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Organic Fertilizers as Partial Substitutes for Chemical Fertilizers Enhance Nitrogen Immobilization and Optimize Nitrogen Fate in Paddy Soils

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Abstract: Organic fertilizers as partial substitutes for chemical fertilizers improve soil nitrogen (N) retention capacity. However, the relative importance of biotic and abiotic N immobilization at different levels of organic N substitution and the subsequent effects on N utilization in paddy soils are not well elucidated. To address these, a combination of ^{15}N incubation experiments and pot experiments were conducted to investigate biotic and abiotic N immobilization features and their effects on N fertilizer fate under long-term different fertilization regimes in paddy soils in China. Test soils that had received chemical fertilization (NPK), chemical N was substituted with 30%, 50%, and 70% organic N (70 F + 30 M, 50 F + 50 M, and 30 F + 70 M, respectively), and no fertilization (control) for 36 years. The results revealed that both abiotic and biotic NH_4^+-N immobilization were enhanced under organic N substitution soils. The highest NH_4^+-N abiotic and biotic N immobilization was observed under 50 F + 50 M soil, significantly increasing by 195.5% and 51.4%, respectively, compared to the NPK soil. In contrast, only abiotic NO_3^--N immobilization increased with rising organic substitution N proportions. N fertilizer utilization efficiency was significantly enhanced in 50 F + 50 M soil (36.7%) compared to the NPK soil (30.3%), which was primarily attributed to the enhanced N pool activity and N immobilization capacity. However, the N fertilizer residue rate was significantly higher in the 30 F + 70 M soil (23.6%) compared to the NPK soil (21.6%), largely attributed to the soil properties improvement. Our results suggest that N immobilization capacity and N fertilizer utilization can be optimized with a 50% organic substitution ratio in our studied soil–crop system.

Keywords: ^{15}N tracer techniques; organic N fractions; N immobilization; N fertilizer fate; paddy soil

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1. Introduction

Rice (*Oryza sativa* L.) is one of the major food crops globally, which feeds more than half of the world's population [1]. Over the past 40 years, the extensive use of chemical fertilizers has been one of the major contributing factors to the increasing rice yields [2,3]. Simultaneously, the massive application of N fertilizers has led to serious environmental pollution (e.g., water eutrophication and greenhouse gas emissions) on a regional and global scale [3,4]. Replacing chemical fertilizers partially with organic fertilizers not only reduces the amount of chemical fertilizers but also makes full use of waste organic resources, which is one of the important approaches to improve the utilization rate of N fertilizers. Rational organic substitution improves soil N retention, reduces soil greenhouse gas emissions, ammonia volatilization, and nitrate leaching, and reduces N losses [5–9]. It reduces losses by the retention of reactive N through fixation and microbial immobilization, at the same time, it improves soil fertility by renewing the soil organic N pool. Given the significance of N immobilization in determining N availability and its environmental impacts, it is necessary to determine the N immobilization pattern as well as the fate of N fertilizer under various proportions of organic substitution in acidic paddy fields.

Nitrogen immobilization includes both biotic and abiotic immobilization mechanisms [10]. Biotic immobilization is primarily influenced by the characteristics of organic materials (e.g., SOC, C/N, activity, etc.). For example, in agricultural soils, organic fertilizer inputs increased N biotic immobilization capacity. Notably, straw with a high C/N ratio exhibits a greater biotic immobilization capacity compared to animal manure with a lower C/N ratio [11,12]. Moreover, soil microbial activity affects N biotic immobilization. For example, soil acidification inhibits biotic immobilization by reducing bacterial abundance and diversity [12]. However, abiotic N immobilization is primarily influenced by SOC content, clay minerals, and phenolic compounds [13]. Forest soils with high C availability present high abiotic N immobilization. In fact, the abiotic immobilization of NH_4^+-N is mainly affected by 2:1 type of clay minerals [14]. The properties of the organic N fraction also play a major role in N immobilization. According to the organic N grouping method [15], acid-hydrolyzable N (AHN) and non-hydrolyzable N (NHN) are present in soil organic N fractions. The AHN fractions were further subdivided into acid-hydrolyzable ammonium N (AHAN), amino acid N (AAN), amino sugar N (ASN), and acid-hydrolyzable unknown N (AHUN). AHN and its four fractions were found to be greatly increased after 15 years of continuous N fertilizer application. Each AHN fraction influenced greatly the N transformation processes by regulating bacterial, fungal, and enzyme activities [16]. Different rates of organic substitution lead to variations in soil characteristics, including SOC, C/N, pH, microbial activity, and organic N fractions. However, it remains unclear how these factors affect the biotic and abiotic N immobilization, and which are their respective roles in N immobilization following long-term organic substitution.

Effective retention of N fertilizer in the soil is crucial for enhancing N fertilizer utilization and mitigating N losses [17,18]. The application of organic amendments is beneficial to N uptake due to the improvement in soil structure and the enhancement of soil fertility level [19]. Highly fertile soils have more tightly coupled C and N cycles and higher microbial population and activity, thus they are sequestering more reactive N [20]. However, increasing soil fertility through organic substitution does not always improve N retention and reduce N losses. Ren et al. [21], suggested that organic substitution levels below 50% are feasible for maintaining crop yield and mitigating N loss, and a higher proportion of organic substitution reduces the N retention capacity and decreases the N utilization rate. Thus, N utilization and residue are closely linked to the proportion of organic substitution. However, there is a gap in research concerning the conversion and destination of exogenous N fertilizer and its correlation with N retention capacity in soils with varying levels of organic substitutions.

We hypothesized that: (1) soil properties (SOM, TN, etc.)and organic N pool activity (organic N fractions) under a long-term fertilization regime may regulate N immobilization capacity; (2) the relative importance of biotic and abiotic N (NH_4^+-N, NO_3^--N) immobilization varies among substitution ratios, with the activity of the organic N pools determining NH_4^+-N biotic immobilization, and the soil properties determining NO_3^--N abiotic immobilization; and (3) the optimal substitution ratio has the highest integrated N retention capacity, optimizing the fate of exogenous N fertilizer and maximizing N availability. Therefore, a ^{15}N incubation experiment was conducted to study the soil biotic and abiotic immobilization characteristics in a long-term experiment with organic substitution over 36 years. As the retention mechanism of soil N is a crucial factor affecting the availability and loss of fertilizer N, a pot experiment using the ^{15}N isotope technique was conducted to investigate how soil N immobilization patterns affect the fate of exogenous N fertilizer, and to clarify a more optimal organic substitution ratio. This study can provide insight into the retention mechanism of soil reactive N and therefore assist in the development of a rational and sustainable fertilization strategy in paddy soil.

2. Materials and Methods

2.1. Long-Term Trial Design and Soil Collection

The field trial was conducted at the experimental field of Jiangxi Provincial Academy of Agricultural Sciences in Nanchang, Jiangxi Province, China (28°57′ N, 115°94′ E). The long-term locational trial was initiated in 1984 with a double-crop rice succession system. The region experiences a subtropical monsoon climate with an altitude of 25 m above sea level in the experimental area, an annual mean temperature of 18.1 °C, and an average annual precipitation of 1617 mm (30-year average). The soil is classified as stagnic anthrosol with a clay-loam texture [22].

