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# Microbial community succession in soil is mainly driven by carbon and nitrogen contents rather than phosphorus and sulphur contents

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## Abstract

Organic manure is widely applied in agricultural systems to improve soil nutrient cycling and other physicochemical properties. However, the biotic and abiotic mechanisms that drive C, N, P, and S cycling following manure application are not completely understood. In this study, soil samples were collected from long-term experimental plots that had been amended with farmyard manure or mineral fertilisers since 1964. Isotope labelling with $^{15}N$, $^{33}P$, and $^{35}S$; metagenomics; and high-throughput sequencing were used to reveal the relationships between C, N, P, and S dynamics and microbial community composition and functions depending on fertilisation. A clear niche differentiation was observed between bacteria and fungi under mineral and manure regimes. A network analysis showed that long-term manure application reduced the complexity and stability of soil microbial network. Furthermore, a variation partitioning analysis based on redundancy analysis indicated that microbial community variation was mainly driven by soil Cand N contents. Dissolved organic C was the most important factor regulating microbial community structure. Soil C and N contents explained 43.5% of bacterial and 37.9% of fungal community variations. In contrast, soil P and S contents explained 29.9% of bacterial and 20.3% of fungal community variations. Long-term manure application increased the abundance of most functional genes related to C, N, P, and S cycling. This led to increased C and N cycling rates under manure application, which provided sufficient substrates for microbial growth. Partial least squares path modelling indicated that soil

physicochemical properties, especially dissolved organic carbon, directly influenced C and S cycling, whereas the N and P cycles were indirectly affected by the changes in microbial community composition. These results provide a new perspective on both direct and indirect effects of organic manure and inorganic fertilisers on the soil nutrient cycling processes mediated by soil microbial community.

Keywords: Manure, Nutrient cycling, Microbial community, Functional genes, Co-occurrence network

## 1 Introduction

Microorganisms drive soil biogeochemical cycles (Jiao et al., 2021). Various factors such as temperature (Li et al., 2021b), precipitation (de Vries et al., 2018), and soil physicochemical properties (Rousk et al., 2010) have been shown to considerably affect the soil microbiota and, thus, microbe-driven nutrient cycling (Wang et al., 2021). Soil nutrient content can also influence microbial community diversity and composition (Eo and Park, 2016; Dai et al., 2020; Tang et al., 2022). For instance, soil with high nutrient content favours copiotrophic taxa, whereas oligotrophic microorganisms are better adapted to nutrient-deficient soils (Fierer and Jackson, 2006; Fierer et al., 2007). In addition, soil nutrients bioavailability not only altered microbial community but also its function expression in nutrient cycling. For example, when the soil C/N or C/S value was high due to manure application, the mineralization process driven by microorganisms will be accelerated to maintain microbial stoichiometry (Ma et al., 2021a).

Microorganisms are not only influenced by environmental factors but also by microbial community interactions, which ultimately affect microbe-driven nutrient cycling (Jiang et al., 2018; Ma et al., 2018). Therefore, elucidating the stability and complexity of the relationships among microorganisms is necessary for attaining a complete understanding of microbial ecology and for the implementation of sustainable agricultural production strategies. Microbial networks are widely applied to reveal the relationships among microorganisms (Deng et al., 2012; de Vries et al.,

2018). Co-occurrence networks allow the evaluation of microbial response to environmental change. Previous studies found that the interactions among microorganisms in networks are influenced by fertiliser regimes (Gu et al., 2019) and climate change, including elevated $CO_2$ levels (Zhou et al., 2011), increased temperatures (Yuan et al., 2021), and increased drought frequency (de Vries et al., 2018). A recent study found that soil fungal richness regulates the soil bacterial community assembly, with stochastic assembly processes decreasing along with an increase in fungal richness (Jiao et al., 2022). Co-occurrence networks also allow the assessment of the functional potential of agricultural ecosystems. Recently, Shi et al. (2020) demonstrated that the functional potential of agricultural ecosystems is highly associated with the abundance of kinless hubs (taxa within highly connected networks) in a network. However, the response of microbial networks to long-term manure application and the relationship between soil nutrient cycling and microbial network response have rarely been reported.

The cycling of C, N, P, and S is strongly associated with agricultural production and ecosystem functions (Ma et al., 2020a; Ma et al., 2020b; Ma et al., 2021a). Fertilisation can influence nutrient cycles directly, through the addition of substrates or products of cycling processes, or indirectly, by altering the soil environment and microbial community (Fan et al., 2019; Dai et al., 2020). For instance, long-term P inputs increase microbial P fixation, while long-term N inputs reduce P mineralisation (Dai et al., 2020). N-fixation rates decrease under long-term fertilisation, and the application of N fertilisers accelerates potential nitrification rates (Yao et al., 2016;

Fan et al., 2019). Mineralisation of organic C is supressed by mineral fertiliser amendment, but this suppression can be alleviated by partial substitution of mineral fertilisers with green manure (Xu et al., 2021). Furthermore, simultaneous long-term use of N fertilisers and manure increases the mineralisation of organic S (Knights et al., 2001). Most studies have investigated the links between element cycling rates and microbial function focusing solely on one element. However, the C, N, P, and S cycles are interconnected, and whether fertiliser application has the same effect on the cycling of various elements and microbial function remains unknown. Various studies have investigated the soil C and N cycles, whereas studies on the P and S cycles are relatively limited (Malik et al., 2018; Dai et al., 2020; Li et al., 2021a; Yu et al., 2021). Previous studies used enzyme activity and functional genes related to the P and S cycles to represent soil P and S processes (Dai et al., 2020; Yu et al., 2021). However, inconsistent relationships have been reported between functional genes and their related soil processes (Graham et al., 2014; Bier et al., 2015; Rocca et al., 2015); these relationships depend on soil physicochemical properties, cycling elements, and climatic factors (Moran et al., 2013; Rocca et al., 2015; Tosi et al., 2020).

