*, which was not significantly affected by land management (Tillage: $F_{1,15} = 1.0$; $p = 0.37$; Fertilizer: $F_{1,15} = 0.65$; $p = 0.6$; Tillage $\times$ Fertilizer: $F_{1,15} = 0.29$; $p = 0.99$), but was significantly affected by block (Fig. 3C; Block: $F_{3,12} = 6.18$; $p = 0.001$). The same pattern was evident in nitrous oxide reductase *nosZ* (Fig. 3D; Tillage: $F_{1,15} = 0.73$; $p = 0.67$; Fertilizer: $F_{1,15} = 1.0$; $p = 0.32$; Tillage $\times$ Fertilizer: $F_{1,15} = 0.56$; $p = 0.92$; Block: $F_{3,12} = 3.58$; $p = 0.001$), suggesting that field-block location is the major determinant of bacterial community and denitrification functional composition.

Spatial and environmental effects on bacterial community composition

Once it became apparent that bacterial communities and denitrification genes appear largely unaffected by land management history, and cluster predominantly by field block, we began investigating the role of environmental factors and spatial location in bacterial community composition. Environmental vector fitting revealed that five soil variables: soil moisture, phosphorous, pH, silt and sand content were significantly related to total bacterial community composition (Fig. 3A; Envfit: Moisture

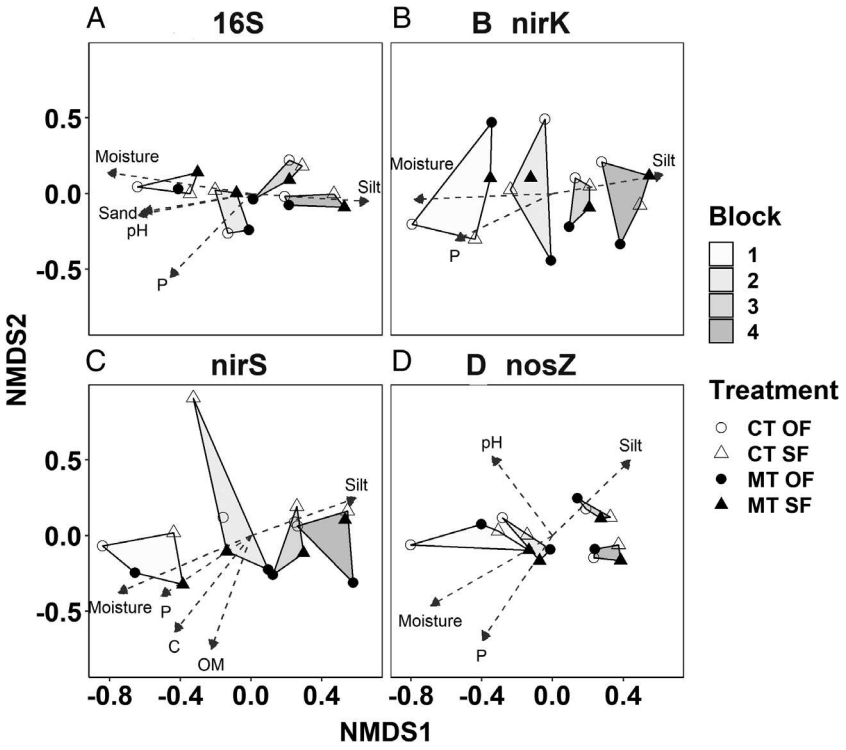

Fig. 3. Non-metric multidimensional scaling (NMDS) of gene amplicon Bray–Curtis dissimilarity from NFSC soils.
A. Whole bacterial community 16S rRNA.
B. *nirK* amplicon variants.
C. *nirS* amplicon variants.
D. *nosZ* amplicon variants. Samples are indicated with points, conventional tillage (CT) plots are shown in white, with minimum tillage (MT) in black; OF is denoted by circles, SF treatments are shown by triangles. Points are grouped by polygons indicating field block, from block 1 shown in white to block 4 shown with the dark grey. Labels indicate environmental variables which significantly explain sample beta diversity.