Five treatments were employed in this study: (1) control, no fertilizer; (2) NPK, chemical fertilizer only (N, P, and K); (3) 70 F + 30 M, 70% chemical N fertilizer plus 30% organic N fertilizer; (4) 50 F + 50 M, 50% chemical N fertilizer plus 50% organic N fertilizer; and (5) 30 F + 70 M, 30% chemical N fertilizer plus 70% organic N fertilizer. All fertilized treatments received equal amounts of N, phosphorus (P_2O_5), and potassium (K_2O) nutrients. The plots were demarcated by a 70 cm deep and 50 cm wide concrete ridge, with a plot area of 33.3 m^2 arranged in a randomized block design, and were replicated three times. N application was designed according to 150 kg ha^{-1} for early rice and 180 kg ha^{-1} for late rice, while phosphorus (P_2O_5) and potassium (K_2O) were applied at the same rate of 60 kg ha^{-1} for P_2O_5 and 150 kg ha^{-1} for K_2O for early and late rice, respectively. Urea was used for N fertilizer, calcium superphosphate (12% P_2O_5) for P fertilizer, and potassium chloride (60% K_2O) for K fertilizer. For early rice, *Astragalus sinicus* L. was used as the organic fertilizer, with nutrient content calculated according to 0.303% N, 0.08% P_2O_5, and 0.23% K_2O. *Astragalus sinicus* L. seeds were sown annually 1 week before the late rice harvest, and the aboveground fresh weight was harvested in April of the following year, weighed, and applied to the counterpart treatments. Pig manure, with a nutrient content calculated at 0.45% N, 0.19% P_2O_5, and 0.23% K_2O, was applied for late rice, and sourced from local farms each year. Calcium superphosphate and organic fertilizers were applied before sowing. Also, 50% of N fertilizer was used as basal fertilizer, 25% as tiller fertilizer, and 25% as panicle fertilizer, and 50% of K fertilizer was used as tiller fertilizer, and 50% of K fertilizer was used as panicle fertilizer.

Before planting rice from each treatment in April 2020, 0–15 cm of bulk soil was gathered. Three replicates of the same treatment were combined to form a composite soil sample. Soil samples were then passed through a 2 mm sieve and were divided into three parts. One portion was kept at 4 °C for the incubation experiment; another portion was used for the determination of soil basal biochemical properties; and the remaining soil was air-dried and sieved for the potting experiment.

2.2. ^{15}N Tracer Incubation Experiment

A 250 mL flask was filled with 20 g (dry basis) of fresh soil sample of each treatment and 25 mL of deionized water, at saturated water holding capacity (WHC), sealed with a sealing film, and punctured with holes to maintain air circulation. For seven days, the flasks were pre-incubated at 25 °C in the dark to simulate the flooding conditions found in paddy fields. Each soil treatment was divided into two labeled groups, 2 mL of either $^{15}NH_4NO_3$ (20.18% abundance) or $NH_4{}^{15}NO_3$ solution (20.30% abundance) was added, respectively, at the end of the pre-incubation. Each treatment was equivalent to an addition of 50 mg kg^{-1} $NH_4{}^+$-N and 50 mg kg^{-1} $NO_3{}^-$-N [12]. The bottles were sealed with a sealing film punctured with holes to maintain aeration, and incubated in the dark at 25 °C for 7 days. A total of 120 bottles (5 soils × 2 markers × 4 sampling times × 3 replications) were combined for this experiment. At 0.5 h, 1 d, 3 d, and 7 d after ^{15}N labeling, soil samples were extracted by a soil/solution ratio of 1:5 with 2M KCl to determine NH_4^+-N and NO_3^--N concentrations and ^{15}N abundance. To determine the insoluble organic N content and ^{15}N abundance, the remaining soil samples were cleaned three times with 150 mL of deionized water, dried at 60 °C to a constant weight, and were then powdered to pass through a 0.15-mm soil sieve [23].

2.3. The ^{15}N Pot Experiment

Potting experiments were conducted for 2 years in a half-open glass greenhouse. Sunlight exposure and controlled irrigation were available. Triplicates of each treatment's soil were used; each one was placed into a plastic bucket with an outer diameter of 25 cm and a height of 30 cm, containing 7.5 kg soil. Equal amounts of fertilizers were applied in each treatment for 2 consecutive years, and the amounts of fertilizer were according to the local standard of N 180 kg ha^{-1}, P_2O_5 90 kg ha^{-1}, and K_2O 150 kg ha^{-1} in the field, and the fertilizer concentration applied in the potting experiment was 1.5 times of that applied in the field experiment. Isotope-labeled urea with a 20.16% abundance (Shanghai Research Institute of Chemical Industry, Shanghai, China) was utilized as N fertilizers, with 120 mg N kg^{-1} soil of ^{15}N applied in proportions of 5:2:3 for the base fertilizer, tillering fertilizer, and panicle fertilizer, respectively. The P fertilizer employed was calcium superphosphate, and 60 mg P_2O_5 kg^{-1} soil was applied at once as a basal fertilizer. K fertilizer consisted of potassium chloride, and 100 mg K_2O kg^{-1} soil, with half applied at the tillering stage and the remaining half at the panicle stage. Rice was transplanted in three holes per plastic bucket with two seedlings per hole on 19 June 2020. During the growth period, the rice was irrigated every 1–2 days and kept in a flooded water layer of 3–5 cm. The treatments were randomly arranged, and the position of the plastic buckets was changed periodically in the greenhouse. After the rice maturation on 30 September 2020, all rice plants were collected and separated into seeds and straw to determine the total N and ^{15}N abundances. Additionally, all soil from the pots was collected, thoroughly mixed, and analyzed for total N and ^{15}N abundance. Following the completion of sampling, the remaining soil was mixed, sieved using a 2-mm mesh, and returned to the original plastic bucket for use in the second year of the trial. Rice transplantation occurred on 10 June 2021, and the harvest was conducted on 30 September 2021. Fertilization, sampling, and management practices for the pot trial remained consistent with those of the previous year.

2.4. Sample Analysis

The results of soil chemical properties were reported in previous studies [24]. Soil pH was determined using a glass pH meter in a soil–water slurry (1:2.5, *w*/*v*). Soil NH_4^+-N and NO_3^--N concentrations were extracted using 2 mol L^{-1} KCL, followed by determination with a Seal AA3 autoanalyzer (Seal Analytical Inc., Mequon, WI, USA). An abundance of ^{15}N of NH_4^+-N and NO_3^--N was measured using Devarda alloy and magnesium oxide distillation to separate NH_4^+-N and NO_3^--N [25]. Briefly, 20 mL of extract was mixed with 0.3 g of MgO for distillation to separate NH_4^+-N, and 0.3 g of Devarda alloy was added to the flask to remove NO_3^--N. The released NH_3 was absorbed in acidified filter paper with 1 M oxalic acid, which was dried in an ammonia-free environment, transferred to tin cups, and wrapped up with an Isoprime100 automated C/N isotope ratio mass spectrometer (Elementar Analysensysteme GmbH, Langenselbold, Germany) to analyze ^{15}N abundance.

