

Article

Effects of Nitrogen Form and Rate on Soil Physicochemical Properties and the *nirS*-Type Denitrifying Bacterial Community Under Straw Addition

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Abstract

Denitrification is a major pathway of reactive nitrogen loss from cold-temperate farmland, but how mineral N form and rate interact with temperature to shape the *nirS*-type denitrifying bacterial community under straw addition is poorly resolved. Maize straw was sealed in mesh bags and buried at a fixed depth in a composite farmland soil from Jiamusi, Heilongjiang Province, and incubated for 240 d through constant-temperature phases of 5, 20, 15 and -15°C (60 d each). Five treatments were compared: straw without mineral N (CK), straw plus full- or half-rate ammonium sulfate (A1, A2), and straw plus full- or half-rate potassium nitrate (B1, B2). The *nirS*-type community, soil chemical properties, and potential enzyme activities were measured at the end of each phase. Community structure differed primarily among the sequential incubation phases. Phase labels the constant-temperature sequence and is not separated from incubation time, straw decomposition or preceding thermal history. Richness of the DNA-detected library was lowest after the 5°C phase and higher later in the sequence, as was the number of ASVs shared by all treatments. Shannon and Simpson diversities differed among treatments only after the 5°C phase, when A1 was lower than CK, A2, B1 and B2. Community composition differed among treatments after the 5°C phase ($p = 0.042$), the 15°C phase ($p = 0.001$) and the -15°C phase ($p = 0.001$), but not after the 20°C phase ($p = 0.060$). *Rhodanobacter* accounted for most *nirS* reads in most samples, but its relative abundance was lower, and that of *Herbaspirillum* and other *Burkholderiales* higher, under A2 after the 5°C phase. These are proportional changes in the sequenced library, not measured changes in gene copies or activity. Soil properties showed significant phase $\times$ treatment interactions. Redundancy analysis described covariation between community structure and invertase and N-related variables after the 20, 15 and -15°C phases, but not after the 5°C phase. Mineral N was associated with changes in *nirS*-type community structure after the 5, 15 and -15°C phases, but not after the 20°C phase. Full-rate ammonium sulfate was associated with lower *nirS* evenness only after the 5°C phase; reduced ammonium and nitrate additions were associated with phase-dependent shifts in relative abundance. Denitrification potential and N_2O were not measured, so these compositional shifts cannot be read as a change in denitrification rate or N loss. Whether matching N form and rate to the early and sub-zero phases would alter field N loss is a hypothesis for trials that measure application timing, crop N uptake and gaseous losses.



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Keywords: *nirS*; straw return; ammonium sulfate; seasonal incubation; freeze–thaw

1. Introduction

Denitrification reduces nitrate (NO_3^-) or nitrite (NO_2^-) to NO, N_2O and N_2 under oxygen-limited conditions and is a major route of reactive nitrogen loss from agricultural soils [1,2]. The gaseous products lower fertilizer-use efficiency and contribute to N_2O accumulation in the atmosphere [3]. The committed step of the pathway is the reduction of NO_2^- to NO, catalyzed either by a copper-type nitrite reductase encoded by *nirK* or by a cytochrome cd₁ enzyme encoded by *nirS* [4,5]. The pathway is modular: organisms that carry *nirS* need not carry *nirK* or *nosZ*. The present study targeted only *nirS* and therefore describes the *nirS*-type denitrifying bacterial community, not the denitrifying community as a whole. Because the two reductases are structurally distinct and rarely coexist in the same genome, *nirK*- and *nirS*-type assemblages are routinely used as markers of denitrifier community structure [6]. Their composition responds to pH, organic carbon, inorganic nitrogen and temperature, but the relative importance of these factors is site-dependent and often differs between the two guilds [7–10].

The Sanjiang Plain is a cold-temperate agricultural region with a short growing season and a long frozen period. Freeze–thaw transitions in spring and autumn release dissolved carbon and nitrogen, lower oxygen availability in soil pores, and can stimulate denitrification and N_2O emission [11,12]. Field surveys in this region have shown that the abundance and membership of *nirS*- and *nirK*-type denitrifiers change between autumn and spring and among land-use types [13], and that *nirS* communities in neighbouring Mollisols are sensitive to both fertilization and season [14]. Such observational designs, however, confound temperature with concurrent changes in moisture, crop growth and substrate supply. Controlled incubations that impose seasonal temperature regimes on the same soil, while holding other variables constant, are therefore needed to isolate the seasonal filter on denitrifier assemblages and to test whether management treatments alter that filter.

Most fertilization studies of denitrifiers have been conducted in warm paddy systems or in multi-year field trials at a single sampling date [7,15,16]. Whether treatment effects that are detectable in one season persist, disappear or reverse under the temperature conditions of another season remains poorly resolved for cold-region soils. Dominant denitrifying lineages in these soils, including *Rhodanobacter*, *Herbaspirillum*, *Cupriavidus* and related *Burkholderiales*, occupy different thermal and redox niches [17,18], so reciprocal shifts among them are a plausible mechanism for season-dependent treatment responses.

Under a single straw addition, it is not known whether ammonium sulfate and potassium nitrate, at full and half rates, reorganize the *nirS*-type denitrifying bacterial community in the same way at each stage of a cold-temperate incubation. A 240 d sequential incubation of one composite farmland soil from Jiamusi was used to follow soil chemistry, potential enzyme activities, and the structure of the *nirS*-type community through constant-temperature phases of 5, 20, 15 and -15°C . The phases are sequential, so temperature is not separated from incubation time. The objectives were (i) to describe how N formed and at what rate, under this common straw addition, was associated with soil properties and potential enzyme activities, and (ii) to describe the corresponding changes in diversity and composition of the *nirS*-type community.

2. Materials and Methods

2.1. Site Description and Sample Collection

Soil and maize (*Zea mays* L.) straw were collected in 2024 from three farmland sites in Jiamusi, Heilongjiang Province, Northeast China: Dongfeng District (46.82°N , 130.26°E), Hexingbao Village (46.75°N , 130.40°E) and Lianjiangkou Town (46.86°N , 130.33°E). The soils are correlated with Mollisols in USDA Soil Taxonomy. The region has a cold-temperate monsoon climate with a long frozen period and a short growing season. Equal masses

of topsoil (0–20 cm) from three farmland sites in Jiamusi were homogenized into one composite before packing. All inference below refers to the response of this composite under the incubation conditions used here. At each site, topsoil (0–20 cm) was sampled at 10 m intervals along a transect. Stones, coarse roots and litter were removed by passage through a 1 cm sieve. A subsample from each bag was air-dried and passed through a 2 mm sieve for determination of baseline properties (Table 1). The remaining soil was stored at 4 °C until the microcosms were established. Equal masses of the three soils were homogenized to obtain a composite used in all incubations.

Table 1. Baseline properties of the composite soil and maize straw (mean $\pm$ SD).

Property	Soil	Straw
pH	5.23 $\pm$ 0.11	7.57 $\pm$ 0.25
SOC (g kg ^{−1})	26.18 $\pm$ 0.25	489.63 $\pm$ 2.88
TN (g kg ^{−1})	0.82 $\pm$ 0.10	7.4 $\pm$ 0.2
Available N (mg kg ^{−1})	101.73 $\pm$ 0.40	19.32 $\pm$ 1.63
Available P (mg kg ^{−1})	49.9 $\pm$ 1.9	387.35 $\pm$ 5.27
Available K (mg kg ^{−1})	215.52 $\pm$ 3.23	376.64 $\pm$ 4.18
SOC/TN	31.92 $\pm$ 2.5	66.16 $\pm$ 14.4

SOC, soil organic carbon; TN, total nitrogen. Straw SOC is reported as the dichromate-oxidizable carbon content of the dried material.

Maize straw was collected at the same three sites. Adhering soil and debris were removed, and the straw was oven-dried at 80 °C. Stems and leaves were combined at a mass ratio of 5:4 and cut to 8 cm segments. The chemical properties of the straw are given in Table 1.

2.2. Experimental Design

Twenty-five polyethylene columns (inner diameter 14.6 cm, height 22.5 cm) were packed with the composite soil, five columns for each treatment. Moist soil at 60% of water-holding capacity was packed to a depth of 3 cm, and the N solution was added at this stage (no N solution in CK). Maize straw (8.55 g dry mass, cut to 8 cm) was sealed in a 100-mesh nylon bag (15 $\times$ 10 cm) and placed on this layer. Further soil and, in the fertilized treatments, the remainder of the N solution were added until the bag lay 20 cm below the final soil surface and the packed column stood 3 cm below the rim. The packed bulk density was 1.06–1.12 g cm^{−3}. The bag kept the straw mass and depth identical across columns, but it confined the residue to one layer rather than mixing it through the soil. Soil water was maintained at 60% of water-holding capacity by weekly weighing and addition of deionized water.

Five treatments received this same straw addition: straw without mineral N (CK), straw plus full-rate ammonium sulfate (A1), straw plus half-rate ammonium sulfate (A2), straw plus full-rate potassium nitrate (B1), and straw plus half-rate potassium nitrate (B2) (Table 2). A1 and A2 therefore added SO₄^{2−} with NH₄⁺, and B1 and B2 added K⁺ with NO₃[−]. The comparison is not a pure contrast of N form. Halving the rate also halved the counterion addition. Available K under B1 and B2 includes K added as KNO₃. Because B1 received KNO₃, this increase includes fertilizer-derived K and is not interpreted as K released from soil or straw.