Fertilisers can considerably affect microbial communities by directly increasing nutrient availability and indirectly changing the aboveground plant growth and soil chemical properties such as pH and electrical conductivity (EC) (Rousk et al., 2010; Yang et al., 2019; Liu et al., 2021). The application of inorganic or organic (manure) fertilisers alone is not favourable to crop growth, because inorganic fertilisers will lead to environmental risk and decline of microbial diversity, whereas, manure

application alone is not sufficient to achieve high crop yields in the context of intensive production systems (Chen, 2006; Garzón et al., 2011; Geisseler and Scow, 2014; Ji et al., 2020). The partial substitution of mineral fertiliser with manure has been proposed to reduce environmental risk while maintaining crop yields (Q. Tang et al., 2021; Xu et al., 2021). However, our knowledge of the effects of long-term mineral fertiliser combined with different amounts of manure on soil microorganisms and nutrient cycles is still relatively poor.

In this study, soil samples were collected from experimental plots treated since 1964 with mineral fertilisers or manure to investigate their effect on the soil microbiota and associated C, N, P, and S cycling. The N, P, and S cycling rates were determined using $^{15}N$, $^{33}P$, and $^{35}S$ isotope pool dilution methods, respectively, while microbial community composition and function were detected using high-throughput sequencing and metagenomics. The specific aims of the study were to 1) assess the response and the interactions of soil microorganisms under long-term fertilisation, 2) investigate the response of functional genes and soil processes to long-term manure application, and 3) analyse the relationships among soil physicochemical properties, soil microorganisms, functional genes, and soil processes.

## 2 Material and methods

### *2.1 Field experiment design and soil sampling*

The field experiment at Woburn Experimental Farm in SE England (51°59′59″ N,

0°36′56″ W) (www.era.rothamsted.ac.uk/WoburnFarm) has been ongoing since 1964 (Mattingly. 1974). The soil texture (excluding organic matter) at the site is classified as sandy loam with 80% sand, 6% silt, and 10% clay (Ma et al., 2020a). Three typical fertilisation treatments were set up as follows: 1) NM: mineral fertiliser with N, P, and K inputs equivalent to those of 25–50 t $ha^{-1}$ $y^{-1}$ of farmyard manure (FYM); 2) MM: mineral fertiliser combined with medium amounts of FYM (10 t $ha^{-1}$ $y^{-1}$); and 3) HM: mineral fertiliser combined with high amounts of FYM (25–50 t $ha^{-1}$ $y^{-1}$). Four replicates were established for each treatment using a randomised block design, wherein each replicate consisted of an 8.83 m × 8.0 m experimental plot. In the early years of the experiment, a five-course arable crop rotation involving potatoes, wheat, sugar beet, barley, and oats (or beans) was conducted. From 2004, the rotation included spring barley, winter beans, winter wheat, maize, and winter rye. A cover crop, mustard, was grown before the spring barley and maize.

The HM treatment consisted of 50 t $ha^{-1}$ $y^{-1}$, 50 t $ha^{-1}$ $y^{-1}$, and 25 t $ha^{-1}$ $y^{-1}$ of FYM amendment during 1966–1971, 1981–1987, and 2003–2018, respectively (28 years in total) (Table 1). The mineral N, P, K, and S fertilisers were applied as nitrochalk (27% N), triple superphosphate, and potassium sulphate at 83 (mean N rate over all plots), 20, 83, and 36 kg $ha^{-1}$ $y^{-1}$, respectively, in the NM and MM treatments between 2003 and 2018. The HM treatments did not include additional P and K fertiliser but did include similar inputs of N fertiliser since 2003. The MM treatment included the application of FYM at a rate of 10 t $ha^{-1}$ $y^{-1}$ over a period of 16 years (2003–2018). Between 1965 and 2003, an amount of mineral fertiliser equivalent to a straw input

178 (7.5 t $ha^{-1}$ $y^{-1}$) was applied to the MM plots. The total nutrient input of each treatment
179 from 1996 to 2018 is listed in Table S1. A detailed description of the NM, MM, and
180 HM treatments has been provided by Ma et al. (2021b).
181 Topsoil (0–23 cm, plough layer) and subsoil (23–38 cm) samples were collected
182 by hand from four plots with each treatment, using a 2.5 cm diameter corer, in June
183 2018. Eighteen cores were collected per plot, and the soil corresponding to each
184 treatment was thoroughly mixed before being transported to the laboratory. There, it
185 was sifted with a 5 mm sieve to remove earthworms and roots. The resulting soil
186 samples were divided into two parts each. The first was stored at 4 °C in a refrigerator
187 for no more than 10 days to determine its physicochemical properties (Table S2).
188 These were measured following a previously reported procedure (Ma et al., 2020a;
189 Ma et al., 2020b; Ma et al., 2021b). The second part was stored at −80 °C and used for
190 high-throughput sequencing.
191
192 *2.2 Analysis of soil physicochemical properties*
193 Soil pH and EC were determined after extraction with deionised water (soil:
194 deionised water = 1:2.5). Soil organic matter (SOM) content was measured using the
195 loss-on-ignition method. Total C and N were detected by dry combustion using a
196 CHN-2000 analyser (Leco Corp., St. Joseph, MI, USA). Soil basal respiration (BS)
197 was measured using an EGM-5 portable infrared gas analyser (PP Systems Ltd.,
198 Hitchin, UK). Dissolved organic carbon (DOC) and total dissolved nitrogen (TDN)
199 were measured using a multi-N/C 2100S TOC-TN analyser (Analytik Jena AG, Jena,