Soil organic N fractions were assessed using Bremner's acid digestion method [15]. This method categorizes organic N into various fractions, including acid-hydrolyzable N (AHN), acid-hydrolyzable ammonium N (AHAN), amino acid N (AAN), amino sugar N (ASN), acid-hydrolyzable unknown N (AHUN), and non-hydrolyzable N (NHN). Plant and soil total N were determined using the Semimicro-Kjeldahl method [26], and the ^{15}N abundance was determined using an Isoprime100 automated C/N isotope ratio mass spectrometer. Soil microbial biomass C and N were determined using the fumigation-extraction method [27].

2.5. Calculation and Statistical Analysis

Biotic and abiotic immobilization of N was performed using the organic ^{15}N recovery method in an incubation experiment [12,22,28–30]. Studies have shown that NH_4^+-N enters the soil partly in the form of insoluble organic nitrogen and that this process is rapid, typically within 10 min, and is approximately equivalent to abiotic NH_4^+-N sequestration in the form of physical condensation of phenolic compounds in humus and immobilization of clay minerals [29,31]. Abiotic fixation of NO_3^--N refers to the rapid conversion (10–15 min) of NO_3^--N to soluble or insoluble organic N [12,29]. Depletion of NH_4^+ or NO_3^- by abiotic processes occurred within the first 15 min of ^{15}N addition, and there was no significant difference in the amount of ^{15}N extracted 15 min and 24 h after the addition of ^{15}N to sterilized soil [12,29,32]. The abiotic immobilization of soil NH_4^+-N and NO_3^--N was quantified as the insoluble ^{15}N recovery in the KCl washed residual soil samples after the addition of ^{15}N for 0.5 h during incubation [12,29]. Biotic immobilization of soil NH_4^+-N and NO_3^--N was expressed as the difference in the insoluble ^{15}N recoveries in the KCl washed residual soil between 0.5 h and 7 d after the addition of ^{15}N during incubation [12,30]. The ^{15}N recovery method for measuring biotic or abiotic immobilization of N proved to be reliable [12,22].

To evaluate the fate of N fertilizer in the pot experiment, ^{15}N plant uptake, ^{15}N use efficiency, soil ^{15}N residue rate, and ^{15}N loss rate were calculated according to the following formulae [33,34].

$$^{15}N\ \text{uptake} = N_{plant} \times (^{15}N_{lp} - {}^{15}N_{natural})/(^{15}N_{fertilzer} - {}^{15}N_{natural}) \quad (1)$$

where N_{plant} (g pot^{-1}) and $^{15}N_{lp}$ (%) represent the total N uptake and ^{15}N abundance of the labeled plant (atom %), $^{15}N_{fertilzer}$ (%) represents the ^{15}N enrichment of the labeled fertilizer (atom 20.15%), and $^{15}N_{natural}$ (%) represents the natural abundance of ^{15}N (atom 0.365%).

$$^{15}N\ \text{residual} = N_{soil} \times (^{15}N_{ls} - {}^{15}N_{natural})/(^{15}N_{fertilzer} - {}^{15}N_{natural}) \quad (2)$$

where N_{soil} (g pot^{-1}) and $^{15}N_{ls}$ (%) represent the soil total N accumulation and ^{15}N abundance (atom %) in the labeled soil.

$$^{15}N\ \text{use efficiency}\ (^{15}NUE, \%) = {}^{15}N\ \text{uptake (g/pot)}/{}^{15}N\ \text{fertilizer rate (g/pot)} \times 100 \quad (3)$$

$$^{15}N\ \text{residual rate}\ (^{15}NRR, \%) = {}^{15}N\ \text{residual (g/pot)}/{}^{15}N\ \text{fertilizer rate (g/pot)} \times 100 \quad (4)$$

$$^{15}N\ \text{loss rate}\ (^{15}NLR, \%) = 100 - {}^{15}N\ \text{use efficiency} - {}^{15}N\ \text{residual rate} \quad (5)$$

where each result is the mean of the triplicate experiments. SigmaPlot 12.0 (version 12.0, Systat, San Jose, CA, USA) was used for plotting, and SPSS 22 (version 24.0, IBM, Chicago, IL, USA) software was used for data statistical analysis. Multiple comparative analyses were conducted using one-way ANOVA and Duncan methods. A correlation map was drawn with Origin Pro 2022 (Origin Lab Corp., Northampton, MA, USA).

Structural equation modeling (SEM) is a multivariate statistical method for hypothesis testing of complex path relation networks [35,36]. Improvement in soil fertility properties (SOM, etc.) after organic replacement directly affects the conversion of reactive N to organic N pools and improves N retention, thereby avoiding N loss. The retention of reactive N further improves N utilization or residue. The quality of soil organic matter also directly affects the rate of its decomposition and dynamic N transformation [37], and the activity of organic N pools associated with soil organic C pools can reflect the rate of soil N immobilization [23]. Soil properties and N pool activity together affect the N immobilization capacity and availability. Here, SEM was used to examine the direct and indirect effects of soil properties, N pool activity, and N immobilization on fertilizer N uptake and residue. The fitting performance of the model was evaluated using the chi-square test (χ^2), degree of freedom (df), p-value, Goodness of Fit (GFI), and Root Mean Square Error of Approximation (RMSEA). The SEM was carried out using AMOS 24.0

(Amos Development Corporation, Chicago, IL, USA). To ensure that the SEM data met the assumptions of normality, soil properties, N immobilization, N pool activity, fertilizer nitrogen uptake, and residue were subjected to standardized transformation.

3. Results

3.1. Crop Yield and Soil Chemical Properties

The average annual yield of rice under the long-term different fertilization regimes was in the following order: control < NPK < 70 F + 30 M < 50 F + 50 M < 30 F + 70 M. The organic fertilizer substitution treatments were significantly higher by 43.8–46.4% ($p < 0.05$) and 4.3–6.9% ($p < 0.05$) than the control and NPK treatments, respectively (Table 1). However, there was no significant difference between the different substitution ratios.

Table 1. Annual rice yield and selected soil properties of buck soil after a 36-year paddy soil experiment in Nanchang, China.