$r^2 = 0.68$, $p = 0.001$; Phosphorus $r^2 = 0.52$, $p = 0.009$; pH $r^2 = 0.43$, $p = 0.021$; Silt $r^2 = 0.44$, $p = 0.025$; Sand $r^2 = 0.38$, $p = 0.04$). Since none of these factors was affected by land management treatment (Table 1), this suggests that innate environmental differences have more effect on bacterial community composition than long-term tillage or fertilizer treatments.

Additionally, the distribution of all the denitrification genes was significantly associated with moisture (Envfit moisture: *nirK* $r^2 = 0.61$, $p = 0.002$; *nirS* $r^2 = 0.70$, $p = 0.001$; *nosZ* $r^2 = 0.69$; $p = 0.001$). Phosphorus and silt were also significantly correlated with denitrification gene distribution (Fig. 3B,D). *NirS* variation was significantly associated with carbon content and organic matter (Envfit *nirS*: carbon $r^2 = 0.59$, $p = 0.005$; organic matter $r^2 = 0.61$; $p = 0.005$), suggesting that *nirS* organisms may be more sensitive to carbon than their *nirK* counterparts.

To further investigate variation in community composition, a spatial analysis was completed. A Euclidean distance matrix (with the distances between the centre of each plot) was compared against the Bray–Curtis dissimilarity matrix using a Mantel test. The test indicated that there was significant positive correlation between the physical distance between plots and their community dissimilarity for all of the amplicons tested (Fig. 4; 16S rRNA: $R^2 = 0.590$, $p = 0.001$; *nirK*: $R^2 = 0.429$, $p = 0.001$; *nirS*: $R^2 = 0.559$, $p = 0.002$; *nosZ*: $R^2 = 0.589$, $p = 0.001$). This result indicates a clear distance decay effect, with increased physical distance being associated by increased dissimilarity between bacterial communities and denitrification gene distributions. Here, spatial distance is found to account for approximately 55% variation in bacterial communities and denitrification genes.

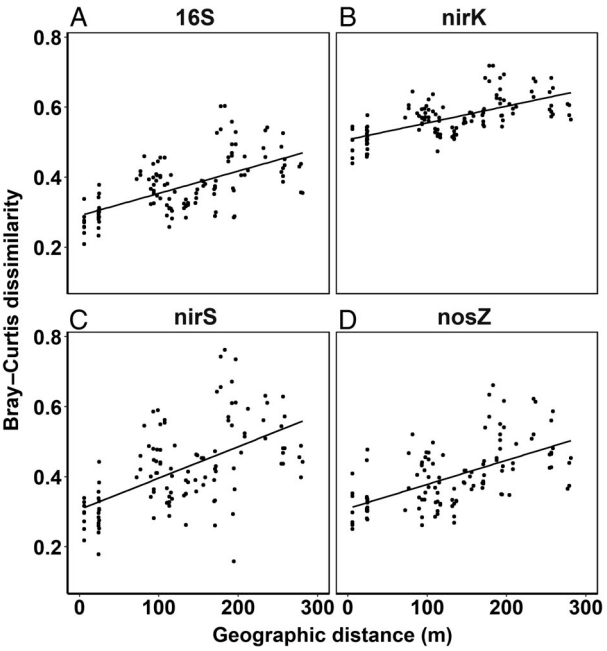

Fig. 4. Distance–decay relationship between geographic distance between samples and Bray–Curtis dissimilarity index for 16S rRNA, *nirK*, *nirS* and *nosZ* genes.

To separate the effects of space alone from environmental gradients on community composition, the calc. relimp function in R was used. This indicated that environmental and spatial variation together accounted for 63.6% variation in 16S rRNA diversity, with 18.9% of this attributable to space alone and 44.7% attributable to environmental variables. Spatial and environmental gradients together accounted for 28.5%, 63.0% and 50.8% of community variation in *nirK*, *nirS* and *nosZ* respectively. Spatial distance had a relatively stronger effect on the denitrification genes than on the overall microbial population, with space alone attributable to 13.1%, 21.1% and 22.6% of variation respectively. Together these analyses indicate that spatial distance is a major determinant of bacterial community similarity and has a much stronger effect than long-term tillage or fertilizer regimes.

