Postprint: Research Advances on Microorganisms in Fluvo-aquic Farmland

Authors: Lin Xiangui, Feng Youzhi

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Abstract

The responses and feedback of soil microorganisms to different fertilization regimes are of paramount importance for evaluating and interpreting scientific fertilization. This paper synthesizes the research findings of our group regarding the responses of microorganisms associated with carbon, nitrogen, and phosphorus cycling in fluvo-aquic soils of the North China Plain to various fertilization practices, particularly the sole application of organic fertilizer, and elucidates the microbiological mechanisms underlying soil fertility improvement in fluvo-aquic soils. Results from long-term stationary experiments demonstrate that long-term balanced fertilization, especially organic fertilizer application, significantly increases organic carbon and nutrient contents—particularly phosphorus content—in fluvo-aquic soils, thereby altering soil microbial community structure and enhancing microbial biomass carbon, invertase activity, respiration intensity, and microbial functional diversity (carbon metabolic activity), while concomitantly reducing the metabolic quotient and metabolic heat of soil microorganisms. Conversely, under nutrient-deficient conditions, especially phosphorus deficiency, soil microorganisms exhibit not only low metabolic efficiency but also dissipate relatively more heat and emit relatively more CO during metabolic processes, resulting in significant soil quality degradation. Long-term nitrogen fertilizer application can enhance nitrification activity in fluvo-aquic soils and increase the abundance and diversity of ammonia-oxidizing bacteria, with inorganic nitrogen fertilizers exerting more pronounced effects than organic nitrogen fertilizers; however, the prerequisite for scientific nitrogen fertilizer application is the rational supplementation of phosphorus fertilizer, which is essential for better promoting crop growth in fluvo-aquic soils, reducing nitrogen loss, and improving soil fertility. For phosphorus-deficient fluvo-aquic soils, long-term phosphorus fertilizer application, particularly balanced fertilization, reduces crop dependency on arbuscular mycorrhizal fungi (AMF), consequently decreasing AMF diversity in soils and reallocating more soil nutrients to other microorganisms, thereby benefiting the sustainability of farmland soil fertility in fluvo-aquic soils. An indigenous microorganism in fluvo-aquic soils, Bacillus asahii, exhibits the most significant response to long-term organic fertilizer application, requiring 2–4 years to become a dominant microorganism in fluvo-aquic soils; it possesses unique physiological characteristics and rich metabolic diversity, capable of accelerating and facilitating the roles of other microorganisms in organic matter accumulation and phosphorus cycling processes in fluvo-aquic soils, thereby playing a “leading” role in crop growth and soil fertility. The aforementioned understanding deepens our knowledge of the microbiological mechanisms through which long-term organic fertilizer application improves soil fertility in fluvo-aquic soils of the North China Plain and helps guide the regulation of soil microorganisms to better serve farmland ecosystems.

Full Text

Preamble

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Research Progresses of Farmland Microorganisms in Fluvo-aquic Soil of China*

LIN Xiangui, FENG Youzhi
(Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, China)

Abstract

Soil microbial responses and feedback to different fertilization regimes are critically important for evaluating and interpreting scientific fertilization practices. This paper summarizes our research team’ s findings on how microorganisms associated with carbon, nitrogen, and phosphorus cycling in fluvo-aquic soil of the North China Plain respond to various fertilization strategies, particularly single organic fertilizer application, and clarifies the microbial mechanisms underlying soil fertility enhancement. Results from long-term field experiments demonstrate that balanced fertilization, especially long-term organic fertilizer application, significantly increases soil organic carbon and nutrient contents—particularly phosphorus—thereby altering soil microbial community structure and enhancing microbial biomass carbon, invertase activity, respiration intensity, and microbial functional diversity (carbon metabolic activity), while markedly reducing microbial metabolic quotient and metabolic heat. Conversely, nutrient deficiency, especially phosphorus deficiency, not only reduces microbial metabolic efficiency but also causes greater heat dissipation and CO emission during metabolic processes, leading to significant soil quality degradation. Long-term nitrogen fertilizer application enhances nitrification activity and increases the abundance and diversity of ammonia-oxidizing bacteria, with inorganic nitrogen fertilizers showing more pronounced effects than organic nitrogen fertilizers. However, rational phosphorus addition is a prerequisite for scientific nitrogen application to better promote crop growth, reduce nitrogen loss, and improve soil fertility in fluvo-aquic soils. For phosphorus-deficient fluvo-aquic soil, long-term phosphorus fertilization, particularly balanced fertilization, reduces crop dependence on arbuscular mycorrhizal fungi (AMF), consequently decreasing AMF diversity in soil and reallocating more soil nutrients to other microorganisms, which benefits the sustainability of farmland soil fertility. An indigenous microorganism, *Bacillus asahii*, showed the most significant response to long-term organic fertilizer application, becoming a dominant species in fluvo-aquic soil within 2–4 years. This species possesses unique physiological characteristics and abundant metabolic diversity, capable of accelerating and facilitating organic matter accumulation and phosphorus cycling processes by other microorganisms, thereby playing a "leading" role in crop growth and soil fertility improvement. These insights deepen our understanding of the microbial mechanisms through which long-term organic fertilizer application enhances fluvo-aquic soil fertility in the North China Plain and provide guidance for regulating soil microorganisms to better serve farmland ecosystems.