Treatments	Control	NPK	70 F + 30 M	50 F + 50 M	30 F + 70 M
SOM (%)	3.08 ± 0.67 c	4.23 ± 0.45 b	4.98 ± 0.42 ab	5.06 ± 0.21 a	5.61 ± 0.13 a
TN (g kg^{-1})	2.35 ± 0.10 e	2.94 ± 0.02d	3.23 ± 0.08 c	3.55 ± 0.07 b	3.88 ± 0.12 a
AN (mg kg^{-1})	154 ± 14 c	193 ± 22 b	210 ± 20 b	225 ± 15 ab	253 ±22 a
AP (mg kg^{-1})	8.95 ± 0.94 d	60.8 ± 0.6 c	75.5 ± 5.9 b	81.0 ± 4.6 ab	84.4 ±3.8 a
pH	5.16 ± 0.20 c	5.12 ± 0.10 c	5.41 ± 0.02 b	5.48 ± 0.02 b	5.74 ±0.03 a
MBN (mg kg^{-1})	35.2 ± 10.3 c	42.0 ± 2.6 c	76.5 ± 9.2 b	72.3 ± 4.9 b	99.0 ± 12.3 a
MBC (mg kg^{-1})	645 ± 198 b	764 ± 184 b	1180 ± 212 a	1149 ± 178 a	1278± 226 a
AHN (mg kg^{-1})	1719 ± 97 d	2076 ± 117 c	2413 ± 59 b	2360 ± 102 b	2777 ± 134 a
NHN (mg kg^{-1})	630 ± 142 c	859 ± 127 b	813 ± 86 bc	1188 ± 73 a	1102 ± 39 a
AHAN (mg kg^{-1})	266 ± 17 c	337 ± 33 b	398 ± 18 a	421 ± 25 a	431 ± 15 a
AAN (mg kg^{-1})	618 ± 22 d	702 ± 23 c	775 ± 15 b	915 ± 38 a	875 ± 26 a
ASN (mg kg^{-1})	92.6 ± 8.1 b	59.9 ± 9.8 c	109 ± 4 b	149 ± 20 a	156 ± 10 a
AHUN (mg kg^{-1})	743 ± 74 d	977 ± 109 c	1131 ± 69 b	875 ± 50 cd	1314 ± 104 a
Annual rice yield (kg·ha^{-1})	6967 ± 203 c	11518 ± 265 b	12009 ± 212 a	12040 ± 282 a	12314 ± 246 a

Control, no fertilization; NPK, chemical fertilization; 70 F + 30 M, 70% of chemical N plus 30% of organic N; 50 F + 50 M, 50% of chemical N plus 50% of organic N; 30 F + 70 M, 30% of chemical N plus 70% of organic N; SOM, soil organic matter; TN, total soil N; AN, available N; AP, available P; MBN, microbial biomass N; MBC, microbial biomass C; AHN, acid-hydrolyzable N; NHN, non-hydrolyzable N; AHAN, acid-hydrolyzable ammonium N; AAN, Amino acid N; ASN, amino sugar N; AHUN, acid-hydrolyzable unknown N. The data represent the mean ± S.D.; n = 3. Different letters within each row indicate significant differences among fertilizer treatments at the $p \leq 0.05$ level (Duncan's test).

Significant alterations were made to the chemical characteristics of the soil following a 36-year continuous fertilization. Compared with the NPK, organic fertilizer substitution significantly improved soil organic matter (SOM), soil total N (TN), available N (AN), available P (AP), microbial biomass N (MBN), and microbial biomass C (MBC) content by 17.7–32.6% ($p < 0.05$), 9.9–32.0% ($p < 0.05$), 8.8–31.1% ($p < 0.05$), 24.2–38.8% ($p < 0.05$), 82.3–135.8% ($p < 0.05$), and 54.5–67.3% ($p < 0.05$), respectively (Table 1). Moreover, soil pH with organic fertilizer substitution treatments increased by 0.29–0.62 units ($p < 0.05$) compared to NPK and increased proportionally with the organic substitutions.

Long-term organic fertilizer substitution also significantly increased the organic N fractions (Table 1). Averaged across all organic fertilizer substitution treatments, acid-hydrolyzable N (AHN), acid-hydrolyzable ammonium N (AHAN), amino acid N (AAN), amino sugar N (ASN), acid-hydrolyzable unknown (AHUN), and non-hydrolyzable N (NHN) were increased by 21.2% ($p < 0.05$), 23.6% ($p < 0.05$), 21.8% ($p < 0.05$), 130.4% ($p < 0.05$), 13.27% ($p < 0.05$), and 20.4% ($p < 0.05$) compared to NPK, respectively. Interestingly, 30 F + 70 M had the highest AHAN, ASN, and AHUN content in fertilization treatment soils, while 50 F + 50 M had the highest AAN content and the lowest AHUN level.

3.2. Recovery of ^{15}N in NH_4^+-N, NO_3^--N and Insoluble Organic N Pools in Incubation Experiment

The recovery of ^{15}N in the insoluble N pools increased gradually throughout the incubation period, regardless of the fertilization treatments (Figure 1a,b). In $^{15}NH_4^+$ labeled soil, 18.8–46.8%, 4.8–26.0%, and 17.4–28.9% were the recoveries of the ^{15}N addition at the end of incubation in the NH_4^+, NO_3^-, and insoluble organic N pools, respectively (Figure 2a). The order of ^{15}N recovery in the NH_4^+ pool was 30 F + 70 M < 50 F + 50 M < 70 F + 30 M < NPK < control, and the organic fertilizer substitution treatments were reduced by 29.10–71.66% ($p < 0.05$) and 22.68–69.10% ($p < 0.05$) compared to the control and NPK, respectively (Figure 2a). The recovery of ^{15}N in the NO_3^- was opposite to that of NH_4^+ pool, and the organic fertilizer substitution treatments significantly increased compared with the control and NPK. The ^{15}N recovery in insoluble N pools followed NPK < control < 70 F + 30 M < 30 F + 70 M < 50 F + 50 M, and organic fertilizer substitution treatments were significantly higher than the control and NPK treatments. The 50 F + 50 M treatment was significantly higher among all other substitution treatment ratios.

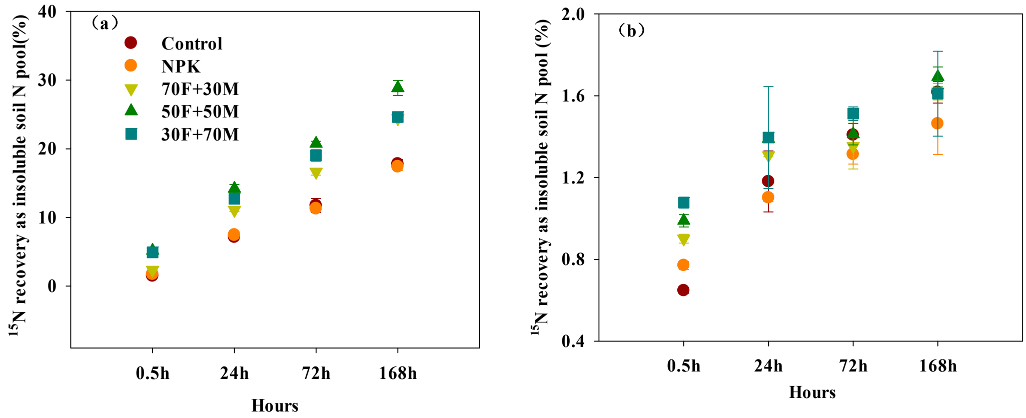

Figure 1. Immobilization of NH_4^+-^{15}N (**a**) and NO_3^--^{15}N (**b**) in insoluble N pool by incubation time under different fertilizer treatments after a 36-year experiment. Control, no fertilization; NPK, chemical fertilization; 70 F + 30 M, 70% of chemical N plus 30% of organic N; 50 F + 50 M, 50% of chemical N plus 50% of organic N; 30 F + 70 M, 30% of chemical N plus 70% of organic N. Error bars represent standard deviations of the means (n = 3).