Columns were pre-incubated at 15 °C for 14 d, then held sequentially at constant 5 t, 20 °C, 15 °C and −15 °C for 60 d each (240 d in total). These set-points approximate long-term seasonal means at Jiamusi. They are not replicated field seasons. Diel variation and repeated freeze–thaw cycles were not imposed, and no thawing transition was monitored during the −15 °C phase. Temperature is confounded with incubation duration, straw

decomposition, and exposure to the preceding phase. In the text, spring, summer, autumn and winter label these four phases. In the figure codes, A, B, C and D denote the 5, 20, 15 and -15°C phases.

Table 2. Experimental treatments.

Treatment	N Source	N Rate	Straw
CK	None	0	8.55 g, 8 cm
A1	$(\text{NH}_4)_2\text{SO}_4$	100% (260 kg ha $^{-1}$)	8.55 g, 8 cm
A2	$(\text{NH}_4)_2\text{SO}_4$	50%	8.55 g, 8 cm
B1	KNO_3	100% (420 kg ha $^{-1}$)	8.55 g, 8 cm
B2	KNO_3	50%	8.55 g, 8 cm

At 60, 120, 180 and 240 d, soil was subsampled from the same three columns per treatment. At each date, approximately 50 g of soil was removed from a point adjacent to, but not inside, the straw bag. Successive samples from one column were taken from different sides of the bag, at least 10 cm apart, so that a previous sampling hole was not reopened. The removed soil was used for chemical analysis, enzyme assays and DNA extraction ($n = 3$). Repeated removal of soil is a disturbance and may have altered local structure, aeration and moisture next to the residue. Those columns were returned to the incubator and continued to the next phase. The other two columns per treatment were not sampled at any date. Samples were not pooled across columns. Observations from the same column across the four phases are repeated measures. Three technical PCR reactions per extract were pooled before sequencing and are not additional biological replicates. Column fate is shown in Figure S1.

In the sample codes used in the figures, the prefix A, B, C or D denotes spring, summer, autumn or winter, respectively.

2.3. Soil Physicochemical Properties

Soil pH was measured in a 1:2.5 (w/v) suspension in deionized water after 30 min of shaking. Soil organic carbon (SOC) was determined by potassium dichromate oxidation with external heating and conversion of organic matter to organic carbon [19]. Total nitrogen (TN) was determined by Kjeldahl digestion. Available nitrogen (AN) was measured by alkaline hydrolysis diffusion. Ammonium ($\text{NH}_4^+\text{-N}$) and nitrate ($\text{NO}_3^-\text{-N}$) were extracted and quantified by Nessler's reagent and phenol-disulfonic acid colorimetry, respectively. Available phosphorus (AP) was extracted with $0.5\text{ mol L}^{-1}\text{ NaHCO}_3$ and determined by the molybdenum–antimony colorimetric method. Available potassium (AK) was extracted with $2\text{ mol L}^{-1}\text{ HNO}_3$ and determined by flame photometry. Water-holding capacity was calculated as (mass of water-saturated soil–mass of oven-dry soil)/oven-dry mass. Bulk density was measured with 100 cm^3 rings. At the end of the -15°C phase, columns were removed from the incubator and subsamples were thawed at 4°C for 12 h before chemical analysis, enzyme assays and DNA extraction.

2.4. Enzyme Activities

Invertase, amylase and urease were assayed by the colorimetric methods of [20]. Air-dried soil was passed through a 0.25 mm sieve, and 5.0 g was weighed for each assay. The values are potential activities under substrate-saturated assay conditions. They are not in situ rates of C or N transformation in the columns. For urease, soil was incubated with 10 mL of 10% urea and 20 mL of citrate buffer (pH 6.7) at 37°C for 24 h; $\text{NH}_4^+\text{-N}$ was measured by the indophenol blue reaction at 578 nm, and activity is expressed as $\text{mg NH}_3\text{-N g}^{-1}\text{ 24 h}^{-1}$. For invertase, soil was incubated with 15 mL of 8% sucrose and

5 mL of phosphate buffer (pH 5.5) at 37 °C for 24 h; reducing sugar was measured with 3,5-dinitrosalicylic acid at 508 nm against a glucose standard, and activity is expressed as mg glucose g⁻¹ 24 h⁻¹. For amylase, soil was incubated with 10 mL of 1% starch and 10 mL of phosphate buffer (pH 5.6) at 37 °C for 24 h and measured at 508 nm by the same DNS reaction. Toluene (1 mL) was added to each assay as a bacteriostat. A substrate-free control was included in every batch. Absorbance was read on a spectrophotometer with a 1 cm cell.

2.5. DNA Extraction and nirS Amplicon Sequencing

DNA was extracted from 0.5 g of the soil subsample. Genomic DNA was extracted from 0.5 g of fresh soil using a commercial soil DNA kit according to the manufacturer's instructions. DNA concentration and purity were checked with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and by electrophoresis on 1% agarose gels.

The cytochrome cd₁ nitrite reductase gene (*nirS*) was amplified with the primers cd3aF (5'-GTSAACGTAAGGARACSGG-3') and R3cd (5'-GASTTCGGRTGSGTCTTGA-3') [21], which yield a fragment of approximately 425 bp. Forward and reverse primers were tagged with sample-specific barcodes. PCR was performed in triplicate in 25 µL reactions containing 2.5 µL of 10× buffer, 2 µL of dNTPs (2.5 mmol L⁻¹), 1 µL of each primer (10 µmol L⁻¹), 0.5 µL of DNA polymerase, 1 µL of template DNA, and nuclease-free water. The thermal programme was 95 °C for 3 min; 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s; and a final extension at 72 °C for 10 min. Amplicons were purified, pooled in equimolar amounts, and sequenced on an Illumina platform using paired-end 2 × 250 bp reads.

2.6. Bioinformatics

Paired-end reads were demultiplexed, quality-filtered, and denoised in QIIME 2 with the DADA2 plugin [22,23]. Primer sequences were removed, and reads were truncated where the median quality score declined. Chimeric sequences were discarded. Raw reads averaged 13,000–25,000 per sample. After filtering, denoising, and chimera removal, 400–800 reads per sample remained. Sequences were rarefied to the lowest retained depth before diversity analyses. Good's coverage at this depth was 0.9968–0.9972.

Taxonomy was assigned with a naive Bayes classifier trained on the FunGene *nirS* reference set (accessed May 2026). Reference sequences were restricted to those annotated as *nirS*, of length 400–800 bp, without ambiguous bases and with a genus name. An ASV was retained at genus level only if the classification confidence was at least 0.7; otherwise, it was labelled unclassified.

Chao1, observed ASVs, and Shannon and Simpson indices were calculated in QIIME 2. Bray–Curtis distances were calculated on the rarefied table and ordinated by principal coordinate analysis. Shared and unique ASVs within each phase were counted with Venn diagrams. Treatment-associated taxa were screened with LEfSe (LDA score > 2).

Redundancy analysis was run on Hellinger-transformed relative abundances, separately for each phase. Candidate predictors were pH, SOC, TN, AN, NH₄⁺-N, NO₃⁻-N, AP, AK, invertase, amylase and urease. Variance inflation factors were calculated, and predictors with VIF > 10 were removed iteratively. Forward selection was applied to the reduced set with ordiR2step (999 permutations, adjusted R² as the stopping criterion). The global model, each constrained axis, and each selected term were tested by permutation (999 permutations). Adjusted R² was calculated for the selected model.

2.7. Statistical Analysis

All statistical analyses were performed in SPSS (version 19.0) and R (version 4.3). Residual normality and homogeneity of variance were checked with the Shapiro–Wilk and Levene tests. Soil properties, enzyme activities and α -diversity were analyzed by repeated-measures ANOVA. Treatment was the between-column factor and was tested against the between-column error ($df = 10$). Phase and the phase $\times$ treatment interaction were within-column terms and were tested against the within-column error ($df = 30$). Sphericity of the within-column covariance was assessed, and Greenhouse–Geisser corrected p -values are reported where sphericity was not met. A significant phase $\times$ treatment interaction ($p < 0.05$) was followed by a one-way ANOVA and Tukey’s HSD within that phase ($p < 0.05$). Letters in Table 3 are from these within-phase tests and are simple effects, not independent replicates. A non-significant interaction was followed by the main effects only; no within-phase treatment contrast was added. Means are reported as mean $\pm$ SD ($n = 3$ columns per treatment).

Table 3. Soil physicochemical properties and enzyme activities (mean $\pm$ SD, $n = 3$) under different treatments in four sequential incubation phases.