Discussion

This study demonstrates that in a 16-year treatment regime there was virtually no effect of long-term tillage and fertilizer regimes on soil microbial community and denitrification gene diversity, but clear field-scale patterns across distance and environmental gradients.

MT was associated with an increase in microbial biomass, which is in accordance with several studies which demonstrate that conservation tillage systems, including NT and MT, have increased microbial biomass (Wallenstein and Hall, 2012; Zhang *et al*., 2012; Murugan *et al*., 2014). The increase is thought to be due to increased levels of microbially available, labile soil carbon and nitrogen in surface soils under conservation tillage (Chen *et al*., 2009). While labile C may represent a relatively small proportion of total soil organic C (Li *et al*., 2018), it plays a key role in driving soil microbial activity. We did not directly measure labile C in this study, but values for soil basal respiration were significantly higher in the MT treatment compared to CT, suggesting a higher proportion of labile C in the MT soils, which were not observed in this long-term field trial. A meta-analysis (Wang and Zou, 2020) indicates that no-tillage treatment leads to an increase in denitrification, N_2O and changes in *nirK*, *nirS* and *nosZ* abundances. Such an analysis has not been done for reduced or minimum-tillage, but the few available studies indicate that the minimum-tillage is not typically different to CT in terms of denitrification or N_2O production, but this varies between studies (Dendooven *et al*., 2012; Tian *et al*., 2012; Tellez-Rio *et al*., 2015; Krol *et al*., 2016).

The headline finding in this article is that there is no significant change in microbial community or denitrifiers gene diversity that can be linked to conventional versus MT in this arable crop setting. While substitution of organic N sources has been proposed as a strategy to reduce losses of mineral N, in this study we found no discernible difference in denitrifier populations due to N source. This may have been due to the application of equivalent levels of total N for each treatment, suggesting that the total nitrogen supplied rather than the nitrogen source has the primary effect on denitrifier community diversity.

Environmental variation through oxygen and nitrate content; organic matter quantity, quality, and availability; redox potential; temperature; pH; and soil type are all known to affect microbial communities and denitrification (Drenovsky *et al*., 2004; Wallenstein *et al*., 2006; Enwall *et al*., 2010; Nadeau *et al*., 2019). Here, we show significant effects of moisture, potassium, pH and soil textural class on all the denitrification gene diversities. Cytochrome cd_1 type nitrite reductase *nirS* is further associated with increased soil carbon and soil organic matter, which may be due to the relatively greater energy demands needed for enzyme assembly for *nirS* type denitrifiers, which requires at least 10 genes for assembly of the mature nitrite reductase enzyme, compared to *nirK* type denitrifiers, which requires only a single gene (Rinaldo *et al*., 2016). Additionally, a combination of other physical factors, including those not measured in this experiment such as oxygen concentration, may also be important drivers of soil microbial community structure. With the majority of heterotrophic denitrifiers utilizing oxygen much of the time, denitrification genes face limited selection pressure. But, as denitrification will at times be a critical response to a bottleneck in electron acceptor availability, the capacity to rapidly pivot to denitrification can prove advantageous.

Whilst environmental gradients can be attributed to approximately 32.6% variation across all the genes studied, it remains clear that geographical distance is also a driver of microbial community and denitrifier gene diversity. Distance-decay and other spatial effects are well-known ecological phenomena (Green *et al*., 2004; Zhou *et al*., 2008; Martiny *et al*., 2011; O'Brien *et al*., 2016) that appear to be driving bacterial and denitrifier gene diversity in this study. Here, community similarity decays as a function of distance, which suggests that dispersal ability is a significant force behind the bacterial diversity observed in this study.

In this study, we used the denitrification genes *nirK*, *niSK* and *nosZI* as indicator genes for the denitrification process. These genes appear to show greater dispersal limitation than the background bacterial population. This may indicate that the genetic backgrounds in which these genes occur have either restricted niches or lower motility – both of which are consistent with the anoxia required for denitrification. The combination of spatial effects and environmental gradients observed here suggests that a combination of niche and neutral processes drives

observed community structure and that the balance of the processes may further be related to function. Future work with greater focus of functional consequences of diversity could explore how other genes in the pathway, such as *nosZII*, respond to environmental change.