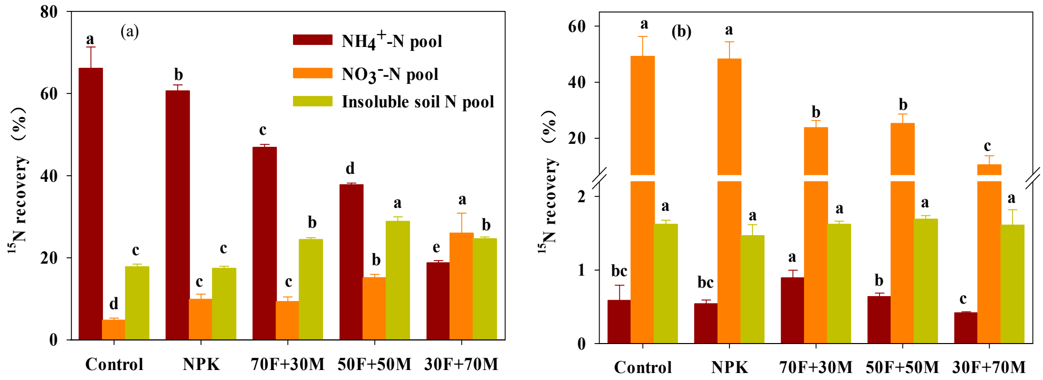

Figure 2. Recovery of ^{15}N in $^{15}NH_4^+$-labeled (**a**) and $^{15}NO_3^-$-labeled (**b**) soils during 168 h incubation under different fertilization treatments after a 36-year experiment. Control, no fertilization; NPK, chemical fertilization; 70 F + 30 M, 70% of chemical N plus 30% of organic N; 50 F + 50 M, 50% of chemical N plus 50% of organic N; 30 F + 70 M, 30% of chemical N plus 70% of organic N. Error bars represent standard deviations of the means (n = 3). Different letters in the bars of the same N pool indicate significant differences among fertilizer treatments at the $p \leq 0.05$ level (Duncan's test).

In $^{15}NO_3^-$ labeled soil, ^{15}N recoveries were 0.42–0.89% and 1.47–1.69% for NH_4^+ and insoluble N pools, respectively (Figure 2b). The ^{15}N recoveries were much greater in the NO_3^- pool (10.5–49.2%) than in the NH_4^+ pool and the insoluble N pools. The ^{15}N recovery in NH_4^+ pools showed that 30% organic substitution treatment was significantly higher than the control and NPK treatments ($p < 0.05$). The recovery rate of ^{15}N in NO_3^- pool followed 30 F + 70 M < 70 F + 30 M < 50 F + 50 M < NPK < control, in which the organic substitution treatment was significantly lower than in the control ($p < 0.05$) and in NPK treatments ($p < 0.05$). Between organic fertilizer substitution treatments, 30%, and 50% organic fertilizer substitution were significantly higher than that of 70% organic fertilizer substitution in the recovery of ^{15}N in NO_3^- pool. However, there were no significant distinctions between fertilizer treatments in the recovery of ^{15}N in insoluble N pools.

3.3. Abiotic and Biotic N Immobilization in Incubation Experiment

Organic fertilizer substitution treatments improved NH_4^+-N abiotic immobilization significantly by 57.51–245.52% ($p < 0.05$) and 34.72–195.52% ($p < 0.05$) than the control and NPK treatments, respectively (Figure 3a). Among the organic fertilizer substitution treatments 50 F + 50 M and 30 F + 70 M were significantly higher than 70 F + 30 M. Similarly, NH_4^+-N biotic immobilization showed the same tendency, and the organic fertilizer substitution treatments increased by 20.88–45.30% ($p < 0.05$) and 25.93–51.36% ($p < 0.05$) compared to the control and NPK treatments, respectively. Biotic immobilization was significantly the highest in 50 F + 50 M in respect of those of 70 F + 30 M, and 30 F + 70 M treatments. In general, the ratio of abiotic to biotic NH_4^+-N immobilization was <1 in all treatments, indicating that NH_4^+-N biotic immobilization played a dominant role in N immobilization (Figure 3c).

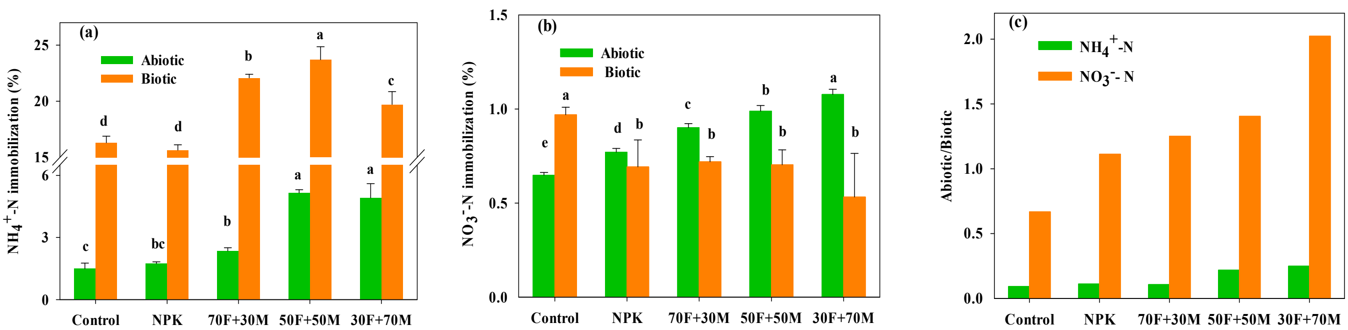

Figure 3. Immobilization of NH_4^+-N (**a**), NO_3^--N (**b**), and abiotic/biotic ratio (**c**) under different fertilization treatments after a 36-year experiment. Control, no fertilization; NPK, chemical fertilization; 70 F + 30 M, 70% of chemical N plus 30% of organic N; 50 F + 50 M, 50% of chemical N plus 50% of organic N; 30 F + 70 M, 30% of chemical N plus 70% of organic N. Abiotic/biotic, soil abiotic N immobilization divided by biotic N immobilization. Error bars represent standard deviations of the means (n = 3). Different letters in the bars of the same variable indicate significant differences among fertilizer treatments at the $p \leq 0.05$ level (Duncan's test).