Germany) after extraction with 0.5 M $K_2SO_4$. $NH_4^+$ and $NO_3^-$ contents were detected colourimetrically using a Power Wave HT microplate spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). Extractable organic N (EON) was calculated by subtracting $NH_4^+$ and $NO_3^-$ contents from the TDN. Soil microbial biomass C (MBC), N (MBN), P (MBP), and S (MBS) were determined using the fumigation-extraction method (Vance et al., 1987). MBC and MBN were calculated using a conversion factor of 2.22 (Jenkinson et al., 2004), while MBP and MBS were calculated using conversion factors of 2.5 and 2.86, respectively (Vong et al., 2003; Spohn et al., 2016). Soil protein content was estimated by measuring the amino acids (AA) produced by acid hydrolysis. Soil peptides and free AA fractions were extracted with 0.5 M $K_2SO_4$ before passing through a 1000 MW ultrafiltration membrane with an Amicon stirred cell (Merck-Milli-pore, Burlington, MA, USA). Subsequently, the AA in the eluent were analysed using the fluorometric o-Phthaldialdehyde-β-Mercaptoethanol method before and after acid hydrolysis (Jones et al., 2002). Soil total P and total S were detected using inductively coupled plasma-optical emission spectrometry (Varian 710 ES, Agilent Corp., Santa Clara, CA, USA). Soil Olsen P was detected colourimetrically, the $PO_4^{3-}$ and $SO_4^{2-}$ contents were measured with ion chromatography, using a Dionex AS14A column (930 Compact IC Flex; Metrohm Ltd, Runcorn, UK). Organic P content was determined as the difference between molybdate reactive-P and total P contents. Organic S content was calculated as the difference between $SO_4^{2-}$ and total S contents.

### *2.3 Analysis of soil gross production and consumption of $NH_4^+$, $NO_3^-$, $PO_4^{3-}$, and $SO_4^{2-}$*

The gross production and consumption of $NH_4^+$, $NO_3^-$, $PO_4^{3-}$, and $SO_4^{2-}$ were determined using $^{15}N$, $^{33}P$, and $^{35}S$ isotope pool dilution techniques, respectively (Eriksen, 2005; Kellogg et al., 2006; Corre et al., 2007). For N cycle determination, 2 ml of 0.1 mM 20% atm $^{15}N$-$NO_3^-$ or $^{15}N$-$NH_4^+$ was added to 20 g of moist field soil. After 3 and 24 h, the soil was extracted with 80 ml of 0.5 M $K_2SO_4$ (180 rpm, 30 min), followed by centrifugation (18,000 *g*, 5 min), and $NO_3^-$ or $NH_4^+$ contents were determined with micro-detection colorimetric methods as described in a previous study (Mariano et al., 2016; Ma et al., 2020b). To determine the P cycle rate, 1 ml $Na_3{}^{33}PO_4$ (99 kBq $ml^{-1}$) was added to 10 g of moist field soil. After 3 and 24 h of cultivation at 20 ºC, the soil was extracted with 40 ml of 0.5 M $NaHCO_3$ (180 rpm, 30 min) and centrifuged (18,000 *g*, 5 min). The $PO_4^{3-}$ content of the supernatant was detected colourimetrically (Murphy and Riley, 1962; Ma et al., 2020a). To determine the S cycle rate, 1 mL $Na_2{}^{35}SO_4$ (99 kBq $mL^{-1}$) was added to 10 g of moist field soil. After 3 and 24 h, the soil was extracted with 50 mL of 0.01 M $CaCl_2$ (180 rpm, 30 min) and centrifuged (18,000 *g*, 5 min). The $SO_4^{2-}$ content was measured by adding 1 M $BaCl_2$ (Ma et al., 2021b). The $^{15}N$ abundance was detected by element analyser-stable isotope mass spectrometry (EA-IRMS) (IsoPrime100; Isoprime Ltd., Cheadle Hulme, UK). $^{33}P$ and $^{35}S$ activity were detected by Wallac 1404 scintillation counter (Wallac EG & G, Milton Keynes, UK) (detailed information is shown in supplementary information Section S1).