Season	Treatment	pH	SOC (g kg ⁻¹)	TN (g kg ⁻¹)	AN (mg kg ⁻¹)	NH ₄ ⁺ -N (mg kg ⁻¹)	NO ₃ ⁻ -N (mg kg ⁻¹)	AP (mg kg ⁻¹)	AK (mg kg ⁻¹)	Invertase (mg g ⁻¹ d ⁻¹)	Amylase (mg g ⁻¹ d ⁻¹)	Urease (mg g ⁻¹ d ⁻¹)
Spring	CK	5.49 $\pm$ 0.09 c	35.20 $\pm$ 1.94 a	1.228 $\pm$ 0.006 b	170.43 $\pm$ 0.93 c	27.00 $\pm$ 3.38 a	26.64 $\pm$ 0.59 a	111.60 $\pm$ 5.08 a	298.65 $\pm$ 4.49 b	11.40 $\pm$ 0.12 a	1.57 $\pm$ 0.08 e	1.49 $\pm$ 0.08 a
	A1	5.82 $\pm$ 0.18 b	34.26 $\pm$ 2.73 a	1.298 $\pm$ 0.012 a	203.80 $\pm$ 7.10 a	28.19 $\pm$ 0.92 a	21.01 $\pm$ 0.72 c	80.58 $\pm$ 2.23 b	234.88 $\pm$ 10.13 d	10.03 $\pm$ 0.11 b	2.20 $\pm$ 0.04 c	1.43 $\pm$ 0.09 a
	A2	6.14 $\pm$ 0.10 a	29.89 $\pm$ 3.08 a	1.160 $\pm$ 0.009 c	189.46 $\pm$ 2.48 b	25.26 $\pm$ 0.55 b	12.04 $\pm$ 0.58 d	92.37 $\pm$ 3.40 b	253.51 $\pm$ 1.75 c	11.17 $\pm$ 0.12 a	2.02 $\pm$ 0.09 d	1.42 $\pm$ 0.07 a
	B1	6.18 $\pm$ 0.10 a	29.82 $\pm$ 1.12 a	1.276 $\pm$ 0.009 a	191.68 $\pm$ 1.16 b	26.97 $\pm$ 2.10 a	22.71 $\pm$ 0.58 b	74.59 $\pm$ 4.63 c	367.38 $\pm$ 15.73 a	9.16 $\pm$ 0.37 c	2.90 $\pm$ 0.05 a	1.15 $\pm$ 0.10 b
	B2	6.09 $\pm$ 0.09 a	30.87 $\pm$ 1.83 a	1.243 $\pm$ 0.013 b	183.08 $\pm$ 2.48 b	32.22 $\pm$ 1.97 a	11.80 $\pm$ 0.60 d	119.50 $\pm$ 8.06 a	265.16 $\pm$ 10.24 c	8.18 $\pm$ 0.23 d	2.61 $\pm$ 0.02 b	1.47 $\pm$ 0.06 a
Summer	CK	5.68 $\pm$ 0.07 b	55.18 $\pm$ 3.12 a	1.280 $\pm$ 0.005 c	180.66 $\pm$ 7.88 d	60.03 $\pm$ 1.03 c	27.72 $\pm$ 1.44 b	91.35 $\pm$ 4.43 a	200.51 $\pm$ 16.54 a	7.90 $\pm$ 0.60 c	2.41 $\pm$ 0.04 c	0.73 $\pm$ 0.04 b
	A1	6.12 $\pm$ 0.15 a	49.33 $\pm$ 1.86 a	1.305 $\pm$ 0.011 b	246.86 $\pm$ 3.05 a	83.83 $\pm$ 2.94 a	25.94 $\pm$ 0.44 b	85.11 $\pm$ 3.64 a	167.69 $\pm$ 11.51 b	8.94 $\pm$ 0.28 b	2.64 $\pm$ 0.06 b	0.58 $\pm$ 0.05 c
	A2	6.19 $\pm$ 0.11 a	47.29 $\pm$ 1.12 b	1.155 $\pm$ 0.024 d	217.54 $\pm$ 4.30 b	71.31 $\pm$ 1.53 b	21.70 $\pm$ 0.85 c	91.21 $\pm$ 2.23 a	149.55 $\pm$ 13.65 b	11.57 $\pm$ 0.30 a	2.24 $\pm$ 0.06 c	0.79 $\pm$ 0.04 b
	B1	6.37 $\pm$ 0.05 a	52.75 $\pm$ 2.63 a	1.439 $\pm$ 0.016 a	226.23 $\pm$ 3.21 b	73.22 $\pm$ 1.11 b	35.25 $\pm$ 0.58 a	76.30 $\pm$ 2.56 b	213.33 $\pm$ 1.75 a	6.48 $\pm$ 0.19 d	3.23 $\pm$ 0.07 a	1.01 $\pm$ 0.07 a
	B2	6.17 $\pm$ 0.07 a	47.17 $\pm$ 2.53 b	1.338 $\pm$ 0.009 b	210.93 $\pm$ 5.20 c	73.99 $\pm$ 1.03 b	16.95 $\pm$ 1.29 d	85.88 $\pm$ 4.58 a	184.21 $\pm$ 3.07 a	9.51 $\pm$ 0.35 b	3.08 $\pm$ 0.11 a	0.70 $\pm$ 0.04 b
Autumn	CK	5.92 $\pm$ 0.03 b	53.92 $\pm$ 1.28 b	1.288 $\pm$ 0.007 a	194.54 $\pm$ 2.95 c	57.49 $\pm$ 0.87 b	22.70 $\pm$ 0.61 d	85.88 $\pm$ 4.58 b	251.77 $\pm$ 18.18 b	11.81 $\pm$ 0.23 c	2.24 $\pm$ 0.11 c	0.76 $\pm$ 0.07 b
	A1	6.11 $\pm$ 0.03 a	59.66 $\pm$ 0.62 a	1.260 $\pm$ 0.003 b	214.35 $\pm$ 4.79 b	63.21 $\pm$ 1.58 a	27.87 $\pm$ 0.62 c	94.75 $\pm$ 1.43 a	246.23 $\pm$ 5.12 b	15.04 $\pm$ 0.07 a	2.99 $\pm$ 0.08 a	1.23 $\pm$ 0.08 a
	A2	6.04 $\pm$ 0.11 a	51.38 $\pm$ 2.16 b	1.237 $\pm$ 0.011 b	199.11 $\pm$ 2.85 c	54.34 $\pm$ 1.13 c	38.92 $\pm$ 0.62 a	85.91 $\pm$ 3.87 b	181.87 $\pm$ 4.63 c	13.21 $\pm$ 0.17 b	2.61 $\pm$ 0.04 b	1.13 $\pm$ 0.03 a
	B1	6.22 $\pm$ 0.06 a	57.61 $\pm$ 1.28 a	1.291 $\pm$ 0.008 a	227.11 $\pm$ 2.85 a	58.80 $\pm$ 1.63 b	28.39 $\pm$ 0.69 c	95.96 $\pm$ 1.60 a	545.32 $\pm$ 2.31 a	15.22 $\pm$ 0.06 a	2.64 $\pm$ 0.06 b	1.11 $\pm$ 0.05 a
	B2	6.01 $\pm$ 0.08 b	47.97 $\pm$ 0.62 c	1.253 $\pm$ 0.014 b	217.47 $\pm$ 4.67 a	62.81 $\pm$ 1.03 a	35.89 $\pm$ 0.69 b	89.74 $\pm$ 1.73 a	250.89 $\pm$ 5.32 b	13.22 $\pm$ 0.11 b	2.50 $\pm$ 0.06 b	1.21 $\pm$ 0.05 a
Winter	CK	5.94 $\pm$ 0.13 a	44.56 $\pm$ 2.18 a	1.205 $\pm$ 0.024 b	180.49 $\pm$ 7.80 b	17.45 $\pm$ 0.52 b	51.82 $\pm$ 1.50 c	75.80 $\pm$ 1.62 a	269.82 $\pm$ 22.95 b	6.61 $\pm$ 0.64 c	1.53 $\pm$ 0.06 c	0.88 $\pm$ 0.07 c
	A1	5.76 $\pm$ 0.05 a	39.59 $\pm$ 2.00 b	1.233 $\pm$ 0.023 a	201.89 $\pm$ 1.85 a	19.23 $\pm$ 0.54 a	43.73 $\pm$ 0.97 e	65.94 $\pm$ 1.85 b	225.27 $\pm$ 19.19 c	9.88 $\pm$ 0.63 a	2.17 $\pm$ 0.10 a	1.24 $\pm$ 0.04 a
	A2	5.93 $\pm$ 0.09 a	36.06 $\pm$ 0.62 b	1.160 $\pm$ 0.024 b	192.27 $\pm$ 1.68 a	16.38 $\pm$ 0.46 b	48.43 $\pm$ 0.96 d	59.07 $\pm$ 1.85 c	185.37 $\pm$ 8.43 d	7.15 $\pm$ 0.39 b	2.11 $\pm$ 0.06 a	1.09 $\pm$ 0.06 b

Table 3. Cont.