The immobilization of NH_4^+-N, both biotic and abiotic, showed a significant positive relationship with organic N fractions and the characteristics of the soil. A stepwise regression using NH_4^+-N abiotic immobilization as the dependent variable and AHN, AHAN, AAN, ASN, NHN, SOM, TN, pH, AN, and AP as the independent variables revealed that AAN, AHAN, TN, and AP together explained 96.0% of the variation in NH_4^+-N abiotic immobilization, with standardized regression coefficients of AAN (1.145; $p < 0.001$), TN (0.747; $p < 0.01$), AP (−0.550; $p < 0.01$), and AHAN (−0.461; $p < 0.05$), respectively. A stepwise regression with NH_4^+-N biotic immobilization as the dependent variable and AHN, AHAN, AAN, ASN, NHN, SOM, TN, pH, AN, and AP as the independent variables indicated that only AAN alone explained 59.4% of the variation in NH_4^+-N biotic immobilization ($p < 0.001$). This suggests that the organic fertilizer substitutes significantly enhanced both abiotic and biotic immobilization of NH_4^+-N. The NH_4^+-N immobilization capacity was primarily influenced by the active fractions (i.e., ANN and AHAN) within the organic N pool.

Long-term fertilization resulted in a significant increase in abiotic NO_3^--N immobilization in the following order: control< NPK < 70 F + 30 M <50 F + 50 M < 30 F + 70 M (Figure 3b). In the organic fertilizer substitution treatments, abiotic immobilization of NO_3^--N was higher than the control and NPK treatments by 39.01–52.56% ($p < 0.05$) and 16.86–39.80% ($p < 0.05$), respectively. The abiotic immobilization of NO_3^--N was significantly higher with the increase in the substitution rates. Long-term fertilization reduced NO_3^--N biotic immobilization compared to the control, and there were no significant differences between fertilization treatments. Abiotic immobilization to biotic immobilization ratios were <1 in the control group and >1 in the other treatments (Figure 3c).

The abiotic immobilization of NO_3^--N increased with the proportion of organic fertilizer substitution, but biotic immobilization decreased. A stepwise regression analysis with NO_3^--N abiotic immobilization as the dependent variable and AHN, AHAN, AAN, AHUN, NHN, SOM, TN, pH, AN, and AP as the independent variables revealed that TN, SOM, and ASN collectively explained 98.8% of NO_3^--N abiotic immobilization ($p < 0.001$). The standardized regression coefficients for TN, SOM, and ASN were 0.575 ($p < 0.001$), 0.283 ($p < 0.01$), and 0.202 ($p < 0.01$), respectively. After stepwise regression with NO_3^--N biotic immobilization as a dependent variable and AHN, AHAN, AAN, AHUN, NHN, SOM, TN, pH, AN, and AP as independent variables, TN explained 57.8% (−0.78, $p < 0.01$) of NO_3^--N biotic immobilization.

3.4. N Fertilizer Fate in the Pot Experiment

The effects of different treatment soils on N fertilizer utilization, residue, and loss are shown in Table 2. The utilization rate of ^{15}N followed the same trend in both years. The two-year values average followed control < NPK < 30 F + 70 M < 70 F + 30 M < 50 F + 50 M, with the 50 F + 50 M soil (36.72%) being significantly higher than both in the control (29.45%) and in the NPK soil (30.24%) ($p < 0.05$). The ^{15}N residual rate was higher in the second year than in the first year, due to the fact that the residuals in the second year included the accumulation from the first year. The two-year average followed control < NPK < 70 F + 30 M < 50 F + 50 M < 30 F + 70 M, with the 30 F + 70 M soil (23.58%) having the highest residual rate, which was significantly higher than in the other treatments. The ^{15}N loss rate was opposite to the trend of the utilization rate of ^{15}N, with the 50 F + 50 M soil possessing the lowest loss rate.

Table 2. Nitrogen fertilization differences in pot experiments of soils under various treatments (%).

Year	Treatments	^{15}N Use Efficiency	^{15}N Residual Rate	^{15}N Loss Rate
^{15}N application in the first year	control	27.43 ± 1.48 c	10.54 ± 1.45 c	62.03 ± 2.67 a
	NPK	29.69 ± 1.04 bc	11.51 ± 1.42 bc	58.79 ± 0.42 ab
	70 F + 30 M	30.53 ± 1.67 ab	13.22 ± 0.97ab	56.25 ± 2.02 b
	50 F + 50 M	33.00 ± 1.76 a	11.55 ± 0.59 bc	55.45 ± 1.85 b
	30 F + 70 M	30.29 ± 1.80 abc	14.17 ± 0.43 a	55.54 ± 2.07 b
^{15}N application in the second year	control	31.47 ± 2.40 b	30.22 ± 3.20 bc	38.31 ± 5.15 ab
	NPK	30.80 ± 7.22 b	28.43 ± 1.50 c	40.77 ± 6.94 a
	70 F + 30 M	30.32 ± 6.22 b	29.92 ± 0.80 bc	39.76 ± 5.58 ab
	50 F + 50 M	40.45 ± 5.70 a	31.93 ± 0.67 ab	27.63 ± 5.74 bc
	30 F + 70 M	34.33 ± 6.94 ab	32.98 ± 0.99 a	32.69 ± 6.46 c
Two-years average ^{15}N application values	control	29.45 ± 0.87 b	20.38 ± 1.24 c	50.17 ± 1.53 a
	NPK	30.25 ± 3.28 b	19.97 ± 0.44 c	49.78 ± 3.66 a
	70 F + 30 M	30.42 ± 2.36 b	21.57 ± 0.30 b	48.01 ± 2.22 a
	50 F + 50 M	36.72 ± 2.70 a	21.74 ± 0.29 b	41.54 ± 2.76 b
	30 F + 70 M	32.31 ± 4.27 b	23.58 ± 0.68 a	44.12 ± 4.13 b

Soil that had received no fertilizer (control soil); soil with chemical fertilization (NPK soil); soil treated with 70% of chemical N plus 30% of organic N (70 F + 30 M soil); soil treated with 50% of chemical N plus 50% of organic N (50 F + 50 M soil); and soil treated with 30% of chemical N plus 70% of organic N (30 F + 70 M soil), for 36 years. The data represent the mean ± S.D.; n = 3. Different letters within each column indicate significant differences among fertilizer treatments at the $p \leq 0.05$ level (Duncan's test).

3.5. Relationships Between Soil Properties, N Immobilization, and N Fertilizer Fate

Correlation analysis showed that organic N fractions (AHAN, AAN, ASN, and NHN), and N immobilization (NH_4^+-N biotic/abiotic immobilization) were significantly positively correlated with the N use efficiency (Figure S1). Soil properties (SOM, TN, pH, AN, AP, MBC, and MBN), organic N fractions (AHN, AAN, ASN, NHN, AHAN, and AHUN), and N immobilization (NH_4^+-N/NO_3^--N abiotic immobilization) were significantly positively correlated with the N residual rate. Conversely, NO_3^--N biotic immobilization was significantly negatively correlated with N residual rate (Figure S1).