244

245 *2.3 Soil DNA extraction and sequencing of bacteria and fungi*

246 DNA was extracted from soil subsamples (0.5 g, stored at −80 °C) using the
247 FastDNA SPIN kit (MP Biomedicals, Irvine, CA, USA) following the manufacturer's
248 instructions. A NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop
249 Technologies, Wilmington, DE, USA) was then used to determine the concentration
250 and quality of the extracted DNA. Primers 515F-806R (Caporaso et al., 2011) for
251 bacteria and ITS1F-ITS2 (Gardes and Bruns, 1993) for fungi were selected for
252 amplification. The sequencing of polymerase chain reaction products was performed
253 using the Illumina Novaseq platform. Bacterial and fungal sequence data were
254 processed using an in-house pipeline (Feng et al., 2017). The same operational
255 taxonomic units (OTUs) were clustered with 97% similarity. Annotation of taxonomic
256 data for representative sequences of bacteria and fungi was performed using the
257 SILVA (Quast et al., 2013) and UNITE (Nilsson et al., 2019) databases, respectively.

258

259 *2.4 Shotgun metagenomic sequencing*

260 Sequencing of the metagenomic DNA shotgun was performed using the Illumina
261 HiSeq 2500 platform (Illumina, Inc., San Diego, CA, USA). For this, ~5.0 Gbp
262 paired-end reads (150 bp in length) were produced after contaminant and quality
263 filtering. The cleaned sequences were de novo assembled into contigs using the
264 Megahit assembler (Zhu et al., 2019). The selected contigs were uploaded to and
265 annotated by the Integrated Microbial Genomes pipeline to derive abundance tables

for genes based on the KEGG database (Kanehisa et al., 2015). Functional genes related to the C, N, P, and S cycles were selected for subsequent analyses. Detailed analysis methods for the shotgun metagenomic sequencing have been previously provided by Ma et al. (2020a).

*2.5 Network analysis*

A microbial network was constructed to reveal the complex microbiome interactions. Each fertilisation treatment consisted of eight replicates of topsoil and subsoil samples. The top 200 abundant bacterial OTUs and top 200 fungal OTUs were selected to avoid spurious correlations; these accounted for 54% and 80% of bacterial and fungal abundance, respectively (Zheng et al., 2021). A Molecular Ecological Network Analyses Pipeline was applied to construct a microbial network based on the random matrix theory (Deng et al., 2012). When the cut-off value was 0.93, the p-values of the Chi-square test for the three treated networks were all greater than 0.05. To make the networks of the three treatments comparable, we chose 0.93 as the cut-off value for each treatment. The network was visualized using Gephi (http://gephi.github.io/). Subsequently, connectivity within a module (*Zi*) and among modules (*Pi*), which were calculated using the 'igraph' package in R, were applied to classify the nodes into peripherals ($Zi < 2.5$ and $Pi < 0.62$), connectors ($Zi < 2.5$ and $Pi > 0.62$), module hubs ($Zi > 2.5$ and $Pi < 0.62$), and network hubs ($Zi > 2.5$ and $Pi > 0.62$) (Guimerà and Nunes Amaral, 2005). *Zi* and *Pi* were calculated following equations (1) and (2), respectively.

$$Zi = \frac{Ki - \overline{K}si}{\sigma_{Ksi}} \quad (1)$$

where $Ki$ is the number of edges between node $i$ and other nodes in module $Si$, $\overline{K}si$ is the average $K$ value of all nodes in module $Si$ ($K$ is the number of connections between this node and other nodes in $Si$), and $\sigma_{Ksi}$ is the standard deviation of the $K$ values of all nodes in module $Si$.

$$Pi = 1 - \sum_{S=1}^{N_M} \left(\frac{Kis}{Ki}\right)^2 \quad (2)$$

where $Ki$ is the degree of node $i$, $M$ represents modules, and $N_M$ is the number of modules in the network. If the edges associated with a node are evenly distributed in all modules, the $Pi$ value of the node is close to 1; if all the edges related to a node are in the module to which it belongs, the $Pi$ value of the node is 0 (Guimerà and Nunes Amaral, 2005).

*2.6 Statistical analysis*

All statistical analyses were performed using R (version 3.4.3). The Shapiro–Wilk test was used to assess normality before testing for significance, and the homogeneity of variance was analysed with an F-test. Differences in soil characteristics between the treatments were analysed with a one-way analysis of variance and least significant difference tests ($p < 0.05$). Permutational multivariate analysis of variance was used to test the significance of changes in the microbial community under the fertilisation treatments. The differences in microbial communities under the fertilisation treatments were analysed with non-metric multidimensional scaling (NMDS), performed with the 'vegan' package in R, based

on Bray–Curtis dissimilarity. Variation partitioning analysis (VPA) based on redundancy analysis and Mantel's test were implemented using the 'vegan' package in R to investigate the contribution of soil C, N, P, and S to the microbial community variation and the drivers of soil functional gene abundance, respectively. Pearson correlation test was carried out to investigate the relationships between the alpha and beta diversities of the microbiome and soil physicochemical properties. The 'pheatmap' package in R was used to generate heatmaps of soil functional genes and processes. Random forest analysis was performed using the 'randomForest' package in R. The partial least squares path model (PLS-PM) based on the 'plspm' package in R was also applied to evaluate the relationships among soil physicochemical properties, microbial community, functional genes, and soil processes. A bootstrap method (1,000 iterations) was used to validate the estimates of the path coefficients and the coefficients of determination ($R^2$). Finally, the goodness-of-fit index was used to evaluate the overall predictive performance of the model.