Keywords: Fluvo-aquic soil; Microorganism; Long-term fertilization; Organic fertilizer; Phosphorus fertilizer; Carbon-nitrogen-phosphorus cycling; *Bacillus asahii*

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Corresponding author: LIN Xiangui, E-mail: xglin@issas.ac.cn
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Population growth, global climate change, and adjustments in food consumption structure have made food security a critical challenge facing humanity today and in the future. Without guaranteed continuous production increases, food supply will become the most important and direct constraint on global economic development and social stability. Fertilizer is the "food" for crops, and it has been proven that among all yield-increasing factors, only fertilizer application shows a linear positive correlation with grain yield. However, unreasonable fertilization leads to low fertilizer use efficiency and causes significant damage to soils and the environment [1]. Therefore, scientific fertilization is the most effective approach to achieve grain yield increases. We must establish a scientific fertilization concept, strive to improve chemical fertilizer use efficiency, and promote sustainable agricultural development.

Scientific fertilization requires scientific evidence. Long-term field experiments represent an irreplaceable research methodology. Many ecological processes in agroecosystems progress rather slowly, and short-term experiments may not reveal trends in ecological processes. Therefore, long-term field experiments are essential for advancing ecological theory, revealing mechanisms of ecological process evolution, and providing scientific parameters for rational fertilization and optimized resource management. Rothamsted Research Station in the UK, with its 170-year history, is the world' s earliest research institution conducting long-term soil experiments, known as "classical field experiments" (Figure 1). Since the 1980s, China has also established a series of long-term field experiments investigating ecosystem structure, function, and dynamics of ecological processes. In 1983, the Fengqiu Agro-ecological Experimental Station of the Chinese Academy of Sciences established long-term positioning experiments, and in autumn 1989, established fertilizer long-term positioning experiments with seven fertilizer treatments: no fertilization control (Control), organic manure (OM), combined chemical and organic fertilizer (1/2OMN), and chemical fertilizers (NPK, NP, NK, and PK). To date, over 20 years of data from these experiments show that balanced fertilization (1/2OMN and NPK) significantly increases maize and wheat yields in fluvo-aquic farmland [2]. Additionally, single organic manure application also demonstrates significant yield-increasing effects, particularly during the maize season.

Soil microorganisms constitute a vital component of soil ecosystems, participating in organic matter decomposition, humus formation, and soil nutrient transformation and cycling. Changes in soil microbial community structure and activity serve as important indicators for assessing soil quality, maintaining soil fertility, and sustaining crop productivity. Consequently, soil microbial responses and feedback to different fertilization regimes are extremely significant for evaluating and interpreting scientific fertilization. Our research team systematically investigated microbial responses to various fertilization strategies, particularly single organic manure application, from three perspectives: carbon, nitrogen, and phosphorus cycling in fluvo-aquic soil, thereby clarifying the microbial mechanisms underlying soil fertility enhancement.

1 Long-term Different Fertilization Effects on Carbon Transformation Microorganisms in Fluvo-aquic Soil

Carbon sources are fundamental substances for microbial growth. Microorganisms decompose organic matter to obtain energy and perform ecological functions. Scientific fertilization not only increases crop yields but also enhances soil organic carbon content, inevitably affecting soil microbial function and diversity. Therefore, we initiated our investigation with carbon cycling-related microorganisms, examining multiple aspects including soil microbial biomass carbon, enzyme activities, carbon source utilization capacity, and community structure.

1.1 Effects of Long-term Different Fertilization on Microbial Biomass Carbon, Enzyme Activity, and Metabolic Activity in Fluvo-aquic Soil

Soil microbial biomass represents the most active and readily changeable fraction of soil organic matter, playing crucial roles in storage and regulation of plant nutrients. Its magnitude and activity directly influence nutrient mineralization, immobilization, and soil enzyme activities. Soil invertase activity, related to soil organic matter content, serves as an important indicator of soil biological activity intensity and soil maturation degree, while dehydrogenase activity reflects overall microbial metabolic activity; thus, microbial metabolic activity is typically calculated as dehydrogenase activity divided by microbial carbon. Figure 2 [Figure 2: see original paper] illustrates soil microbial biomass carbon, invertase and dehydrogenase activities, and microbial metabolic activity under different fertilization treatments [3-4]. Organic manure application resulted in the highest soil microbial biomass carbon (Figure 2A) and enzyme activities (Figures 2B, 2C), followed by the 1/2OMN treatment. Among chemical fertilizer treatments, those containing phosphorus showed higher values than phosphorus-deficient treatments, while the NK treatment showed no significant difference compared to the control. Microbial metabolic activity results demonstrated that organic manure and balanced NPK fertilization enhanced soil microbial metabolic activity (Figure 2D).

1.2 Effects of Long-term Different Fertilization on Microbial Metabolic Quotient and Metabolic Heat in Fluvo-aquic Soil

Soil respiration intensity refers to the rate of O absorption and CO release, with microbial activity being the primary source of soil respiration; thus, soil respiration intensity can characterize total microbial metabolic capacity. The ratio of respiration intensity to microbial biomass is termed metabolic quotient, an important indicator for assessing ecosystem evolution and health. Soil basal respiration intensity and metabolic quotient under different fertilization treatments are shown in Figure 3 [Figure 3: see original paper] [4]. Compared with the control, all phosphorus-containing treatments significantly increased soil basal respiration intensity (Figure 3A) while decreasing microbial metabolic quotient (Figure 3B), with organic manure treatments (1/2OMN and OM) showing particularly prominent changes. This phenomenon indicates that organic manure application enables soil microorganisms to serve ecosystems more “efficiently with low emissions,” signifying substantially improved soil health quality. The NK treatment without phosphorus fertilizer, along with the control, exhibited the highest metabolic quotient, indicating severe nutrient deficiency stress (particularly phosphorus) on microbial metabolism.