The United Nations promote the use of reduced tillage for improved and sustained crop production (FAO, 2015) and the use of OFs to maintain and enhance soil productivity. This experiment demonstrates that long-term reduced tillage and OF regimes have no significant negative effects on soil physicochemical properties. Indeed, OF use led to an increase in soil potassium which may improve crop yield (Pettigrew, 2008) and MT led to increased active microbial carbon biomass, which can improve crop yield through increased nutrient cycling, suppression of soil-borne pathogenic microorganisms and the decomposition of organic matter, which is closely associated with the aboveground performance of crops (Marschner, 2007; Berg and Smalla, 2009).

This study evaluated the role of agricultural management practices on microbial and denitrification gene diversity through the use of a long-term randomized complete block field trial. Although this study focussed on the diversity of bacterial denitrifiers possessing indicator genes *nirK*, *nirS* and *nosZI*, further work may focus on a broader scope of genes and organisms as well as elucidating the link between genetic diversity and functional behaviour. Here, whilst land management had a limited effect on microbial community abundance, distance and physicochemical properties were the main drivers of total bacterial and specific denitrification gene diversity. Therefore, whilst the microbial community can be identified and its genetic potential characterized, it is unlikely that long-term land management strategies will significantly alter the community to improve nitrogen use efficiency. Strategies for optimizing nitrogen-use efficiency and minimizing N_2O release should be grounded on knowledge about the properties of specific, innate below-ground microbial communities, which are geographically determined and resistant to land-management interventions.

Experimental procedures

Field experiment and sampling

Experiment 3 of the Nafferton Factorial Systems Comparison (NFSC) study is a long-term factorial field experiment established in 2001 in the Tyne Valley, UK (54:59:26.3 N; 1:54:37.4 W) to compare the effects of tillage and fertilizer regimes on crops in an 8-year crop rotation (Orr *et al*., 2011). The soils were sampled from the site in April 2017 when all plots were planted with spelt and rye and were at the same stage in the crop rotation. The 24 m $\times$ 6 m plots were treated with synthetic mineral (SF) or organic (OF) fertilizer (100 kg total N ha^{-1} $year^{-1}$ ammonium nitrate fertilizer or composted dairy manure respectively) and deep inversion CT or shallow non-inversion MT, with each treatment replicated four times in blocks distributed across the field site (Fig. 5). The topsoil of the 4 ha trial site is a uniform sandy loam formed in slowly permeable glacial till deposits: Dystric Stagnosol (Soil Survey Staff, 2014).

The SF was applied in mid-April 2016, with P and K in the SF plots only added at maintenance levels based on soil analysis. Compost was added in October 2016 for the 2016/17 season, at the same total N-input level as the SF. The compost was produced onsite at Nafferton Farm from cow manure and straw and applied prior to sowing, with a fresh batch used each year. The SF used ammonium nitrate, triple superphosphate and muriate of potash as the source of NPK respectively. Treatments for fertilizer varied each year depending on the crop but have been organic or conventional for the full 16-year experiment.

The two tillage levels were also applied prior to sowing: minimum and CT. MT was shallow (<20 cm depth) non-inversion tillage using a Dyna-Drive cultivator (Bomford Turner Ltd.) while CT treatments were mouldboard ploughed to a depth of 25–30 cm with soil inversion. All plots were sown with a 1.5 m wide Sow-Lite seed drill (Jordan Engineering Ltd.). Plots were not sprayed with pesticides during the 16-year trial to remove possible impacts on microbial and denitrifier populations. To allow for within-plot variability, 10 cores of soil (0–10 cm depth) were randomly sampled within each plot and immediately mixed to form one composite sample per plot. Soils were sieved through a 4 mm sieve then stored at $-20^{\circ}C$ prior to DNA extraction.