## 3 Results

### *3.1 Microbial alpha diversity under long-term manure fertilisation*

The Shannon and Chao1 indices were used to reflect the response of microbial alpha diversity to long-term manure application. The Chao1 index accounts for species richness and reflects rare species change, whereas the Shannon index reflects both evenness and abundance of species (Shannon, 1948; Chao, 1984). Compared to

NM application, long-term HM application significantly ($p < 0.05$) increased the bacterial Shannon index but had no significant effect ($p > 0.05$) on fungal Shannon index (Fig. 1A and 1C). Overall, microbial Shannon index significantly decreased ($p < 0.05$) with increasing soil depth. The bacterial Chao1 index showed no significant differences ($p > 0.05$) to the fertilisation regime and soil depth (Fig. 1B). HM application significantly ($p < 0.05$) increased the fungal Chao1 index of the subsoil (Fig. 1D). The bacterial Shannon index was significantly ($p < 0.05$) increased in soils with high N, SOM, and $SO_4^{2-}$ contents (Fig. 1E). However, the fungal Shannon index was independent of ($p > 0.05$) soil physicochemical properties (Fig. 1E).

*3.2 Microbial community succession under long-term manure fertilisation*

Long-term manure application reduced the relative abundance of Proteobacteria and favoured the growth of Firmicutes (Fig. S1A). Correspondingly, the relative abundance of Proteobacteria decreased with increasing soil nutrient contents (Fig. S1C), while the relative abundance of Actinobacteria and Firmicutes increased (Fig. S1C). Ascomycota accounted for more than 60% of the total fungal population (Fig. S1B). Basidiomycota growth was supressed by manure application, and it decreased with soil nutrient content (Fig. S1D). NMDS results further suggested that the effects of fertilisation treatments on microbial community structure were not significant ($p > 0.05$) (Fig. 2A and 2B). The HM treatment significantly ($p < 0.05$) reduced bacterial β-diversity, whereas it had no significant ($p > 0.05$) effect on fungal β-diversity (Fig. 2D and 2E). Soil C and N contents explained 43.5% of bacterial and 37.9% of fungal

community variations. In contrast, soil P and S contents explained 29.9% of bacterial and 20.3% of fungal community variations (Fig. 2C). Random forest analysis results suggested that microbial community succession was most highly associated with DOC and was more strongly influenced by soil C and N contents than by P and S contents (Fig. 2F and 2G).

*3.3 Response of soil functional gene abundance to long-term manure application*

In general, long-term manure application led to enrichment in the functional genes involved in C, N, P, and S cycling (Fig. 3). However, the relative abundance of methane oxidation and nitrification genes was reduced under the HM treatment. High manure application rates stimulated soil C and N cycling rates (Fig. S2). Regarding soil P cycling, the $PO_4^{3-}$ production (mineralisation) and consumption (immobilisation) rates in topsoil were highest under the HM treatment. In the case of the subsoil, MM application led to the highest $PO_4^{3-}$ production and consumption rates among all treatments (Fig S2). $SO_4^{2-}$ consumption was highest under MM fertilisation, while $SO_4^{2-}$ production increased in the topsoil but decreased in the subsoil as manure application rate increased. The soil $CO_2$ respiration rate significantly ($p < 0.001$) increased with SOM content (Fig S3). $NH_4^+$ production and consumption significantly ($p < 0.001$) increased with soil $NO_3^-$ content. $NO_3^-$ production significantly ($p < 0.05$) increased with soil $NH_4^+$ content. In contrast to those in C and N cycling, the $PO_4^{3-}$ production rate significantly ($p < 0.01$) decreased with increasing organic P content, whereas the $SO_4^{2-}$ production rate was independent

($p > 0.05$) of soil organic S content.

Mantel analysis indicated that functional genes involved in the C and N cycles were significantly ($p < 0.05$) associated with soil EON, $NH_4^+$, and MBN (Fig. S4). Pearson correlation analysis further demonstrated that the functional genes involved in the N cycles were more affected by soil physicochemical properties than those involved in the C, P, and S cycles (Fig. S5). The relative abundance of most genes involved in the N cycle increased with soil N content. Notably, the abundance of functional genes significantly ($p < 0.05$) decreased with an increase in fungal α-diversity (Fig. 4). However, functional gene abundance was independent ($p > 0.05$) of bacterial α-diversity.

*3.4 Microbial networks under the three fertilisation regimes*

Microbial network analysis of the two soil layers showed that the network nodes were mainly composed of the phyla Ascomycota (23–26%), Proteobacteria (16–22%), Actinobacteria (12–15%), Acidobacteria (10–11%), and Firmicutes (5–6%) (Fig. 5A). The network nodes and links decreased with the manure application rate (Table 2). Moreover, the interaction between bacteria and fungi showed the lowest positive correlation ratio (87%) under the HM treatment. Compared to the NM treatment, HM application produced a less complex network with low average degree (3.10) and path distance (4.58) (Table 2). Furthermore, the average degree of bacteria in the networks was higher than that of the fungi, except in the HM treatment (Table S3). The average eigencentrality of bacteria was higher than that of fungi (Table S3). Nodes with higher

within-module connectivity ($Zi > 2.5$) or higher among-module connectivity ($Pi > 0.62$) were identified as key taxa. $Zi > 2.5$ indicates that node *i* is highly connected within a module, and these taxa may function through the exchange of energy and matter within the module. $Pi > 0.62$ means that node *i* is highly connected among modules, mediates inter-module interactions, and functions through the inter-module exchange of energy and matter (Guimerà and Nunes Amaral, 2005; Deng et al., 2012; Deng et al., 2016). Across the three networks, the identified key taxa were classified as the phyla Ascomycota, Proteobacteria, and Acidobacteria (Fig. 5B and 5C). The relative abundance of key taxa was also found to be highly associated with soil cycling rates and functional gene abundance (Fig. S6).