Season	Treatment	pH	SOC (g kg ⁻¹)	TN (g kg ⁻¹)	AN (mg kg ⁻¹)	NH ₄ ⁺ -N (mg kg ⁻¹)	NO ₃ ⁻ -N (mg kg ⁻¹)	AP (mg kg ⁻¹)	AK (mg kg ⁻¹)	Invertase (mg g ⁻¹ d ⁻¹)	Amylase (mg g ⁻¹ d ⁻¹)	Urease (mg g ⁻¹ d ⁻¹)
Winter	B1	5.94 ± 0.07 a	45.59 ± 1.90 a	1.288 ± 0.020 a	197.87 ± 1.87 a	19.56 ± 0.96 a	76.01 ± 0.89 a	70.38 ± 1.76 b	338.55 ± 4.31 a	9.33 ± 0.22 a	2.27 ± 0.03 a	1.16 ± 0.02 a
	B2	5.92 ± 0.03 a	38.84 ± 0.93 b	1.209 ± 0.038 b	192.11 ± 1.88 a	16.79 ± 1.01 b	57.74 ± 0.68 b	65.94 ± 1.62 b	271.86 ± 5.32 b	7.96 ± 0.50 b	2.00 ± 0.09 b	1.22 ± 0.05 a

Different lowercase letters within a phase indicate significant differences among treatments (Tukey HSD, $\alpha = 0.05$). Cell shading is scaled independently within each phase and each column (light = lowest mean; dark = highest mean) and is intended only as a visual guide; statistical differences are indicated by lowercase letters. The three field sites were homogenized before analysis. The standard deviation is among analytical replicates of that composite, not among sites.

Community composition was tested by PERMANOVA on Bray–Curtis distances (999 permutations). Each term used the permutation scheme that matches its experimental unit. Treatment is a between-column factor. It was tested on the column centroid, the mean relative abundance of the four phases from each column ($n = 15$ columns). Treatment labels were permuted freely among columns. Phase is a within-column factor. It was tested on the full sample table. The phase and treatment main effects were fitted first and removed. The interaction was then tested by permuting phase labels within columns on the residuals, so that a phase main effect is not carried into the interaction statistic. Within-phase treatment tests are simple effects. Within one phase, the three columns are independent, and treatment labels were permuted freely among those columns. PERMDISP was run on the same distances for phase, for treatment, and for treatment within each phase. A PERMANOVA term was read as a difference in centroid only when the matching PERMDISP test was not significant. Figures were prepared in R (version 4.3) and Origin (version 10.0).

3. Results

3.1. Soil Chemical Properties and Enzyme Activities

Repeated-measures ANOVA, with column as the experimental unit, detected significant effects of phase, treatment and the phase $\times$ treatment interaction for every measured soil property and potential enzyme activity (phase $F_{3,30} = 20.56$ – 5843.09 ; treatment $F_{4,10} = 13.74$ – 644.66 ; interaction $F_{12,30} = 6.03$ – 252.23 ; all Greenhouse–Geisser $p < 0.01$; Table S1). Sphericity was not met (Greenhouse–Geisser $\epsilon = 0.54$ – 0.87).

Soil pH was lowest under straw without mineral N (CK) in spring (5.49 ± 0.09), summer (5.68 ± 0.07) and autumn (5.92 ± 0.03) and was significantly higher under ammonium sulfate (A2) and both nitrate treatments (B1, B2) in spring and under A1, A2 and B1 in summer and autumn. No treatment difference in pH was detected in winter (Table 3).

SOC was higher in summer and autumn than in spring. In summer, SOC under A2 and B2 was lower than under CK and B1. In autumn, A1 and B1 had the highest SOC and B2 the lowest. In winter, CK and B1 retained more SOC than A1, A2 and B2. TN was consistently reduced under A2 relative to B1. The highest TN occurred under B1 in summer (1.439 ± 0.016 g kg⁻¹) and winter (1.288 ± 0.020 g kg⁻¹).

Available N and inorganic N varied strongly with phase. AN was highest under full-rate ammonium sulfate (A1) in spring and summer and under B1 and B2 in autumn. NH₄⁺-N increased from spring (25.3 – 32.2 mg kg⁻¹) to summer (60.0 – 83.8 mg kg⁻¹) and declined to 16.4 – 19.6 mg kg⁻¹ in winter; A1 had the highest NH₄⁺-N in summer. NO₃⁻-N showed the opposite seasonal pattern: values were lowest under A2 and B2 in spring (12.04 and 11.80 mg kg⁻¹) and highest in winter, particularly under B1 (76.01 ± 0.89 mg kg⁻¹).

AP was highest under CK and B2 in spring and lowest under A2 in winter. AK was highest under B1 in all phases, with a marked peak in autumn ($545.32 \pm 2.31 \text{ mg kg}^{-1}$). AK was highest under B1 in all phases, with a marked peak in autumn. Because B1 received KNO_3 , this increase includes fertilizer-derived K and is not interpreted as K released from soil or straw. Invertase activity peaked in autumn (up to $15.22 \text{ mg g}^{-1} \text{ d}^{-1}$ under B1) and was highest under A2 in summer. Amylase was highest under B1 in spring and summer. Urease was lower in summer than in the other phases and was reduced under B1 in spring and under A1 in summer.

Collectively, CK soils were more acidic and often richer in SOC in spring, A2 was associated with lower TN and spring NO_3^- -N, and B1 was associated with elevated TN, AK and, in winter, NO_3^- -N. Potential invertase and urease activities under assay conditions were highest in autumn and lowest in summer or winter, depending on treatment. These chemical and enzymatic measurements describe soil status at sampling; they are not rates of denitrification.

3.2. Alpha-Diversity of the nirS-Type Community

Repeated-measures ANOVA, with column as the experimental unit, detected a phase effect and a phase $\times$ treatment interaction for Chao1 (phase $F_{3,30} = 30.78$, $p < 0.01$; interaction $F_{12,30} = 3.98$, $p < 0.01$), observed ASVs ($F_{3,30} = 35.52$, $p < 0.01$; $F_{12,30} = 4.44$, $p < 0.01$), Shannon ($F_{3,30} = 6.06$, $p = 0.002$; $F_{12,30} = 3.50$, $p < 0.01$) and Simpson (interaction $F_{12,30} = 3.48$, $p < 0.01$) (Table S2). The treatment main effect was not significant for any index (all $p > 0.08$). Sphericity was not met (Greenhouse–Geisser $\epsilon = 0.55$ – 0.77); the phase and interaction terms remained significant after the Greenhouse–Geisser correction, except the phase main effect for Simpson.

Richness was lowest in spring. Chao1 values ranged from 458.6 to 719.8 in spring, compared with 600.0–909.9 in summer, 541.3–877.4 in autumn, and 717.0–913.2 in winter. Observed species richness displayed the same seasonal ranking. Pairwise comparisons revealed phase-specific treatment effects on richness. In spring, Chao1 was significantly lower under A1 (458.6 ± 63.2) than under CK, A2, B1 and B2, and observed species richness was significantly higher under B1 and B2 than under CK, A1 and A2. In autumn, observed species richness under A1 (467.3 ± 59.3) was significantly lower than under the remaining treatments. No significant treatment differences in Chao1 or observed species richness were detected in summer or winter. Shannon and Simpson diversities were reduced by A1 only in spring (Shannon: 4.434 ± 0.857 ; Simpson: 0.855 ± 0.075), when both indices were significantly lower than those of CK, A2, B1 and B2. Treatment means did not differ within summer, autumn or winter following Tukey adjustment. Diversity under A1 increased from spring to winter (Shannon: 4.434 to 5.710; Simpson: 0.855 to 0.933), whereas the other treatments fluctuated within a narrower seasonal range. Overall, *nirS* richness in the DNA library was lowest in spring and was most consistently lower under A1, whereas evenness indices differed among treatments only after the 5 °C phase (Table 4). These indices describe membership of the sequenced *nirS* assemblage, not denitrification activity.

Table 4. α -diversity of soil *nirS*-type communities under different treatments.

	Treatment	Chao1	Observed Species	Shannon	Simpson
Spring	CK	$567.3 \pm 85.7 \text{ a}$	$471.0 \pm 78.4 \text{ b}$	$5.244 \pm 0.133 \text{ a}$	$0.917 \pm 0.015 \text{ a}$
	A1	$458.6 \pm 63.2 \text{ b}$	$399.8 \pm 55.0 \text{ b}$	$4.434 \pm 0.857 \text{ b}$	$0.855 \pm 0.075 \text{ b}$
	A2	$559.0 \pm 86.0 \text{ a}$	$478.3 \pm 64.2 \text{ b}$	$5.471 \pm 0.252 \text{ a}$	$0.940 \pm 0.009 \text{ a}$
	B1	$681.3 \pm 101.2 \text{ a}$	$550.9 \pm 57.0 \text{ a}$	$5.131 \pm 0.251 \text{ a}$	$0.911 \pm 0.018 \text{ a}$
	B2	$719.8 \pm 93.3 \text{ a}$	$579.6 \pm 63.8 \text{ a}$	$4.981 \pm 0.481 \text{ a}$	$0.898 \pm 0.043 \text{ a}$

Table 4. Cont.