To examine the impacts of soil with different substitution proportions on exogenous N fertilizer uptake and residue, this study conducted structural equation modeling (SEM) based on soil properties, N pool activity, N immobilization, N fertilizer uptake (2-year average ^{15}N uptake), and N fertilizer residue (2-year average ^{15}N residue) indicators (Figure 4). This model explained 62.9% and 77.3% of the variance in N fertilizer uptake and residue, respectively. The results showed that the soil properties had a direct positive effect on N pool activity ($p < 0.001$). Both soil properties ($p < 0.05$) and N pool activity ($p < 0.001$) had a direct positive effect on N immobilization, with a greater factor loading for the N pool activity. N immobilization had a direct positive effect on N fertilizer uptake ($p < 0.05$), while soil properties had a direct positive effect on N fertilizer residue ($p < 0.05$). Soil properties and N pool activity affected N fertilizer uptake ($p < 0.01$) indirectly through their effect on N immobilization.

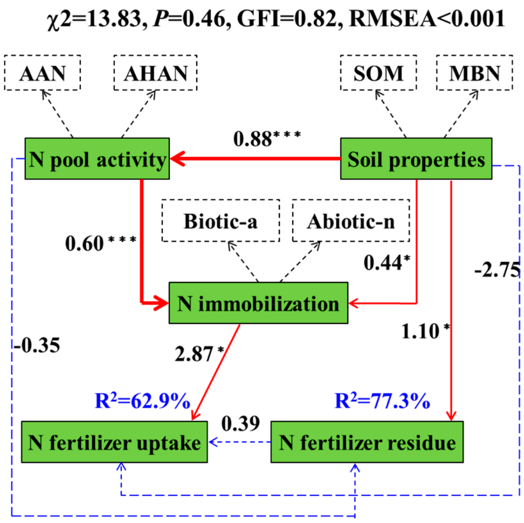

Figure 4. Structural equation modeling (SEM) analysis of the relationship between different soil properties, N pool activity, N immobilization, N fertilizer uptake, and residue. Indicators in the box specify observed or potential variables, respectively. Soil properties include soil organic matter (SOM) and microbial biomass nitrogen (MBN); N pool activity includes acid-hydrolyzable ammonium N (AHAN) and amino acid N (AAN); N immobilization includes biotic immobilization of NH_4^+-N (Biotic-a) and abiotic immobilization of NO_3^--N (Abiotic-n). R^2 indicates the explanatory power of the variables. The path coefficients with significant effects are designated by solid red lines, and the thickness of the lines indicates significance. "*" and "***" specify $p < 0.05$ and $p < 0.001$, and path coefficients with no significant difference are indicated by dashed blue lines.

4. Discussion

4.1. Effects of Long-Term Organic Fertilizer Substitution on Biotic and Abiotic N Immobilization

Microbial N immobilization is a crucial mechanism in soil N immobilization. Studies have indicated that long-term application of organic fertilizer can enhance N biotic immobilization. Biotic immobilization of N in agricultural soils is mainly limited by C availability [12]. Similar conclusions were obtained in this study, that NH_4^+-N biotic immobilization was significantly positively correlated with SOM, TN, and organic N fractions

(Figure S1). These results are consistent with the first hypothesis of this study, that the soil properties regulate N biotic immobilization. Notably, the ANN fraction presents as an organic-inorganic compound was found to be the primary influencing factor of NH_4^+-N biotic immobilization in this study's stepwise regression analysis. AAN occurs in the form of polymers, is closely related to the metabolism of soil microorganisms, and is an important repository of N immobilization [38], which plays an important role in soil organic N mineralization and immobilization [39]. Lu et al. [40] argued that the newly synthesized organic N and ANN are mostly derived from N fertilizer. A significant role for ANN in N immobilization is also suggested by its increased N turnover rate when compared to other organic N fractions. The proportion of ANN in AHN was found to be significantly positively correlated with the gross mineralization rate and gross NH_4^+-N immobilization rate in a previous study [23]. Cao et al. [37] point out that the chemical quality of organic C is determined by the three carbon pools (i.e., recalcitrant, intermediate, and labile) rather than the C/N ratio, and is a key factor in regulating the response of microorganisms N immobilization to organic C addition. Numerous studies have also demonstrated that the activity of soil organic C pools controls microbial N immobilization; however, there are not any particular metrics to gauge C activity when evaluating N cycling [37,41,42]. This study indicates specific measures where the quality of the soil organic N pool active fractions (i.e., ANN) relative to organic C pool plays an important role in the determination of NH_4^+-N biotic immobilization. The present study offers valuable insights into NH_4^+-N biotic immobilization, showcasing improvement through the modulation of the N pool activity with a suitable substitution ratio (i.e., 50%). The highest level of ANN was found in 50% organic fertilizer substitution soil and in stepwise regression analysis, AAN alone explained 59.4% of the variation in NH_4^+-N biotic immobilization ($p < 0.001$). This result partially supports the second hypothesis of this study.

In this study, our results show that NH_4^+-N abiotic immobilization (1.5–5.2%) was lower than the NH_4^+-N biotic immobilization (15.7–23.7%; Figure 3) because abiotic immobilization of NH_4^+-N in agricultural soils is much smaller than biotic immobilization of NH_4^+-N [10], and it is a common phenomenon in soils mainly due to the physical condensation reaction of NH_4^+-N with phenolic compounds and its immobilization in clay minerals [12,43]. Long-term straw return or pig manure application enhanced the abiotic immobilization of NH_4^+-N, showing a significant positive correlation with organic C, TN, and pH [12]. High pH favors abiotic substitution and sequestration of NH_3 or NH_4^+ by humus and clay minerals [12,44]. This is in line with our study, in which the abiotic immobilization of NH_4^+-N showed a significant positive correlation with SOM, TN, organic N fractions, and pH (Figure S1). However, Barrett et al. [45] found no relationship between abiotic NH_4^+-N immobilization and soil organic C content, which was attributed to the fact that the composition of the organic C pool played a more important role than its amount. The composition of the organic N pool may also influence the abiotic immobilization due to the close coupling of C and N. The finding of the present study were similar to the previous findings, and the stepwise regression results indicated that NH_4^+-N abiotic immobilization was jointly influenced by the active organic N fractions (ANN, AHAN), soil TN, and P contents.