All living organisms produce heat during metabolism; therefore, heat release can represent an organism' s active status. Microcalorimeters can sensitively detect environmental heat changes and have been applied to measure microbial metabolic activities in complex systems such as soil. Microcalorimetric characterization of kinetic curves approximates a “death line” in phosphorus-deficient treatments (Control, NK), manifested as low microbial heat release values and delayed peak appearance (Figure 4a [Figure 4: see original paper] and Table 1) [5]. This phenomenon demonstrates that severe phosphorus deficiency in fluvo-aquic soil can almost inhibit microbial metabolic activity. Meanwhile, several core indicators characterize soil microbial metabolic activity: microbial growth rate, total metabolic heat, heat release peak value, and peak time. Reaching a high heat release peak in a relatively short time indicates strong metabolic activity. Conventional methods divide curves into lag, ascending, stationary, and descending phases. Microcalorimetric studies on fluvo-aquic soil under different long-term treatments revealed that phosphorus-deficient treatments showed slow microbial growth rates, high total metabolic heat (Table 1), indicating more wasted work and higher energy consumption by soil microorganisms under phosphorus deficiency, which is unfavorable for emission reduction effects. Moreover, adding phosphorus sources could significantly activate NK and Control treatments (Figure 4b), confirming that phosphorus is a key limiting factor for microbial activity in fluvo-aquic soil.

Heat dissipation per cell (QT/N0) serves as a powerful indicator of soil microbial activity. Figure 5 [Figure 5: see original paper] shows that NK and Control treatments exhibited significantly higher heat dissipation per cell than balanced fertilization. Long-term balanced fertilization resulted in low heat release values per unit microorganism (metabolic quotient) in fluvo-aquic soil. These results indicate that phosphorus-containing fertilization (particularly balanced fertilization) promotes the "carbon sequestration and emission reduction" effect in fluvo-aquic soil.

1.3 Effects of Long-term Different Fertilization on Microbial Carbon Metabolic Functional Diversity in Fluvo-aquic Soil

BIOLOG ECO plates, which utilize soil microbial carbon source analogs, are widely employed to study soil microbial community functional diversity. Table 2 demonstrates that balanced fertilization enhanced soil microbial carbon utilization capacity and functional diversity; the NP treatment also significantly increased Shannon and Simpson indices [4]. PCA analysis revealed (Figure 6a [Figure 6: see original paper]) that on the PC1 axis, phosphorus-containing treatments separated significantly from the control (Control), organic manure treatments separated from nutrient-deficient treatments (NP, PK, and NK), and NPK and NP also separated from NK. Redundancy analysis (RDA) further showed (Figure 6b) that organic manure application substantially increased soil microbial biomass and invertase activity by elevating soil organic carbon and total nitrogen contents. Organic manure or phosphorus application significantly improved soil microbial diversity and metabolic activity, with diversity indices also significantly correlated with organic carbon and total nitrogen contents. Organic manure or phosphorus application significantly reduced soil microbial metabolic quotient, which showed significant negative correlations with organic carbon, total nitrogen content, and microbial diversity indices and carbon source utilization activity. These comprehensive results indicate that organic manure application improves microbial function in fluvo-aquic soil.

1.4 Effects of Long-term Different Fertilization on Microbial Structural Diversity in Fluvo-aquic Soil

Phospholipid fatty acid (PLFA) analysis can characterize both microbial biomass and community structure. As shown in Figure 7a, compared with nutrient-deficient fertilization and no fertilization, balanced fertilization (OM, 1/2OMN, and NPK) increased soil microbial biomass and structural diversity, with organic manure showing superior effects to chemical fertilizers. PLFA analysis also revealed that fungi and Gram-positive bacteria were more sensitive to balanced fertilization than Gram-negative bacteria, actinomycetes, and arbuscular mycorrhizal fungi. PCA analysis further demonstrated (Figure 7b) that PC1 contributed 81.76% to the differentiation of microbial community structure in fluvo-aquic soil, representing the primary differentiating factor: organic manure treatments (OM and 1/2OMN) separated significantly from chemical fertilizer treatments. Additionally, phosphorus-containing chemical fertilizer treatments (NPK, NP, and PK) separated from phosphorus-deficient treatments (Control and NK).

1.5 Effects of Long-term Different Fertilization on Microbial Genetic Diversity in Fluvo-aquic Soil

To reveal the effects of long-term different fertilization on fluvo-aquic soil microbial communities at high resolution, we employed Illumina MiSeq sequencing technology to conduct high-throughput sequencing analysis of bacterial 16S rRNA genes. Sequencing yielded 159,455 sequences, with each soil sample obtaining 15,191–33,266 bacterial 16S rRNA gene sequences. Based on 97% similarity, the 159,455 sequences formed 47,207 OTUs, with each DNA sample forming 6,996–14,224 OTUs (Table 3). Sample library coverage reached 65%–69%, basically reflecting the actual bacterial community structure.