	Treatment	Chao1	Observed Species	Shannon	Simpson
Summer	CK	858.5 ± 104.2 a	685.4 ± 83.2 a	5.603 ± 0.141 a	0.930 ± 0.010 a
	A1	600.0 ± 164.6 a	515.2 ± 135.7 a	5.152 ± 0.251 a	0.904 ± 0.031 a
	A2	794.5 ± 100.0 a	663.9 ± 84.7 a	4.934 ± 0.265 a	0.870 ± 0.012 a
	B1	909.9 ± 216.6 a	769.3 ± 146.5 a	5.626 ± 0.400 a	0.929 ± 0.011 a
	B2	821.4 ± 129.3 a	680.3 ± 99.4 a	5.276 ± 0.018 a	0.910 ± 0.002 a
Autumn	CK	841.1 ± 175.3 a	700.4 ± 140.4 a	5.715 ± 0.180 a	0.941 ± 0.008 a
	A1	541.3 ± 63.0 a	467.3 ± 59.3 b	5.001 ± 0.087 a	0.878 ± 0.007 a
	A2	777.2 ± 122.4 a	625.4 ± 73.6 a	5.225 ± 0.162 a	0.902 ± 0.017 a
	B1	877.4 ± 88.7 a	711.2 ± 73.6 a	5.366 ± 0.286 a	0.914 ± 0.015 a
	B2	791.0 ± 109.3 a	667.7 ± 98.6 a	5.619 ± 0.398 a	0.928 ± 0.019 a
Winter	CK	717.0 ± 80.0 a	575.8 ± 59.4 a	5.318 ± 0.086 a	0.932 ± 0.003 a
	A1	840.1 ± 151.6 a	699.8 ± 139.9 a	5.710 ± 0.431 a	0.933 ± 0.012 a
	A2	913.2 ± 129.3 a	738.4 ± 77.0 a	5.830 ± 0.146 a	0.942 ± 0.005 a
	B1	794.7 ± 127.9 a	653.5 ± 86.8 a	5.270 ± 0.115 a	0.919 ± 0.013 a
	B2	911.2 ± 141.9 a	736.7 ± 103.2 a	5.334 ± 0.364 a	0.902 ± 0.021 a

Different lowercase letters within a phase indicate significant differences among treatments (Tukey HSD, $\alpha = 0.05$).

3.3. Beta-Diversity of the nirS-Type Community

PCoA plots of soil denitrifying bacterial communities were generated separately for each season (Figure 1). The first two principal coordinates accounted for 68.0% of the variation in spring (PCo1 57.0%, PCo2 11.0%), 70.5% in summer (55.9% and 14.6%), 65.5% in autumn (54.8% and 10.7%) and 54.9% in winter (32.9% and 22.0%). PCoA showed treatment-related separation after the 5, 15 and -15°C phases. After the 20°C phase, the treatments occupied different regions of the ordination, but this separation was not significant ($p = 0.060$). The strongest visual separation of A2 was after the 5°C phase.

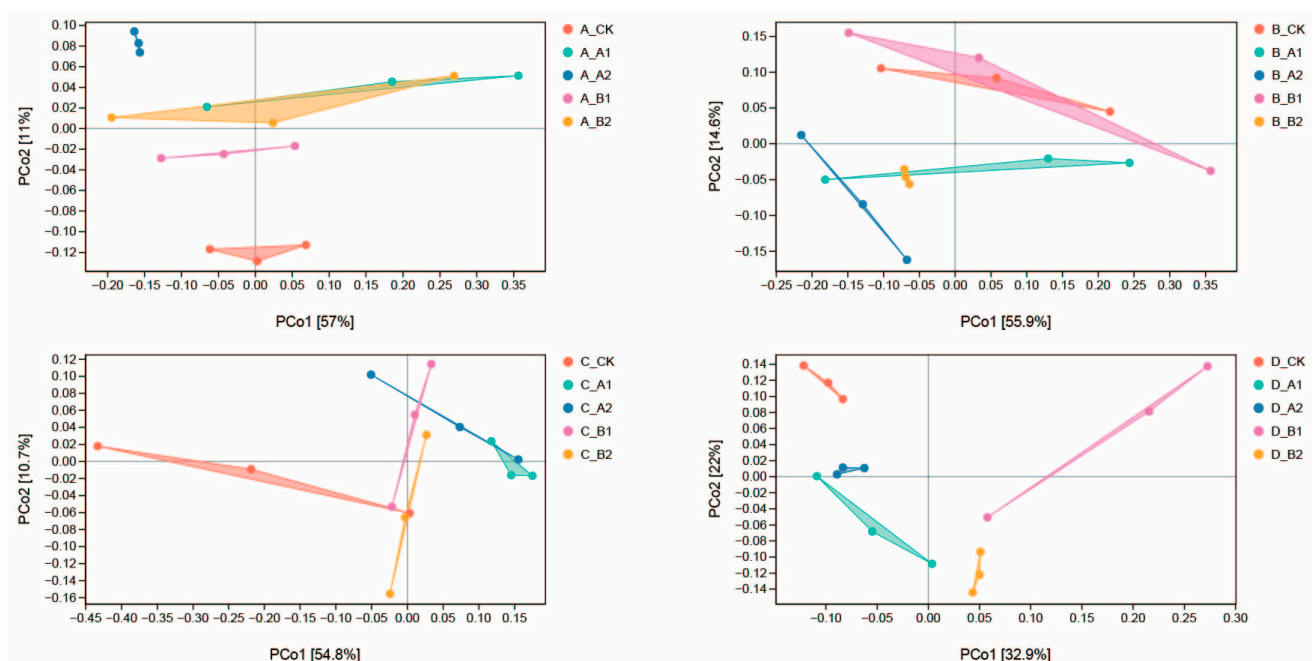


Figure 1. β -Diversity of soil *nirS*-type communities under different treatments. Treatment separation was significant after the 5, 15 and -15°C phases ($p = 0.042$, 0.001 and 0.001) and not after the 20°C phase ($p = 0.060$).

PERMANOVA with permutations matched to the experimental unit detected a treatment effect (pseudo- $F = 2.94$, $R^2 = 0.541$, $p < 0.01$; 15 column centroids, unrestricted among columns), a phase effect (pseudo- $F = 2.85$, $R^2 = 0.133$, $p = 0.001$; permutations within column) and a phase $\times$ treatment interaction (pseudo- $F = 2.04$, $R^2 = 0.337$, $p < 0.01$; phase labels permuted within column; 999 permutations). Within-phase tests, for which the three columns are independent, separated treatments after the 5 °C phase (pseudo- $F = 2.31$, $R^2 = 0.480$, $p = 0.042$), the 15 °C phase (pseudo- $F = 3.21$, $R^2 = 0.562$, $p = 0.001$) and the −15 °C phase (pseudo- $F = 4.30$, $R^2 = 0.632$, $p = 0.001$), but not after the 20 °C phase (pseudo- $F = 1.96$, $R^2 = 0.440$, $p = 0.060$).

3.4. Soil Taxonomic Composition of the nirS-Type Community of Different Treatments

The *nirS* libraries were dominated by *Pseudomonadota* at the phylum level in all phases and treatments, accounting for the great majority of sequences; *Chloroflexota*, candidate division NC10, *Actinomycetota*, and other phyla were present only at low relative abundance (Figure 2).

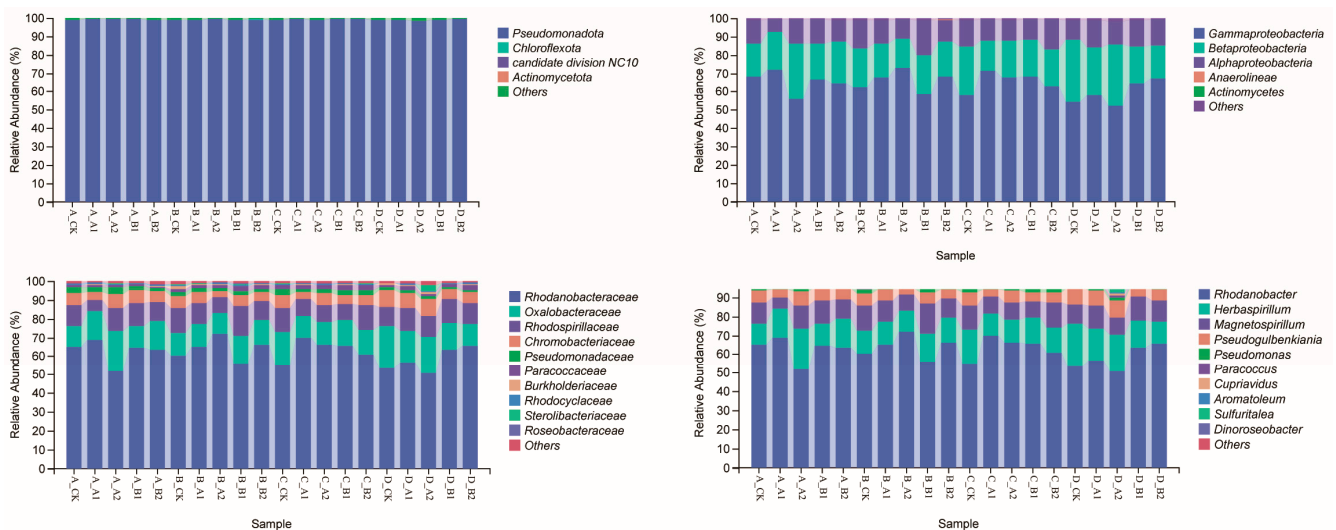


Figure 2. Relative abundance of *nirS*-type taxa at the phylum level under the five treatments after the 5 °C, 20 °C, 15 °C and −15 °C phases. Bars are means of $n = 3$ columns per treatment.