In general, microorganisms prefer to use NH_4^+ as a N source because more energy is required to assimilate NO_3^--N, especially in low C/N agroecosystems [30,46–48]. The present study showed that the biotic immobilization of NO_3^--N (<1%) was minimal (Figure 3b). Moreover, there was no difference in the biotic immobilization of NO_3^--N in the organic fertilizer substitution treatments compared with the chemical fertilizer. This observation aligns with the findings of previous studies [12,49]. The biotic immobilization of NO_3^--N was negatively correlated with SOM and TN, with TN being the most important factor to affect NO_3^--N biotic immobilization. Inevitably, the farmland ecosystem may be a carbon-limited system, and the soil's available C content was not enough to support the energy demand of the NO_3^--N reduction and assimilation processes [10,50]. Furthermore, the high supply of NH_4^+-N under organic fertilizer substitution conditions inhibits

NO_3^--N assimilation [47,51], which may be the reason why NO_3^--N biotic immobilization was found negatively correlated with SOM, TN, etc., in the present study. Applying high C/N organic materials, such as straw, in field or forest soils can improve NO_3^--N biotic immobilization [22]. The organic materials applied in this study were all of low C/N ratio materials, and thus did not significantly affect the NO_3^--N biotic immobilization. The mechanism of NO_3^--N abiotic immobilization by organic matter compared with the biotic immobilization is still not clear [12,52], while a large number of studies have demonstrated that soil organic C and total N content were significantly positively correlated with the abiotic immobilization of NO_3^--N [12,53]. Furthermore, acidic conditions favor the incorporation of NO_3^--N through abiotic processes such as the ferrous wheel hypothesis [54]. This study consistently concluded that abiotic immobilization of NO_3^--N exhibited a significantly positive correlation with SOM, TN, pH, and organic fractions (Figure S1). This indicates that the abiotic immobilization of NO_3^--N was mainly increased under organic substitution conditions, and it played a more important role than biotic immobilization in the acidic paddy soil (Figure 3b). These results also partially support the second hypothesis of this study. Similar results were demonstrated by Fitzhugh et al. [31] and Wang et al. [12].

The order of total ^{15}N recovery of NH_4^+-N, NO_3^--N, and insoluble N from the different treatment soils in the incubation experiment, was 30 F + 70 M < 50 F + 50 M < 70 F + 30 M < NPK < control (Figure 2), with the highest value being in the control due to lower soil microbial activity and N cycling rate [55]. The conversion of NH_4^+-N to both NO_3^--N and insoluble N pools exhibited a significant increase in NH_4^+-N labeled treatments with the substitution ratio (Figure 2a). Contrarily, the conversion of NO_3^--N to both NH_4^+-N and insoluble N pools was limited by the organic fertilizer substitution treatments in NO_3^--N labeled soils (Figure 2b). Denitrification, utilizing NO_3^--N as the substrate, stands out as a crucial N loss pathway in rice fields [56]. Therefore, the increase in NH_4^+-N immobilization under organic substitution also increases NO_3^--N loss. By regulating the substitution ratios, we may control these two processes and increase the capacity to retain N. In this study, NH_4^+-N immobilization capacity was the highest under 50% organic substitution, which improved N retention capacity. When the substitution ratio reached 70%, nitrification capacity and NO_3^--N loss were at the highest, and N retention capacity decreased.

4.2. Mechanisms of Long-Term Organic Fertilizer Substitution Affect on N Retention and N Fertilizer Fate

In crop–soil systems, the conversion of N fertilizer to plant-available soil organic N is essential to improve N use efficiency, reduce N losses, and achieve the goal of sustainable agriculture [40]. The present study demonstrated that the substitution of organic matter into the soil enhanced soil fertility properties and elevated the conversion of N fertilizer to soil organic N pools. Additionally, it led to an augmented plant uptake of N fertilizer, aligning with the findings from previous studies by Zhao et al. [16] and Pan et al. [55]. Organic N is an important N pool in agricultural soils, and immobilization of N fertilizer to organic N is an important N retention mechanism to improve crop N utilization. Soil amino acid N (ANN) is a major active fraction of soil organic N, which builds a N fertilizer immobilization transit pool for crop N uptake [40]. Wu et al. [57] showed that soil ANN and fixation of ammonium can be used as a predictor of soil N retention capacity.

Consistent with previous work, the SEM in this study demonstrated that organic fertilizer substitution enhanced exogenous N fertilizer immobilization capacity and further promoted rice N uptake, mainly by increasing the accumulation of active fractions of soil organic N pools (ANN and AHAN) (Figure 4). Additionally, soil immobilization of N fertilizer, through the mineralization of organic N for crop uptake, and the residue in the soil stable N pools may improve soil N fertility. The SEM result also indicated that improved soil properties (SOM and MBN) promoted N fertilizer immobilization and residuals. This result is consistent with the findings of Elrys et al. [58]. The introduction of organic fertilizer contributes to increased immobilization of inorganic N within microbial N, resulting in improved N retention, and reduced N losses [59–61]. Increased soil organic matter content promotes nitrogen fertilizer residues while reducing crop uptake of nitrogen fertilizer [62]. Crop N fertilizer uptake decreases as a result of microorganisms and crops competing with one another for the available N from the organic fertilizer co-application [11,60]. Subsequent transformation of N fertilizer residue is so low that it does not affect the nutrition of the next season's crop considerably [63]. The end point of these two processes (crop uptake and soil residue) is mainly determined by the mineralization–immobilization turnover (MIT) and the activity of the organic N pools. This is the reason why 70% organic fertilizer substitution soil had the highest ^{15}N residue but lower ^{15}N uptake than 50% organic fertilizer substitution soil in this study. The disadvantages caused by the application of organic and inorganic N fertilizers can be avoided by suitable substitution ratios. This study further develops the previous conclusions that specific substitution ratios can improve N availability, confirming our third hypothesis. Focusing on the activity of the soil N pool, suitable substitution ratios can improve N fertilizer retention, promote N fertilizer uptake, and relatively reduce N fertilizer residuals in the soil's stable N pool.

5. Conclusions

This study highlights the crucial role of biotic immobilization in NH_4^+-N over abiotic immobilization in paddy fields subjected to long-term organic fertilizer substitution. The organic N fraction AAN emerges as a key determinant of the biotic immobilization of NH_4^+-N. In contrast, abiotic immobilization of NO_3^--N played a more important role than biotic immobilization under fertilization conditions and increased with SOM and TN. Organic fertilizer substitution in soils improved exogenous N fertilizer utilization and residuals. The optimal 50% organic N substitution exhibits superior N fertilizer utilization due to high N pool activity level and N immobilization capacity, while 70% of organic N substitution leads to increased fertilizer N residue owing to high soil properties (SOM and MBN). These findings suggest that N immobilization capacity and N fertilizer utilization in our studied soil–crop system can be improved simultaneously by a suitable organic substitution ratio (i.e., 50%). Future studies on microbial community patterns and on the positive regulation of N immobilization and fate under long-term organic substitution conditions could elucidate the best pathway strategies in sustainable fertilization practices in paddy soils.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14122300/s1, Figure S1: Correlation heatmap of soil properties, organic N fractions, N immobilization, and fertilizer N fate.

Author Contributions: H.H.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, software, writing—original draft, writing—review and editing; J.J.: funding acquisition, project administration, resources; X.L. (Xianjin Lan): supervision, visualization; M.D.: writing—review and editing; X.L. (Xiumei Liu): investigation, software; Z.L.: funding acquisition, writing—review and editing; Y.L.: investigation, validation; Z.C.: visualization, writing—review and editing; and W.Z.: conceptualization, supervision, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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