Long-term different fertilization significantly affected bacterial genetic diversity in fluvo-aquic soil (Table 3). The effects on Shannon index and PD index were consistent: the PK treatment showed the highest values, while phosphorus-deficient NK treatment showed the lowest. Organic manure treatments (OM and 1/2OMN) and NPK treatment had lower Shannon indices than the control (Control) and other nutrient-deficient treatments (except NK), while nutrient-deficient NP and PK treatments were significantly higher than NK and Control. Unlike these indices, the Chao1 index was highest in 1/2OMN treatment, followed by PK and NP, while NK treatment showed the lowest Chao1 index. Among nutrient-deficient treatments, phosphorus application resulted in significantly higher Chao1 indices than phosphorus-deficient treatments.

Dominant bacteria in fluvo-aquic soil (relative abundance >5%) were primarily Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Alphaproteobacteria, and Betaproteobacteria, accounting for 62% of total sequences (Figure 8 [Figure 8: see original paper]). Additionally, Chloroflexi, Nitrospira, Planctomycetes, Gammaproteobacteria, Deltaproteobacteria, other Proteobacteria, and 12 additional bacterial populations existed at lower relative abundances. The proportions of Proteobacteria and Bacteroidetes in NK and Control treatments were significantly lower than in other treatments, indicating that long-term phosphorus-deficient fertilization substantially reduced the proportions of these bacterial groups. Firmicutes proportions increased in OM and 1/2OMN treatments, demonstrating that long-term organic manure application enhanced Firmicutes abundance in fluvo-aquic soil.

Based on 2,000 bacterial 16S rRNA gene sequences, we performed PCA analysis (Figure 9a [Figure 9: see original paper]) and nonmetric multidimensional scaling (NMDS) analysis (Figure 9b) to visually demonstrate bacterial community responses to different fertilization. PCA analysis showed that organic manure treatments separated significantly from chemical fertilizer treatments along PC1 (Figure 9a). Unifrac distance-based NMDS analysis yielded consistent results: balanced fertilization (OM, 1/2OMN, and NPK) significantly altered bacterial community distribution, while phosphorus application also promoted gradient succession of soil bacterial populations in nutrient-deficient soils (Figure 9b). Thus, carbon and phosphorus contents represent the primary factors influencing bacterial community distribution in fluvo-aquic soil.

For fluvo-aquic soils in the North China Plain, long-term balanced fertilization, particularly organic manure application, significantly increases soil organic carbon and nutrient contents—especially phosphorus—consequently altering soil microbial community structure and enhancing microbial biomass carbon, invertase activity, respiration intensity, and microbial functional diversity (carbon metabolic activity), while markedly reducing microbial metabolic quotient and metabolic heat. These microbial responses indicate that under balanced fertilization, microorganisms serve ecosystems "efficiently with low emissions," ensuring soil nutrient transformation and crop nutrient uptake, thereby creating obvious comprehensive carbon sequestration effects. Conversely, under nutrient deficiency, particularly phosphorus deficiency, soil microorganisms exhibit not only low metabolic efficiency but also dissipate relatively more heat and emit more CO during metabolic processes, causing significant soil quality degradation.

2 Long-term Different Fertilization Effects on Nitrogen Transformation Microorganisms in Fluvo-aquic Soil

Nitrogen is one of the most important nutrients in farmland ecosystems, playing crucial roles in crop and microbial growth and ecological function performance. Long-term different fertilization affects carbon cycling in fluvo-aquic soil and inevitably alters nitrogen transformation processes. Soil microorganisms drive nitrogen transformation in farmland ecosystems; for example, ammonia-oxidizing microorganisms execute the rate-limiting step of ammonia oxidation in the nitrogen cycle. Therefore, long-term different fertilization must affect the diversity and function of nitrogen cycling-related microorganisms. In addition to studying nitrogen orientation and nitrogen-related microbial characteristics, we focused on revealing ammonia-oxidizing microbial responses to different fertilization.

2.1 Effects of Long-term Different Fertilization on Soil Total Nitrogen and Its 15N Abundance

After nearly 20 years of the Fengqiu long-term nutrient balance fertilization experiment, soil total nitrogen contents under different treatments are shown in Figure 10 [Figure 10: see original paper]. Balanced fertilization treatments (OM, 1/2OMN, and NPK) exhibited significantly higher soil total nitrogen contents than nutrient-deficient treatments (NK, PK) and the unfertilized control [6]. Compared with the initial soil total nitrogen content of 0.45 g·kg ^{1} in 1989 (Table 4), balanced fertilization treatments significantly increased soil total nitrogen content, with increases of 148%, 79%, and 32% after 18 years for OM, 1/2OMN, and NPK, respectively. Organic manure treatments (OM and 1/2OMN) introduced substantial organic nitrogen into soil, resulting in significantly higher total nitrogen contents than NPK treatment (Table 4). Additionally, since Fengqiu fluvo-aquic soil is naturally potassium-rich but phosphorus-deficient, NP treatment effects on soil total nitrogen were similar to NPK treatment.