*3.5 PLS-PM analysis*

The relationships between soil properties, the microbiome, and functional genes and processes were elucidated using PLS-PM analysis (Fig. 6). Soil properties had a direct positive effect ($p < 0.05$) on bacterial community, whereas they had a direct negative effect ($p < 0.05$) on fungal community. Compared to bacteria, fungi were more closely correlated with functional genes. In addition, soil properties had a greater effect on bacteria than on fungi. The microbial community had limited effect ($p > 0.05$) on soil C and S cycling. However, fungi had a direct negative effect ($p < 0.05$) on N cycling, and bacteria had a direct positive effect ($p < 0.05$) on P processes. Soil properties also had direct effects ($p < 0.05$) on C and S cycling while indirectly ($p < 0.05$) influencing N and P cycling through the microbiome. Regarding standardised

total effects, soil properties contributed most to C and S cycling (Fig. 6E), while the N and P cycles were mainly driven by fungi and bacteria, respectively. The PLS-PM model explained 83%, 66%, 32%, and 44% of the variance in soil C, N, P, and S processes, respectively.

## 4 Discussion

### *4.1 Soil microbial community succession under long-term manure application*

Bacterial and fungal communities exhibited divergent responses to long-term manure application (Figs. 1 and 2). Compared to the MM treatment, HM application significantly ($p < 0.05$) increased the bacterial Shannon index and decreased the fungal Shannon index. Previous studies also showed that bacterial α-diversity increased, whereas fungal α-diversity first increased and then decreased, with the ratio of organic substitution (Ji et al., 2018; Ji et al., 2020). Generally, the bacterial Shannon index was more sensitive to fertilisation treatments than was the Chao1 index (Fig. 1A and 1B). The Shannon index reflects both evenness and abundance of species, whereas the Chao1 index accounts for species richness and reflects rare species change (Shannon, 1948; Chao, 1984). Therefore, the presence of rare species of bacteria was independent of fertilisation regimes ($p > 0.05$).

Soil nutrients (C, N, P, and S) accounted for 73% of the variation in the bacterial community and 58% of the variation in the fungal community (Fig. 2C). This observation indicates that microbial community succession is mainly regulated by soil

nutrient content rather than soil pH which has been widely reported as one of the most important factors influencing microbial communities (Fierer and Jackson, 2006; Geisseler and Scow, 2014; Glassman et al., 2017). This is likely because the fertilisation treatments had limited effects on soil pH in this study, and pH conditions (pH = 7.1–7.5) were in a suitable range for microbial growth. Conversely, long-term fertiliser application significantly ($p < 0.05$) altered soil nutrient content. Soil nutrients not only directly provide nutrients for microorganisms but also play an important role in electron transfer (Marschner, 2021). DOC is an active substrate and is easily decomposed by microorganisms which has been demonstrated to regulate nutrient cycling by altering microbial communities (Wang et al., 2021). The random forest analysis results suggested that DOC was the most important factor that regulated microbial community succession and was more influenced by soil C and N contents than by P and S contents in this sandy soil (Fig. 2F and 2G).

*4.2 Long-term manure application upregulated the functional genes and reduced the complexity of the microbial network*

Most of the genes involved in the C, N, P, and S cycles were enriched under the manure application regimes (Fig. 3). As a large amount of C enters the soil following manure application, soil microorganisms govern nutrient cycle rates to maintain microbial substrate stoichiometry (Luo et al., 2020; Ma et al., 2021a). We found that manure application increased the abundance of N fixation genes. Furthermore, it increased the soil C content, thus stimulating microbial N fixation and stabilizing the

464 microbial substrate C/N ratio (Fan et al., 2019; Schleuss et al., 2021). Moreover,
465 manure application was reported to improve soil physicochemical properties, thus
466 favouring microbial growth and functional genes expression (Gu et al., 2019; Ma et
467 al., 2020b). Compared with the relationship between the genes related to C, P, and S
468 cycles and soil nutrients, gene responsible for N cycle showed higher correlations
469 with soil nutrients (Fig. S5). In this study, the substrates or products of the processes
470 involved in the genes related to N cycle were more comprehensively measured. For
471 example, $NH_4^+$ and $NO_3^-$ are important substrates or products of the N cycle process
472 (Kuypers et al., 2018). However, the specific substrates involved in C, P, and S cycles
473 were not detected in this study, so the genes related to N cycle resulted a high
474 correlation with soil nitrogen content (Fig. S5).
475 Manure application is generally considered to generate a more complex co-
476 occurrence network than that obtained with mineral fertiliser application (Ling et al.,
477 2016; Ji et al., 2020). In this study, long-term manure application reduced network
478 complexity (lower degree and density) (Table 2). Manure application increase the
479 nutrient abundance but reduced the microbial nutrient acquisition (Ji et al., 2020). In
480 contrast, NM led to enhance microbial interaction under the relatively less soil
481 nutrient content (Zhao et al., 2019; Yuan et al., 2021). The reduced average path
482 length of the network under the HM treatment indicates faster information
483 transmission, inducing a faster microbial community response to environmental
484 change (Zhou et al., 2010). Interestingly, bacteria showed a higher average degree
485 than fungi in the network constructed under the NM and MM treatments, while the

opposite was observed under the HM treatment (Table S3). This can be ascribed to niche differentiation between bacteria and fungi. While mineral fertilisers supply labile soil nutrients, which tend to favour bacterial growth, fungi tend to decompose recalcitrant C released from manure (Zhao et al., 2019; Xu et al., 2021). The recalcitrant C from high manure application favoured fungal growth and increased the fungal degree in the network.