At the class level, *Gammaproteobacteria* were the most abundant taxon, followed by *Betaproteobacteria*. *Alphaproteobacteria*, *Anaerolineae* and *Actinomycetes* each contributed a smaller fraction of the community. The relative abundance of *Gammaproteobacteria* declined, and that of *Betaproteobacteria* increased, under A2 in spring and under several winter treatments, particularly A1 and A2. Family-level profiles were consistent with this pattern. *Rhodanobacteraceae* predominated in most samples, with *Oxalobacteraceae* as the second most abundant family. The relative abundance of *Rhodanobacteraceae* was reduced, and that of *Oxalobacteraceae* was elevated, under A2 in spring and under CK, A1 and A2 in winter. These are proportional changes within the sequenced *nirS* library. Remaining families, including *Rhodospirillaceae*, *Chromobacteriaceae* and *Pseudomonadaceae*, together constituted a minor and relatively stable fraction of the community. The same structure was recovered at the genus level. *Rhodanobacter* was the dominant genus across phases, followed by *Herbaspirillum*, *Magnetospirillum* and *Pseudogulbenkiania*. *Rhodanobacter* decreased and *Herbaspirillum* increased under spring A2 and under winter CK, A1 and A2. Other genera (*Pseudomonas*, *Paracoccus*, *Cupriavidus*, *Aromatoleum*, *Sulfuritalea* and *Dinoroseobacter*) remained at low relative abundance and showed no consistent treatment-related pattern. Collectively, taxonomic composition was stable at the phylum level but shifted at finer ranks, mainly through

Standardized relative abundances of 20 predominant bacterial species were compared across phases and treatments (Figure 4). The selected taxa were almost exclusively *Pseudomonadota* and included several putative *nirS*-harbouring lineages (*Rhodanobacter*, *Herbaspirillum*, *Pseudogulbenkiania*, *Cupriavidus necator*, *Thauera mechernichensis*, *Aromatoleum tolulyticum*, *Sulfuritalea hydrogenivorans*, *Dechlorospirillum*, *Magnetospirillum*, *Paracoccus*, *Pseudomonas* and *Dinoroseobacter shibae*). Hierarchical clustering separated the species into two main groups that differed in their seasonal response.

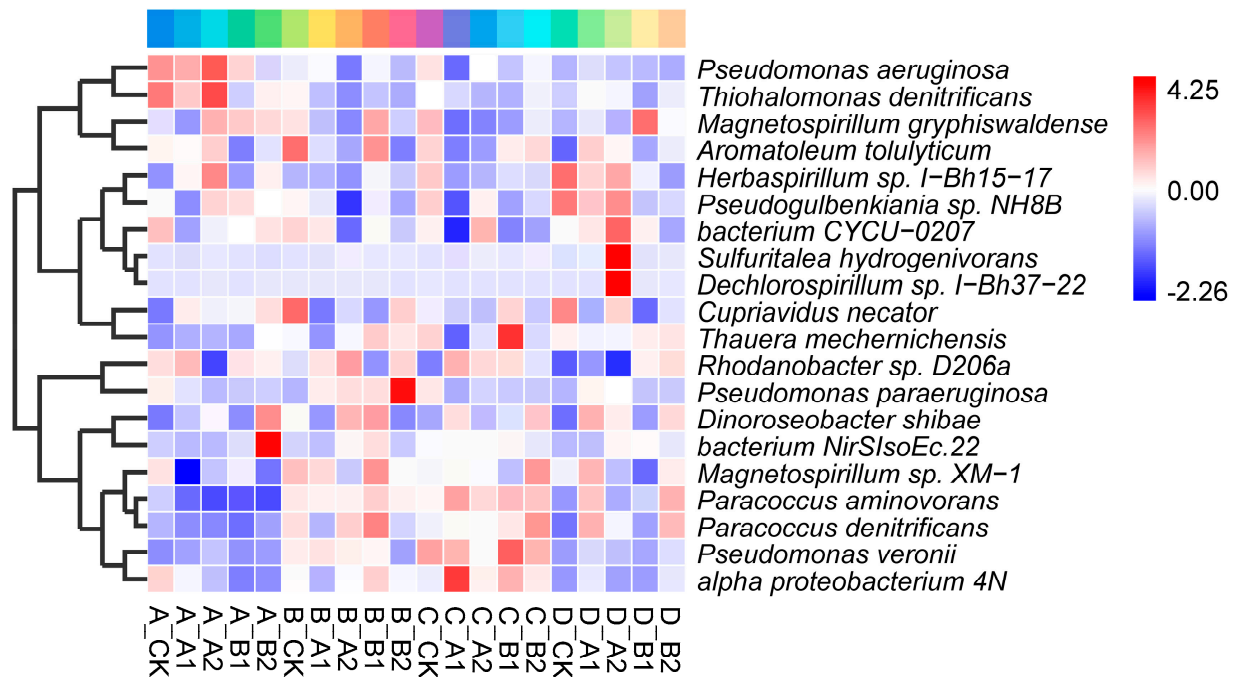


Figure 4. Heatmap of genus-level relative abundance in the *nirS*-type community. Note: Columns are samples and rows are genera. The upper annotation bar denotes phase: pink, 5 °C (A); olive, 20 °C (B); green, 15 °C (C); blue, −15 °C (D). The lower annotation bar denotes treatment: dark grey, CK; red, A1; orange, A2; yellow, B1; pale green, B2. Cell colour is the row-wise z-score of relative abundance (blue, below the genus mean; red, above it). Values are proportions of the sequenced library.

Abundance profiles were more strongly structured by season than by a uniform treatment effect. Several taxa showed elevated standardized abundance under A2 in spring, consistent with the enrichment of *Burkholderiales*-affiliated lineages detected by LEfSe. In summer and autumn, most species remained near the mean standardized value, with only sporadic treatment-specific peaks. Winter samples displayed the greatest contrast among treatments: *Dinoroseobacter shibae* and related taxa were higher under A1, *Sulfuritalea hydrogenivorans* and allied lineages were higher under A2, and selected actinobacterial and betaproteobacterial species were higher under B2, matching the winter LEfSe biomarkers.

Rhodanobacter, the dominant genus in the community-level profiles, did not exhibit a single treatment of consistent enrichment across all phases. Treatment-associated increases in relative abundance were instead concentrated in co-occurring denitrifying genera (*Herbaspirillum*, *Cupriavidus*, *Dechlorospirillum*, *Thauera* and *Sulfuritalea*), and the identity of the enriched species changed with phase.

3.5. Indicator Species of Soil Denitrifying Bacterial Community Composition of Different Treatments

LEfSe (LDA score > 2) was used to identify treatment-associated taxa within each season (Figure 5). Each class comprised $n = 3$ biological replicates. Discriminant clades

were detected for only a subset of treatments, and the identity of biomarkers changed with season. With this replication and with five treatments compared within each phase, the calls are sensitive to single samples and are not treated as confirmed indicators.