Notably, despite long-term nitrogen input, the phosphorus-deficient NK treatment showed soil total nitrogen content similar to the long-term unfertilized control, actually decreasing by 8.8% compared to 1989 levels (Table 4). Furthermore, soil total nitrogen abundance in NK treatment was significantly higher than in the control. This occurs because higher soil pH in NK treatment facilitates ammonia volatilization, intensifying nitrogen fractionation. Simultaneously, severe phosphorus deficiency strongly inhibits crop growth, leaving substantial inorganic nitrogen in soil and exacerbating other nitrogen loss pathways, particularly gaseous nitrogen losses that cause fractionation, such as denitrification producing N O and ammonia volatilization. Therefore, from perspectives of nitrogen use efficiency and environmental protection, rational phosphorus addition is essential when applying nitrogen fertilizers to phosphorus-deficient soils to better promote crop growth and reduce nitrogen loss.

2.2 Effects of Long-term Different Fertilization on Soil Microbial Biomass Nitrogen and Urease Activity

Balanced fertilization, particularly organic manure application, increased organic nitrogen input, promoting microbial growth and consequently increasing soil microbial biomass nitrogen and urease activity, with organic manure showing more significant effects than inorganic fertilizers (Figure 11 [Figure 11: see original paper]). Among chemical fertilizer treatments, balanced NPK fertilization significantly increased both microbial biomass nitrogen and urease activity, while NP treatment also significantly enhanced urease activity; however, nitrogen- or phosphorus-deficient treatments showed no significant effects. The phosphorus-deficient NK treatment not only failed to significantly increase urease activity (Figure 11B) but actually showed declining microbial biomass nitrogen (Figure 11A). This phenomenon aligns with the decreased soil total nitrogen content in NK treatment shown in Table 4, again demonstrating that rational phosphorus addition is necessary when applying nitrogen fertilizers to phosphorus-deficient soils to promote crop growth and maintain soil fertility.

2.3 Effects of Long-term Different Fertilization on Soil Nitrification Intensity and Ammonia-Oxidizing Bacteria Abundance

Figure 12 [Figure 12: see original paper] reveals that nitrogen fertilizer application greatly enhanced soil nitrification activity, with inorganic nitrogen fertilizers showing more significant effects than organic nitrogen fertilizers, and balanced NPK application demonstrating the most pronounced effect. Soils without nitrogen fertilizer showed nitrification activity not significantly different from the control [7]. Additionally, nitrogen fertilizer application substantially increased ammonia-oxidizing bacteria abundance, with inorganic nitrogen fertilizers again showing more significant effects than organic nitrogen fertilizers (Figure 13A) [8]. Nitrogen in organic fertilizers exists primarily as organic nitrogen that cannot be directly utilized by plants and requires mineralization by soil microorganisms, making its availability significantly lower than chemical fertilizers. Consequently, NPK fertilization produced the highest nitrification intensity and greatest ammonia-oxidizing bacteria abundance. However, nitrification activity per cell was significantly higher with organic nitrogen than inorganic nitrogen fertilizers (Figure 13B). Soils deficient in nitrogen showed ammonia-oxidizing bacteria abundance and activity not significantly different from the control.

2.4 Effects of Long-term Different Fertilization on Ammonia-Oxidizing Bacterial Community Structure

Ammonia-oxidizing bacteria are key microorganisms in soil nitrogen transformation. Previous results consistently showed that nitrogen fertilizer application, particularly balanced fertilization, substantially affected soil nitrogen transformation, enzyme activities, microbial biomass nitrogen, and ammonia-oxidizing bacteria. How does ammonia-oxidizing bacterial community diversity change? Which ammonia-oxidizing bacteria are more sensitive? We analyzed ammonia-oxidizing bacterial community structure using PCR-DGGE technology targeting the functional gene *amoA* encoding ammonia monooxygenase -subunit [7]. DNA fingerprinting showed that nitrogen-treated soils had increased DGGE band numbers (Figure 14a [Figure 14: see original paper]), indicating higher diversity. Meanwhile, a distinctive band (band 3) present in the control disappeared in all fertilized treatments. Sequencing results revealed that nitrogen fertilization made unculturable *Nitrosospira* Cluster 3 the dominant genus (Figure 14b), suggesting this species may play a key role in nitrogen transformation in fluvo-aquic soil.

Long-term balanced fertilization significantly increased soil total nitrogen content, microbial biomass nitrogen, and urease activity. Nitrogen fertilizer application greatly enhanced soil nitrification activity, ammonia-oxidizing bacteria abundance and diversity, with inorganic nitrogen fertilizers showing more significant effects than organic nitrogen fertilizers, while nitrogen-deficient soils showed no significant differences from the control. Notably, for phosphorus-deficient fluvo-aquic soils, rational phosphorus addition is a prerequisite for scientific nitrogen application to better promote crop growth, reduce nitrogen loss, and improve soil fertility. These findings help guide future scientific fertilization practices, improve nitrogen fertilizer use efficiency and yield effects in the North China Plain, and mitigate farmland nitrogen loss and environmental pressure.

3 Long-term Different Fertilization Effects on Phosphorus Transformation Microorganisms in Fluvo-aquic Soil

Soil microorganisms engage in "biological underground trading." Scientific fertilization increases soil nutrients, affecting soil microbial diversity and function through this "underground trading" process. Due to phosphorus deficiency in fluvo-aquic soil, phosphorus addition in fertilizers influences the diversity and function of phosphorus transformation microorganisms. Based on the "biological underground trading" concept, we selected arbuscular mycorrhizal fungi (AMF), microorganisms closely related to phosphorus cycling, for investigation.