Our study showed that the key fungal taxa belonged to the phylum Ascomycota, while the identified bacterial key taxa were Acidobacteria and Proteobacteria (Fig. 5). Ascomycota, Acidobacteria, and Proteobacteria were also the major phyla in the network, accounting for 46.4% to 54.3% of the total nodes. Previous studies have reported that key taxa play a vital role in soil nutrient cycling, including SOM mineralisation and N cycling (Shi et al., 2020; Zheng et al., 2021). The key taxa Botu55 was classified as Rhizobiales, which plays a vital role in N fixation (Fan et al., 2019). Pearson correlation analysis results also suggested that the relative abundance of Botu55 was significantly ($p < 0.05$) correlated with soil C, N, and S cycling gene abundance (Fig. S6).

*4.3 Relationships among soil physicochemical characteristics, microbiome, functional genes, and processes*

There is increasing evidence that soil microbial diversity is significantly associated with soil multifunctionality (Jiao et al., 2021). We found that functional gene abundance was independent ($p > 0.05$) of bacterial diversity but significantly ($p$

508 < 0.05) decreased with an increase in fungal diversity (Figs. 4 and 6). This is
509 inconsistent with the findings of some existing studies (Jing et al., 2015; Chen et al.,
510 2020). Compared to NM, high manure application increased functional gene
511 abundance and reduced the fungal Shannon index. A previous study also found that
512 manure incorporation reduces fungal richness by increasing extinction (Sun et al.,
513 2020). This phenomenon could be ascribed to the disparate nutrient characteristics of
514 manure and mineral fertilisers. Mineral fertilisers release fast-acting, broad-spectrum
515 nutrients, which help alleviate nutrient limitation and inter-species competition,
516 thereby favouring many fungal taxa (Sun et al., 2020). Conversely, the nutrients
517 released from manure are narrow-spectrum, favouring the growth of only specific
518 fungal species because of the differential utilisation of carbon substrates (Hanson et
519 al., 2008; Rinnan and Baath, 2009). Thus, compared to mineral fertilisers, manure
520 induces a substantial selection pressure on soil fungi.

521 Understanding the factors and pathways that influence soil nutrient processes
522 could help farmers regulate nutrient cycles by adjusting field practices and meet crop
523 nutrient demands while also reducing environmental risks. Fungi and bacteria were
524 found to be the most highly correlated with N and P processes, respectively (Fig. 6E).
525 The N processes in this study including $NH_4^+$/$NO_3^-$ production and consumption
526 which involved in organic N transformation, nitrification, and denitrification; soil
527 fungi have been played a vital role in N cycling (Kuypers et al., 2018; Zhong et al.,
528 2018; Zheng et al., 2020). And the results of Figure 4 also showed the Shannon index
529 of fungi was significantly ($p < 0.01$) correlated with abundance of genes responsible

for N cycling. Bacteria play a vital role in soil P cycling and the regulation of P availability (Dai et al., 2020; Wei et al., 2021). Based on the results, explanatory models were well fitted to C and N processes which explained 83% and 66% respectively, than explanatory models for P (32%) and S (44%), indicating the measured soil physicochemical and microbial properties in this study were inadequately explained the observed variations in P and S cycling. Soil C and N cycling, including mineralisation, N fixation, nitrification, and denitrification, are mainly driven by microorganisms (Kuypers et al., 2018; Naylor et al., 2020). In contrast to those in the C and N cycles, both biological and physicochemical processes, including sorption/desorption and precipitation/dissolution, which were not covered in this study, are considered to strongly affect the P and S cycles (Yan et al., 2018; Ma et al., 2021b). Manure application can improve soil fertility and structure while reducing the complexity and stability of the microbial network which deserves more attention.

## 4.4 Conclusions

In this study, we investigated the effects of long-term manure application on soil physicochemical characteristics, microbial communities, and nutrient cycling. The responses of P and S cycling rates to long-term manure application were decoupled with those of C and N. High manure application rates reduced the fungal Shannon index but increased the bacterial Shannon index. Manure application also reduced bacterial and fungal β-diversity. Soil C and N contents explained the microbial

552 community variation to a greater extent than did P and S contents. Functional genes
553 related to C, N, P, and S cycling were enriched under high manure application.
554 Functional gene abundance was independent of ($p > 0.05$) bacterial α-diversity,
555 whereas it significantly ($p < 0.05$) decreased with fungal α-diversity. In addition, long-
556 term manure application reduced the complexity and stability of the microbial
557 network. Furthermore, the C and S cycles were mainly regulated by soil
558 physicochemical properties, whereas the N and P cycles depended on
559 microorganisms. Our results provide new insights into the mechanisms of C, N, P, and
560 S cycling under long-term soil fertilisation regimes.
561

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572

573 **Author contributions**

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892 **Table captions**

893 Table. 1 Mineral fertiliser or farmyard manure (FYM) application of treatments. NM,
894 MM, and HM indicate zero, medium, and high rates of farmyard manure application,
895 respectively. *Mineral fertilisers equivalent to total content of N, P & K in FYM.