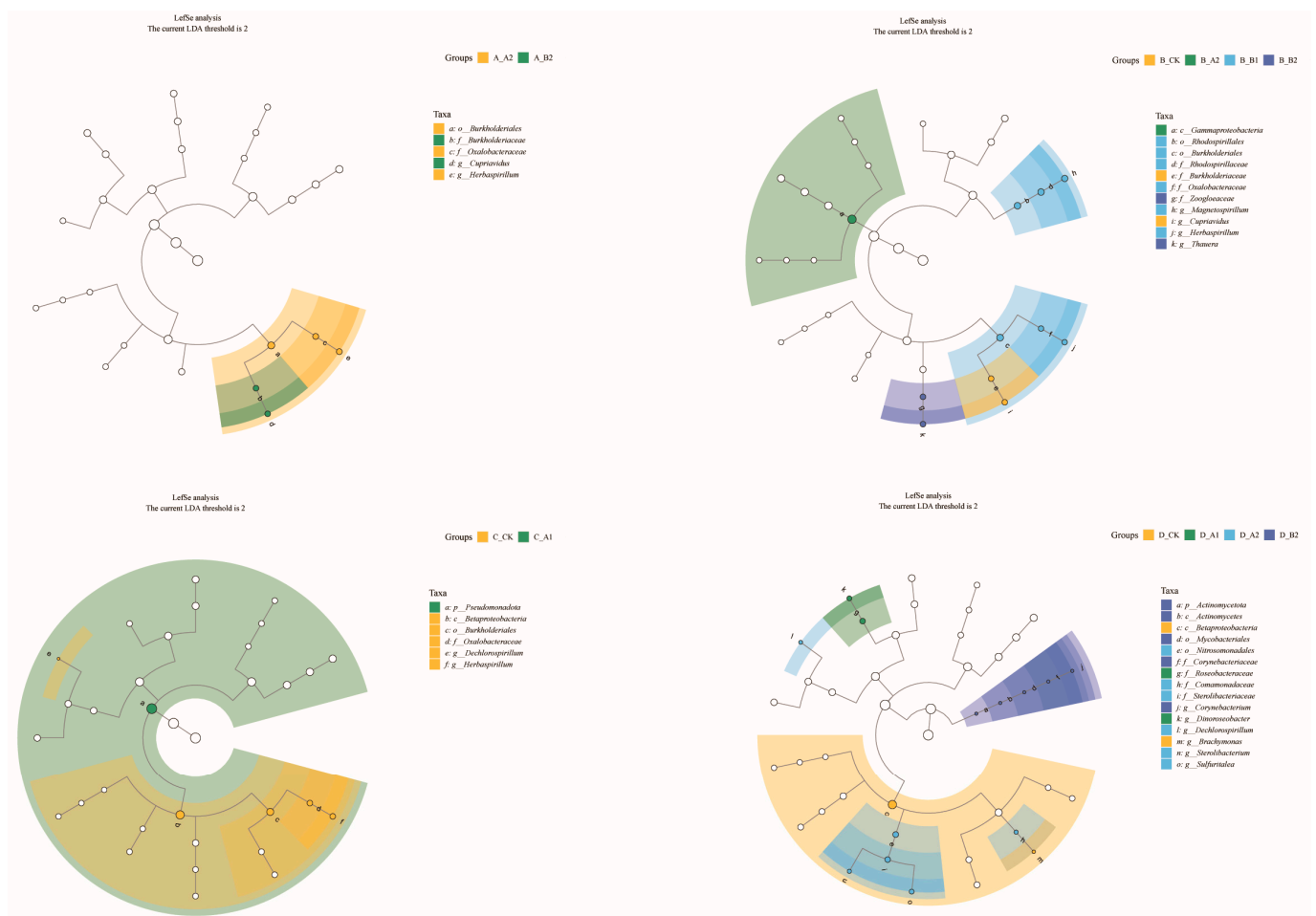


Figure 5. LEfSe cladograms of treatment-associated taxa in the *nirS*-type community under different treatments.

In spring, treatment-associated taxa were recovered only for A2 and B2. A2 was associated with a higher relative abundance of *Burkholderiales*, *Burkholderiaceae*, *Cupriavidus* and *Herbaspirillum*, whereas B2 was enriched in *Oxalobacteraceae*. In summer, four treatments were distinguished. A2 was associated with *Gammaproteobacteria*. CK was enriched in *Burkholderiaceae* and *Cupriavidus*. B1 was enriched in *Rhodospirillales*, *Burkholderiales*, *Rhodospirillaceae*, *Oxalobacteraceae*, *Magnetospirillum* and *Herbaspirillum*. B2 was enriched in *Zoogloeaceae* and *Thauera*. In autumn, biomarkers were confined to CK and A1. A1 was associated with *Pseudomonadota* and *Betaproteobacteria*. CK was enriched in *Burkholderiales*, *Oxalobacteraceae*, *Dechlorospirillum* and *Herbaspirillum*. In winter, a broader set of lineages was discriminative. CK was associated with *Betaproteobacteria*, *Comamonadaceae*, *Dechlorospirillum* and *Brachymonas*. A1 was enriched in *Roseobacteraceae* and *Dinoroseobacter*. A2 was enriched in *Sterolibacteriaceae*, *Sterolibacterium* and *Sulfuritalea*. B2 was enriched in *Actinomycetota*, *Actinomycetes*, *Mycobacteriales*, *Nitrosomonadales*, *Corynebacteriaceae* and *Corynebacterium*.

Across seasons, *Burkholderiales* and related genera (*Herbaspirillum*, *Cupriavidus*, *Dechlorospirillum*) recurred as treatment-associated taxa, but the treatment to which they were assigned was not constant. Winter was the only season in which *Actinomycetota* and several alphaproteobacterial lineages were identified as treatment-specific indicators.

3.6. Effects of Soil Physicochemical Properties on Soil nirS-Type Community Structure of Different Treatments

All candidate predictors had VIF < 10; none was removed for collinearity. Redundancy analysis was used to describe covariation between related *nirS* community structure and soil chemical properties and enzyme activities within each season (Figure 6). The first two RDA axes explained 41.8% of the constrained variation in spring (26.8% and 15.0%), 51.3% in summer (36.0% and 15.3%), 52.4% in autumn (39.7% and 12.8%) and 47.1% in winter (28.9% and 18.1%). Monte Carlo permutation tests indicated that the environmental model was not significant in spring ($p = 0.592$) but was significant in summer ($p = 0.014$), autumn ($p = 0.010$) and winter ($p = 0.036$).

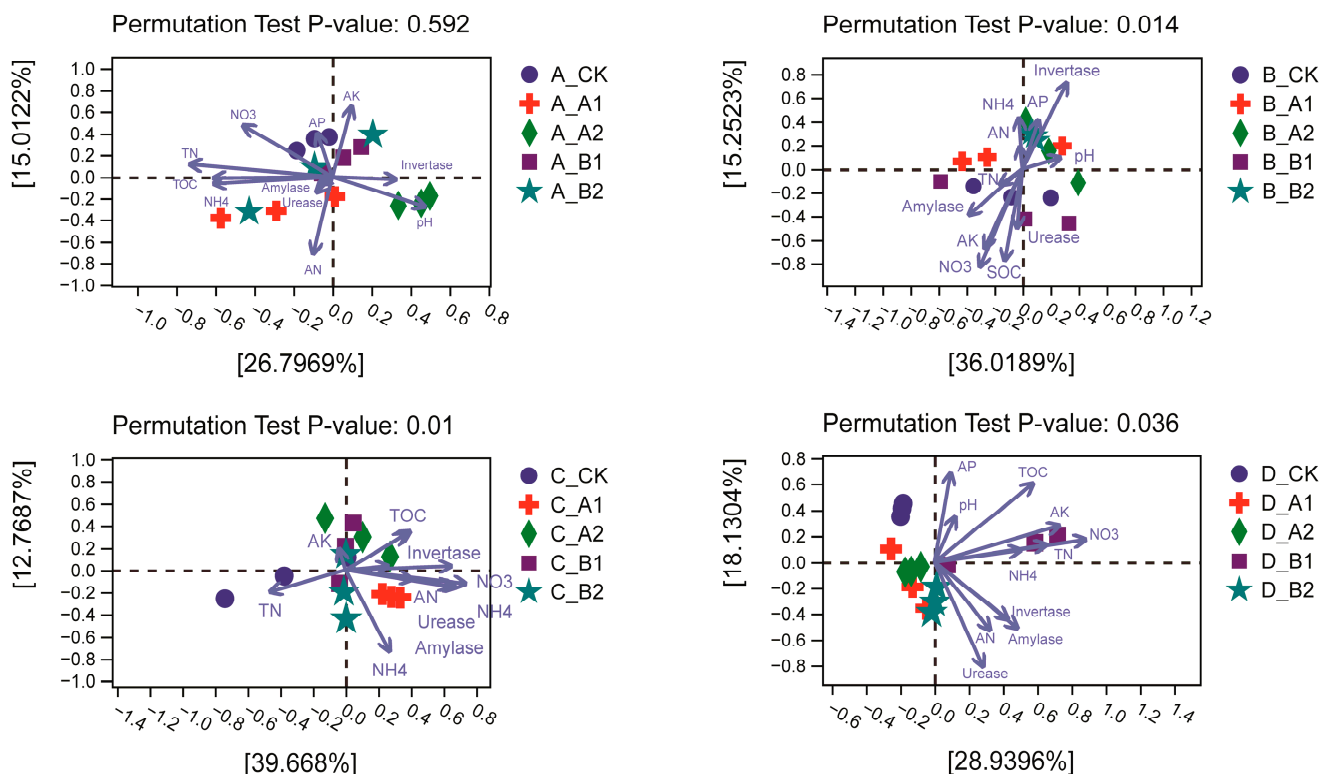


Figure 6. RDA analysis of soil denitrifying bacterial communities and soil physicochemical properties under different treatments.

In spring, samples were weakly ordered along RDA1. A2 was associated with higher pH and invertase activity, whereas A1 and B2 lay toward higher TN, TOC and NH₄⁺-N; CK and B1 occupied intermediate positions. Because the permutation test was not significant, these alignments should be interpreted with caution.

In summer, invertase, AP, NH₄⁺-N and pH pointed toward the positive RDA2 axis, whereas TN, TOC, NO₃⁻-N, AK and urease pointed toward negative RDA2. A1 was displaced toward the upper-left quadrant, and B1 toward negative RDA2.

In autumn, invertase, urease, AN, TOC and NO₃⁻-N aligned with positive RDA1, TN with negative RDA1, and NH₄⁺-N with negative RDA2. CK was separated on the negative RDA1 axis, whereas A1 clustered with invertase, urease and AN on the positive side.

In winter, most samples were concentrated on the negative RDA1 axis. AP and pH pointed upward, TOC, AK, TN, NO₃⁻-N and NH₄⁺-N toward positive RDA1, and invertase, amylase, AN and urease downward. Treatment groups were therefore less clearly partitioned along the measured gradients than in summer and autumn, although the overall constrained model remained significant.