3.1 Effects of Long-term Different Fertilization on Arbuscular Mycorrhizal Fungal Spore Diversity

AMF are key microorganisms in terrestrial ecosystems, primarily participating in phosphorus biogeochemical cycling and forming mutualistic symbioses with over 90% of terrestrial plants. Through "biological underground trading," plants and AMF exchange materials and energy: plants deliver photosynthetic products as carbon sources to AMF, and in return, AMF absorb more phosphorus and other nutrients for plants to promote growth. Additionally, host plants "selectively" abandon certain AMF according to environmental changes, allocating more photosynthetic products to other microorganisms to promote sustainable soil fertility development. Therefore, AMF community changes can not only characterize soil quality and health but also sensitively reflect changes in soil ecosystems.

We first isolated spores using sucrose centrifugation-wet sieving-decantation and observed AMF spore diversity under a stereomicroscope [9]. Organic manure application significantly reduced AMF spore species richness, with average species numbers decreasing from 22.8 in the control to 15.3 (OM) and 16.3 (1/2OMN) (Table 5). Similarity analysis showed (Figure 15 [Figure 15: see original paper]) that AMF spore diversity in Control and NK treatments had the highest similarity, forming one cluster separate from other fertilization treatments. This phenomenon indicates that phosphorus application substantially affects AMF diversity in soil.

3.2 Effects of Long-term Different Fertilization on Arbuscular Mycorrhizal Fungal Genetic Diversity

Since traditional observation methods have certain subjectivity and one-sidedness in AMF diversity identification, we objectively and comprehensively revealed long-term fertilization effects on AMF communities in fluvo-aquic soil using 454 high-throughput gene sequencing technology in 2011 [10]. High-throughput sequencing yielded 59,611 AMF-specific fragment sequences, forming 70 OTUs (Figure 16a [Figure 16: see original paper]), primarily distributed in two families: Glomeraceae (52,130 sequences, 55 OTUs) and Gigasporaceae (4,334 sequences, 3 OTUs) (Figure 16b).

We constructed rarefaction curves for four diversity indices—phylogenetic diversity (PD) index, Shannon index, Chao1 estimator, and observed species number—to compare AMF diversity differences among fertilization treatments (Figure 17 [Figure 17: see original paper]). When sequencing depth exceeded 1,000 sequences, all four indices stabilized with reduced variation, indicating that our sequencing depth captured the complete AMF species pool in fluvo-aquic soil. Comparison among treatments revealed that phosphorus application reduced AMF diversity: compared with NK and Control, NP, NPK, and OM treatments significantly decreased PD and Shannon diversity indices, as well as Chao1 and observed species numbers. Specifically, PD and Shannon indices decreased from 2.0–4.0 in NK and Control to 1.7–3.0 in phosphorus treatments, while Chao1 and observed species numbers decreased from 42–38 to 35. Moreover, no differences in AMF diversity indices existed among NP, NPK, and OM treatments.

AMF community composition under different fertilization is shown in Figure 18 [Figure 18: see original paper]. Overall, long-term phosphorus application (NP, NPK, and OM) significantly reduced AMF richness compared with phosphorus-deficient NK and unfertilized Control. Specifically, Glomerales consistently dominated across all treatments, while NP, NPK, and OM fertilization significantly reduced proportions of Acaulosporaceae and Gigasporaceae.

To more intuitively display AMF community structural responses to different fertilization, we performed PCA analysis (Figure 19a [Figure 19: see original paper]). The first principal component (PCA horizontal axis) contributed 88.2%, indicating it basically explained the reasons for AMF community structure differentiation. Along this axis, phosphorus-deficient NK and unfertilized Control separated significantly from phosphorus-containing NP, NPK, and OM treatments, demonstrating that phosphorus fertilizer is the primary factor causing AMF community structure differentiation in fluvo-aquic soil. Additionally, along the vertical axis (second principal component, contributing 9.9%), AMF community structures in Control and NK also differed, indicating that nitrogen and potassium inputs also influence AMF community structure to some extent. We subsequently associated environmental variables—including soil pH, total carbon (SOC) and total nitrogen (TN) contents, available potassium (AK) and available phosphorus (AP) contents, soil microbial biomass, and enzyme activities—

with AMF community structure changes (Figure 19b) and performed canonical correspondence analysis (CCA) to reveal which soil physicochemical properties most strongly influenced AMF community structure changes and how AMF community changes subsequently affected soil microbial characteristics. CCA showed that phosphorus application, particularly balanced fertilization (NPK and OM), improved soil fertility and provided more nutrients for crops, consequently reducing crop dependence on AMF. Crops then "abandoned" certain AMF species, such as *Gigaspora*, *Acaulospora*, *Acaulospora-environmental*, and *Scutellospora* identified in this study, reallocating more photosynthetic products to other soil microorganisms. This process increased soil microbial biomass carbon and various enzyme activities (Figure 19b), promoting soil nutrient cycling. Additionally, easily extractable glomalin and total glomalin, which are closely related to soil physical structure and organic matter accumulation, significantly increased under phosphorus application, particularly balanced fertilization (Figure 20) [11], with organic manure treatments showing higher values than chemical fertilizer treatments. This indicates that balanced fertilization, especially organic manure application, can improve soil physical structure and promote soil organic matter accumulation through microbial processes.