DNA extraction and amplicon sequencing

Genomic DNA was extracted from all 16 soil samples for community 16S rRNA gene and denitrification gene amplicon sequence analysis. The denitrification genes used were nitrite reductases *nirK* and *nirS*, and nitrous oxide reductase *nosZ* (clade I). Details of DNA extraction and PCR amplification of 16S rRNA and denitrification genes are described in Supplementary Materials and Methods.

PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and quantified using NanoDrop (Thermo Fisher Scientific). PCR products were sequenced using the Illumina MiSeq platform with 2 $\times$ 300 bp reads by the Bioscience Technology Facility, University of York.

Raw fastq files were demultiplexed, then trimmed to 100 000 reads per sample and quality-filtered using QIIME2 version 2018.2 (Bolyen *et al*., 2019) to a quality

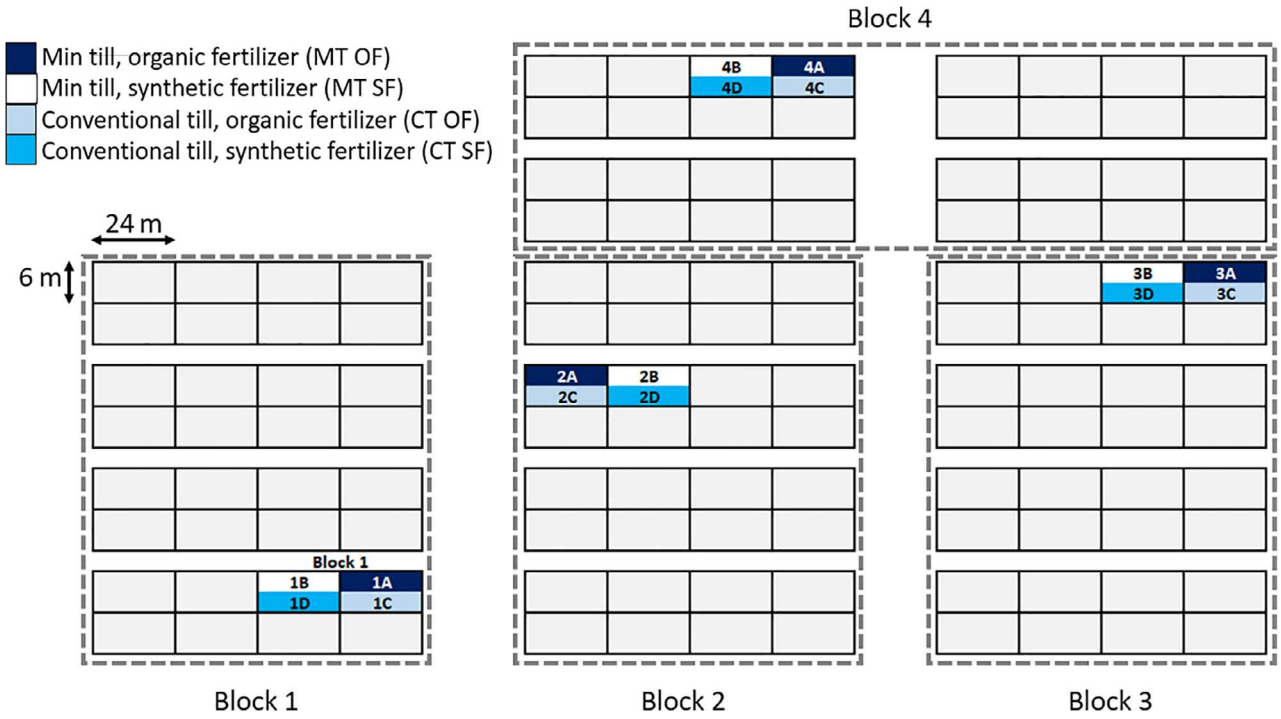

Fig. 5. Schematic of Nafferton Factorial Systems Comparison (NFSC) East Hemmel Experiment 3 Plots indicating plots used in this study. MT = minimum tillage, CT = conventional tillage, OF = organic fertilizer and SF = mineral fertilizer. Each individual plot measured 6 × 24 m (four blocks of 32 plots are separated from other blocks by 7 m). [Color figure can be viewed at wileyonlinelibrary.com]

score above 25. Sequences were denoised, dereplicated and chimaeras removed using the dada2 denoise-paired function. Feature IDs were summarized before taxonomy was assigned using the Greengenes 16S rRNA database (DeSantis *et al*., 2006). For denitrification genes, exact sequence variants (ESVs) were used, with reads manually verified using the FunGene database (Fish *et al*., 2013). Sequence data was uploaded to the European Nucleotide Archive, under accession number PRJEB49432.