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897 Table. 2 The topological properties of networks in soils under long-term manure
898 application. NM, MM, and HM indicate zero, medium, and high rates of farmyard
899 manure application, respectively.

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914 **Figure legends**

915 Fig. 1 Alpha diversity of bacteria (A, B) and fungi (C, D) under long-term manure
916 fertiliser application. Different letters above the box indicate significant differences ($p$
917 $< 0.05$) between treatments. NM, MM, and HM indicate zero, medium, and high rates
918 of farmyard manure application, respectively. Topsoil: 0-23cm, Subsoil: 23-38cm. (E)
919 Pearson correlation between soil chemical properties and microbial alpha diversity.
920 EC: electric conductivity; BS: basal respiration; SOM: soil organic matter; DOC:
921 dissolved organic carbon; AA: amino acids; MBC, MBN, MBP, and MBS: microbial
922 biomass carbon, nitrogen, phosphorus, and sulphur; EON: extractable organic N. *, $p$
923 $< 0.05$; **, $p < 0.01$.

924

925 Fig. 2 Non-metric multidimensional scaling (NMDS) based on the Bray–Curtis
926 dissimilarities of (A) bacterial and (B) fungal communities in the topsoil (0–23 cm)
927 and subsoil (23–38 cm) under long term manure application. NM, MM, and HM
928 indicate zero, medium, and high rates of farmyard manure application, respectively.
929 Variation partitioning analysis based on redundancy analysis of bacterial and fungal
930 communities (C). The C pool consists of total C, SOM, DOC, protein, peptide, AA,
931 and MBC. The N pool consists of total N, EON, $NO_3^-$, $NH_4^+$, and MBN. The P pool
932 consists of total P, organic P, Olsen-P, $PO_4^{3-}$, and MBP. The S pool consists of total S,
933 organic S, $SO_4^{2-}$, and MBS. Dissimilarity based on Bray-Curtis of bacteria (D) and
934 fungi (E) among different fertilisation treatments. Different letters above the boxes
935 indicate significant differences ($p < 0.05$) between treatments. Random forest analysis

for the determination of factors affecting bacterial (F) and fungal community (G). EC: electric conductivity; BS: basal respiration; SOM: soil organic matter; DOC: dissolved organic carbon; AA: amino acids; MBC, MBN, MBP, and MBS: microbial biomass carbon, nitrogen, phosphorus, and sulphur; EON: extractable organic N.

Fig. 3 Functional gene abundance under long-term manure application. The values are centered and scaled by treatment. NM, MM, and HM indicate zero, medium, and high rates of farmyard manure application, respectively. Topsoil: 0–23 cm, Subsoil: 23–38 cm. Pentose phosphate pathway[1]: Pentose phosphate pathway (Pentose phosphate cycle), Pentose phosphate pathway[2]: Pentose phosphate pathway, archaea, fructose 6P => ribose 5P.

Fig. 4 Pearson correlation analysis between functional genes abundance and fungal Shannon index.

Fig. 5 Network of co-occurring bacterial and fungal operational taxonomic units (OTUs) after long-term application of manure or mineral fertilisers (A). NM, MM, and HM indicate zero, medium, and high rates of farmyard manure application, respectively. (B) Classification of nodes to identify keystone species within the fertilisation networks. (C) The classification of key taxa identified by the microbial network.

Fig. 6 Partial least squares path modelling (PLS-PM) analysis of the relationships between soil properties, bacteria and fungi functional genes, and cycles of C (A), N (B), P (C), and S (D). E: Standardized total effects of soil characters, bacteria, fungi, and functional genes on soil processes. Soil properties include total C, soil organic matter, and dissolved organic carbon. Bacteria: bacterial Shannon index and non-metric multidimensional scaling axis 1 value. Fungi: bacterial Shannon index and non-metric multidimensional scaling axis 1 value. C genes: the citrate cycle, the glyoxylate cycle, the general L-amino acid transport system, and xylene degradation selected from the KEGG database. N genes: the urea transport system, dissimilatory nitrate reduction, assimilatory nitrate reduction, and nitrate assimilation selected from the KEGG database. P genes: the pentose phosphate cycle, the reductive pentose phosphate cycle, the phosphate transport system, and the pentose phosphate pathway selected from the KEGG database. S genes: dermatan sulphate degradation, chondroitin sulphate degradation, and keratan sulphate degradation selected from the KEGG database. C processes: soil basal respiration. N processes: production and consumption of $NH_4^+$ and $NO_3^-$. P processes: $PO_4^{3-}$ production and $PO_4^{3-}$ consumption. S processes: $SO_4^{2-}$ production and $SO_4^{2-}$ consumption. Observed or latent variables are illustrated in the box. 1,000 bootstraps were conducted to estimate the path coefficients. Positive and negative effects are represented by blue and red arrows, respectively. Path coefficients that were not significantly different from zero are shown as grey dashed lines; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Percentages above the boxes represent the explanatory power of the variables. The goodness-of-fit

was used to assess the model.