For phosphorus-deficient fluvo-aquic soils, long-term phosphorus application, particularly balanced fertilization, affects the "biological underground trading" process, especially for symbiotic microorganisms closely related to crop growth: balanced fertilization increases soil nutrients, reducing crop dependence on AMF and consequently decreasing AMF diversity and differentiating community structure. This process reallocates more photosynthetic products to other microorganisms, thereby improving soil chemical, physical, and biological properties and ultimately contributing to sustainable fluvo-aquic farmland soil fertility. This study represents the first in-depth revelation of how arbuscular mycorrhizal fungi in typical farmland soils respond and feedback to different fertilization regimes, providing theoretical basis and scientific guidance for rational fertilization in the North China Plain.

4 Microbial Mechanisms of Soil Fertility Enhancement in Fluvo-aquic Soil

Our previous research demonstrated that long-term balanced fertilization, particularly organic manure application, promotes carbon, nitrogen, and phosphorus transformation processes in fluvo-aquic soil, improves characteristics of related microorganisms, and helps enhance and sustain soil fertility. However, what are the specific microbial mechanisms? Which soil microorganisms respond most sensitively to organic manure application—that is, which microorganisms become dominant populations under organic fertilization (Who)? Where do these dominant microorganisms originate from—are they indigenous microorganisms induced by fertilization or introduced from the organic manure (Where)? If they are induced indigenous microorganisms, how long after organic manure application do they become dominant (When)? Why do they become dominant microorganisms (Why)? And what functions do they perform (What)? Answering these five W questions will help reveal the microbial mechanisms underlying soil fertility enhancement in fluvo-aquic soils of the North China Plain and guide us in better fertilization practices.

Related work began in 2004 when we used PCR-DGGE fingerprinting technology based on bacterial 16S rRNA gene fragments to study long-term fertilization effects on fluvo-aquic soil bacterial communities (Figure 21a [Figure 21: see original paper]) [3]. We found that inorganic fertilizer application did not affect band numbers or intensities, but organic manure treatments exhibited specific bands (band 1). Phylogenetic tree analysis revealed this specific band belonged to a *Bacillus* sp. (Figure 21b). However, due to technical limitations at that time, we could not clearly answer the five W questions.

4.1 (Implied) The Five W Questions

In 2011, we systematically investigated the scientific question of how fluvo-aquic soil bacteria, particularly *Bacillus*, respond to organic manure application [12]. First, we analyzed fluvo-aquic soil bacteria in 2010 using high-throughput sequencing technology. We constructed a heatmap and performed hierarchical clustering analysis of bacterial OTU composition at the phylum level (Figure 22 [Figure 22: see original paper]). Clustering of different fertilization treatments showed that organic manure treatments and chemical fertilizer treatments fell into two major branches, indicating that organic manure input significantly altered fluvo-aquic soil bacterial community structure and composition. From the perspective of bacterial species clustering, long-term OM and 1/2OMN applications significantly increased the relative abundance of Firmicutes compared with Control, suggesting that species within Firmicutes are most sensitive to long-term exogenous organic carbon input.

We performed LEfSe (least discriminant analysis effect size) analysis on different fertilization treatments. Different colors represent significantly different species among samples or groups, with each dot indicating a bacterial taxon. Circles from inner to outer represent Kingdom, Phylum, Class, Order, Family, and Genus classifications (Figure 23 [Figure 23: see original paper]). LEfSe analysis revealed that differences in fluvo-aquic soil bacterial composition mainly concentrated among OM, 1/2OMN, NK, and Control treatments, specifically in Firmicutes, Chloroflexi, Acidobacteria, and Nitrospirae, with Firmicutes showing the most significant differences. At the outermost genus level, OM treatment primarily increased proportions of *Bacillus*, *Microvirga*, *Arenimonas*, *Rhodococcus*, *Streptomyces*, and *Adhaeribacter*; 1/2OMN treatment also primarily increased *Bacillus* proportions.

To further increase resolution, we focused on Firmicutes in the high-throughput data (Figure 24 [Figure 24: see original paper]). We found that *Bacilli* class accounted for up to 92% of Firmicutes under OM treatment, while other treatments did not exceed 85%, indicating that organic manure application promoted

Bacillus growth in fluvo-aquic soil. Meanwhile, *Bacilli* community composition under OM treatment differed substantially from other treatments. Although high-throughput sequencing clearly showed that *Bacillus* species within Firmicutes were most sensitive to organic manure application, the gene fragments obtained (~400 bp) contained insufficient biological information for precise species identification. Therefore, we further used a 1,250 bp specific fragment from the *Bacillus* ribosome to continue tracking its response to organic manure application.

4.2 ***Bacillus asahii*** is an Indigenous Microorganism in Fluvo-aquic Soil

To determine whether this species was introduced from organic manure or induced from indigenous microorganisms, we analyzed the *Bacillus* community in organic manure itself using clone library analysis and found no *B. asahii* (OTU1 and OTU2) (Figure 26 [Figure 26: see original paper]), confirming that this microorganism is indigenous to fluvo-aquic soil.