Quantification of 16S rRNA

A 16S rRNA gene copy number quantification technique (Smets *et al*., 2016) was carried out for soils by spiking them with an internal DNA standard (*Thermus thermophilus* DSM 46338) prior to DNA extraction at an estimated 0.1% of total DNA. This strain was selected as it is unlikely to be found in UK soils as it grows optimally at 72°C. Details of culture conditions and DNA extraction procedure are described in Supplementary Materials and Methods.

To determine the number of 16S rRNA genes found in each sample, the equation given by Smets *et al*. (2016) was used. Briefly, the number of reads assigned to each taxon is multiplied by a constant, made by multiplying the 16S copy number of the internal standard by the weight of DNA added to the sample, divided by the genome weight of the internal standard. This value is then divided by the number of reads assigned to the internal standard. A gene copy number of two and a weight of 2.16×10^{-15} g per *T. thermophilus* genome were assumed (Hallin and Ussery, 2004).

Determination of soil properties

Soil was chemically analysed for carbon and nitrogen content using the Vario MACRO Cube CN Elemental Analyser (Elementar). Extractable phosphorus was determined using the Olsen P test (Olsen *et al*., 1954) and extractable potassium was determined following extraction with 1 M ammonium nitrate using a flame photometer. pH was measured in H_2O in 1:2.5 wt./vol. suspensions using a pH probe.

Gravimetric water content was calculated by dividing the mass of water lost when soil was dried at 105°C for 24 h by the mass of the dry soil. Particle size distribution was determined by a Low Angle Laser Light Scattering technique using a Malvern Mastersizer 2000 optical bench with recirculating wet cell enhancement and a Hydro 2000MU sample introduction unit. Organic matter content was calculated using the Loss on Ignition method (Ball, 1964). Active soil bacterial carbon biomass was determined by substrate-induced respiration (Beare

et al., 1990) through addition of glucose to the MicroResp™ system. CO_2 respiration rates were converted to ml CO_2 100 g^{-1} of dry soil weight h^{-1} using the ideal gas law equation. Microbial carbon biomass was calculated using the formula described by Anderson and Domsch (1978).

Statistical and diversity analysis

Statistical and diversity analyses were performed using R version 3.5.1 (R Core Team, 2013) implemented in RStudio version 1.2.1335 1 (RStudio Team, 2018). Physicochemical soil data were analysed using two-way analysis of variance (ANOVA) using the lmer function from the lmerTest package (Kuznetsova *et al*., 2017), with tillage method and fertilizer type as factors and block as a random factor. Percentage data were arcsine-square root transformed prior to analysis.

The multipatt function from the indicspecies package (De Cáceres and Legendre, 2009) was used to perform Dufrene–Legendre indicator species analysis to identify bacteria that were specifically associated with different treatments. Alpha diversity was compared across samples using species richness (observed OTUs/ESVs), the Shannon index and Pielou's evenness index (Shannon, 1948; Pielou, 1966). Differences in alpha diversity were tested using two-way ANOVA as described above. Beta-diversity was represented by Bray–Curtis distance matrices generated from the OTU/ESV table (Roger Bray and Curtis, 1957). Function adonis2 in the R package vegan (Oksanen *et al*., 2013) was used to quantify the possible effects of tillage, fertilizer and field block on beta-diversity, using the Bray–Curtis distance matrix and NMDS plots were used to visually represent community dissimilarities between samples based on the Bray–Curtis index using the metaMDS function in vegan. Distance matrices were composed using the vegdist function in vegan before Mantel tests were conducted to measure the correlation between spatial distance and Bray–Curtis dissimilarity and also between environmental data and Bray–Curtis dissimilarity using the mantel function in vegan.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supporting Information.