4.3 ***Bacillus asahii*** Becomes Dominant 2-4 Years After Organic Manure Application

After confirming that *B. asahii* became a dominant indigenous microorganism induced by organic manure, we further analyzed the *Bacillus* community succession in organic manure-treated soil from 1989-2009 using PCR-DGGE fingerprinting to determine how long after organic manure application it became dominant (Figure 27 [Figure 27: see original paper]). Clustering analysis revealed four stages of *Bacillus* community structure over 21 years. Stage 1 was 1989, when organic manure input caused the greatest change to the native soil *Bacillus* community structure. Stage 2 was 1990-1995. Stage 3 was 1996-2003. Stage 4 was 2004-2009. Stages 3 and 4 showed similar community structures, forming one cluster separate from Stage 2, suggesting major changes occurred during 1990-1995. Analysis of specific bands revealed that band 4 appeared in 1993 and became dominant after 1994. Phylogenetic tree analysis identified this band as *B. asahii*, indicating that DGGE fingerprinting showed *B. asahii* became dominant in the fourth year of organic manure application.

To verify PCR-DGGE fingerprinting reliability, we constructed 1,250 bp clone libraries for soil *Bacillus* communities from 1989-2009 (coverage 82.4%). Species accumulation curves showed that OTU1 and OTU2, i.e., *B. asahii*, became dominant microorganisms in fluvo-aquic soil in 1991, fluctuating between 40%-72% of the *Bacillus* community over the subsequent nearly 20 years (Figure 26). Integrating PCR-DGGE fingerprinting and clone library results, we infer that 2-4 years of organic manure application causes an indigenous *Bacillus* species, *B. asahii*, to become dominant in fluvo-aquic soil.

4.4 Functional Studies of *Bacillus asahii*

After clarifying Who, Where, and When, we investigated why *B. asahii* became dominant and what functions it performs in fluvo-aquic soil. Physiological and biochemical characterization of the wild-type *B. asahii* strain (Figures 28a, b, c) revealed rich metabolic capabilities, strong lipid degradation ability, and phosphorus solubilization capacity. This microorganism differs from other *Bacillus* species by not producing organic acids during metabolism. These unique properties may enable it to become dominant in alkaline soils with high organic matter input. Inoculation experiments further demonstrated that this microorganism can increase soil polyphenol oxidase and lipase activities (Figure 28d), which facilitates nutrient release from organic manure and enhances soil fertility.

Integrating multiple molecular biology techniques and traditional microbiological methods, we discovered that an indigenous microorganism, *Bacillus asahii*, showed the most significant response to long-term organic manure application, becoming dominant in fluvo-aquic soil within 2–4 years. This species possesses unique physiological characteristics and abundant metabolic diversity, capable of accelerating and facilitating organic matter accumulation and phosphorus cycling processes by other microorganisms, thereby playing a "leading" role in crop growth and soil fertility improvement. These results deepen our understanding of microbial mechanisms underlying long-term organic manure effects on soil fertility enhancement in the North China Plain and provide guidance for regulating soil microorganisms to better serve farmland ecosystems.

Future Directions

In recent years, research on soil microbial diversity and function has flourished with rapid progress. People increasingly recognize the critical roles soil microorganisms play in sustainable agricultural development, ecological environmental protection, and global change research, hoping to develop soil microbial regulation technologies as a new approach for environmentally friendly and economically efficient agriculture. This presents us with more important scientific tasks requiring our response and challenge.

First, soil microbial theoretical systems need innovation. The most urgent current problem is that soil microbiology theoretical development lags behind experimental development. With continuous introduction of new technologies and methods, farmland soil microbial research has yielded abundant results, but these are mostly fragmented, lacking theoretical synthesis and advancement. This requires us to develop interdisciplinary approaches, integrate and expand existing theoretical frameworks, and construct new theoretical frameworks to better reveal the important roles of soil microorganisms in farmland ecosystems.

Second, deeper understanding of soil microorganisms needs expansion. Coupling diversity and function is fundamental for comprehensively evaluating soil microbial roles in farmland soils. Current research primarily focuses on soil microbial diversity, while studies on microbial community species interaction networks, soil microbial functional gene diversity and functional responses, particularly directed regulation of individual microbial functions, lag behind or remain blank. Due to the difficulty of observing soil itself, we know little about interactions between soil microstructure and microorganisms, requiring in-situ observation at microscales of interactions among microorganisms, plants, and various complex interfaces among water, gas, and solid phases in soil. Moreover, current research mostly focuses on correlations between soil microorganisms and environmental factors, while causality studies are limited, primarily due to lack of strain-level verification experiments. Soil microbiology research must ultimately interpret soil problems at the microbial strain level.

Third, soil microbial research methodology needs advancement. Although new technologies and methods have invigorated soil microbial research in recent years, soil microbiology still lags behind other disciplines, partly due to relatively backward research methods. This requires us to continue borrowing advanced technologies and concepts from physics, chemistry, mathematics, engineering, and computer science to develop more applicable technologies and methods for soil microbial research. Since all methods have limitations, we need to integrate and corroborate multiple techniques. Furthermore, we must devote greater effort to developing new methods and technologies specific to our discipline to achieve greater discoveries and breakthroughs.

Fourth, long-term experimental platform construction needs emphasis. As stated at the beginning of this paper, many ecological processes in agroecosystems progress rather slowly, and short-term experiments cannot reveal trends in ecological processes. Only sustained, stable long-term field experiments can promote ecological theory development, reveal mechanisms of ecological process evolution, and provide scientific parameters for rational fertilization and optimized resource management. Therefore, we must emphasize the construction and maintenance of long-term experimental platforms.

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Note: Figure translations are in progress. See original paper for figures.