Taken together, soil nutrients and potential enzyme activities therefore covaried with *nirS* community structure in summer, autumn and winter, whereas no significant environment–community coupling was detected in spring. Invertase and nitrogen-related variables (TN, NH_4^+ -N, NO_3^- -N, AN and urease) were the most recurrent correlates, but the treatment that aligned with a given vector changed with season.

4. Discussion

4.1. Seasonal Temperature as the Primary Filter of the *nirS* Community

Because the same columns were opened four times, part of the later-phase pattern may reflect cumulative sampling disturbance as well as temperature and incubation time. The experiment demonstrates temporal change across a sequential temperature regime; it cannot isolate temperature from incubation history. Season is used as a shorthand for this combined thermal-temporal filter. The design isolates four constant thermal regimes in sequence. The four thermal phases were imposed sequentially on the same soil–straw system. Spring, summer, autumn and winter therefore represent 60, 120, 180 and 240 d of incubation, successive stages of straw decomposition and fertilizer transformation, and cumulative exposure to preceding temperatures, as well as the set-point temperature of each phase.

Richness of the DNA-detected *nirS* library was lowest after the 5 °C phase. This is not interpreted as a temperature effect alone. That sampling date was also the earliest point after sieving, homogenization, cold storage, straw addition and fertilizer addition. Succession after this disturbance, substrate release from the straw, and a fertilizer shock are confounded with the 5 °C set-point and with the short incubation time (60 d). A reversed or parallel temperature series would be required to separate these factors. The spring-phase reduction under full-rate ammonium sulfate is therefore associated with the earliest post-amendment stage, not shown to be caused by 5 °C.

Denitrification is carried out by taxonomically scattered bacteria whose nitrite reductases are encoded by *nirS* or *nirK* [1,2]. The present study sequenced only *nirS* and did not measure denitrification potential, N_2O or *nirS* transcript abundance. Interpretation is therefore restricted to the structure of the DNA-detected *nirS*-type assemblage. Observed ASVs and Chao1 were lowest after the 5 °C phase and higher toward the end of the sequence, and ASVs shared by all five treatments rose from 327 in spring to 454 in winter. The winter increase is a property of the DNA-detected *nirS*-type community. Extracted DNA does not distinguish active, dormant, and recently dead cells, and no RNA or viability assay was used. A higher ASV count after −15 °C is therefore not evidence of a more active denitrifier assemblage. It is also compatible with a decline in the relative abundance of *Rhodanobacter*, which would leave a larger share of reads for rarer variants. Phase, rather than N form or rate, was the stronger organizer of *nirS* richness: treatment differences in Shannon and Simpson diversities were confined to the 5 °C phase. N form and rate were associated with shifts in community composition within phases, and those shifts were not the same after each phase. Observed ASVs and Chao1 were lowest in spring and increased toward winter, and the number of ASVs shared by all five treatments rose from 327 in spring to 454 in winter. Shannon and Simpson indices responded to N addition only in spring. These patterns are consistent with field surveys in the Sanjiang Plain and in neighbouring Mollisols, where *nirS* abundance and composition change between autumn and spring [13,14], and with laboratory work showing that temperatures near freezing reorganize denitrifier assemblages and their activity [11]. Freeze–thaw and low-temperature periods in North-east China also alter bulk bacterial communities and N-cycling potential [12,24]. Because moisture and straw mass were held constant, the seasonal trajectory observed here can be

attributed mainly to temperature and to the substrates released or immobilized as the same soil passed from 5 °C through 20 °C and 15 °C into frost.

The contrast with Yu [14], who found land use to outweigh season in a field Mollisol, is informative. In the field, temperature covaries with crop growth, rainfall and residue quality. Isolating temperature in sequential microcosms made the seasonal filter visible: α -diversity was constrained in spring, when the soil was cold but unfrozen, and the core *nirS* assemblage expanded as the cycle proceeded. Winter did not collapse richness. Instead, *Actinomycetota* appeared as LEfSe indicators only at −15 °C, a pattern compatible with the relative persistence of Gram-positive lineages during frozen or near-frozen periods [24].

4.2. N Form and Rate Modify *nirS* Structure Rather than a Measured Denitrification Rate

All treatments received the same maize straw. The experimental contrast was therefore the presence, form and rate of mineral N: straw without mineral N (CK), straw plus full- or half-rate ammonium sulfate (A1 and A2, respectively) and straw plus full- or half-rate potassium nitrate (B1 and B2, respectively). Treatment effects on α -diversity were confined to spring, when full-rate ammonium sulfate (A1) reduced Shannon and Simpson indices relative to CK, reduced ammonium sulfate (A2) and both nitrate treatments (B1, B2). Full-rate ammonium sulfate was therefore associated with a spring-phase reduction in evenness of the *nirS* library, not with a general suppression of denitrification. A1/A2 received $(\text{NH}_4)_2\text{SO}_4$ and B1/B2 received KNO_3 . Treatments therefore differ in counterion (SO_4^{2-} versus K^+) as well as in NH_4^+ versus NO_3^- . The higher available K under B1, including the autumn peak, is expected from the K added with KNO_3 and is not evidence of a treatment effect on soil K supply. Differences between the A and B series cannot be attributed to N form alone; SO_4^{2-} input, K^+ input, and ionic strength are confounded with the N source. Full- versus half-rate contrasts are likewise confounded with the amount of counterion added.

The sensitivity of soil microbial communities to fertilizer regime is consistent with evidence from long-term fertilization experiments showing that fertilizer type and application intensity can modify microbial diversity and functional profiles through concomitant changes in the soil chemical environment [25]. That evidence is consistent with the phase-dependent chemical differences observed here. It does not, however, identify which *nirS*-type taxa respond, and the present contrasts remain associations between N addition and *nirS* relative composition. In summer, autumn, and winter, the same N treatments left richness and evenness statistically unchanged, although PCoA still separated treatments. Composition, not α -diversity, was the more sensitive response once temperatures rose above the spring regime. This season-conditional response helps to reconcile reports that mineral N and organic amendments sometimes restructure *nirS* communities and sometimes do not [7,14–16]. Sampling date is not a nuisance variable. An ammonium effect that is clear at 5 °C can be invisible at 15–20 °C. Reduced ammonium (A2) and nitrate (B1, B2) did not reproduce the spring diversity loss seen under A1, indicating that the suppression was linked to the combination of a high ammonium load and low temperature rather than to straw C or to N addition per se. A1 versus A2, and B1 versus B2, are rate comparisons, but the microbial differences cannot be attributed to fertilizer intensity alone. The full rate supplied approximately twice the mineral N of the half rate and, with it, twice the counterion. That package changes inorganic N availability, the potential for acidification under ammonium sulfate, ionic strength, and the C:N balance against a fixed straw input. The lower Shannon and Simpson diversities under A1 in spring, relative to A2, are therefore associated with the full ammonium-sulfate addition as a whole, not with a separable effect of rate. The same limit applies to every A1-A2 and B1-B2 contrast in community composition.

Field studies often report that straw return increases *nirS* abundance and shifts denitrifier composition relative to straw removal [13,15,16]. That contrast is absent here. Every column, including CK, received the same maize straw in a mesh bag, so a straw effect cannot be estimated and disagreement with those studies on the size of a straw response is expected. Long-term field trials also differ in kind. Chen et al. [7] found that chemical fertilizer altered *nirK*-type composition more than *nirS*-type composition, and other work has found *nirS* to be the more responsive marker [26]. The present data set contains only *nirS* amplicons from a 240 d sequence, not *nirK* gene copies from a multi-year field. Within that limit, N form and rate did not impose one community across all phases: full-rate ammonium sulfate was associated with lower Shannon and Simpson diversities only after the 5 °C phase, and the treatment assigned to *Burkholderiales* by LEfSe changed from phase to phase. Surveys in the Sanjiang Plain likewise show *nirS* membership changing between autumn and spring [13,14], but those samples come from independent field dates, whereas the phases here are the same soil at 60, 120, 180 and 240 d. The shared pattern is a strong temporal signal. The point of difference is that this signal cannot be assigned to temperature, and that relative composition is not the abundance those field studies measured by qPCR.

4.3. Reciprocal Shifts Between *Rhodanobacter* and *Burkholderiales*

Pseudomonadota dominated the *nirS* libraries in every season, as is typical when cd3aF/R3cd primers are used on agricultural soils [21,27]. At the genus level, the community was organized around a persistent *Rhodanobacter* core and a seasonally expanding *Burkholderiales* fraction (*Herbaspirillum*, *Cupriavidus* and related taxa). *Rhodanobacter* is repeatedly recovered as a dominant denitrifier in acidic, nitrate-bearing soils and can complete denitrification at pH values near 4 [17,28]. The baseline pH of the Jiamusi composite (5.23) and the still-acid CK soils in spring lie within that niche, which accounts for the numerical dominance of *Rhodanobacter* under most treatments. The same genus is a major *nirS* taxon in N-fertilized soils of semi-arid Northeast China [27].

The exception was reduced ammonium sulfate (A2) in spring, and to a lesser extent CK, A1 and A2 in winter, when *Herbaspirillum* increased and *Rhodanobacter* declined. Amplicon sequencing reports composition, not population size. Without *nirS* qPCR, a higher relative abundance of *Herbaspirillum* does not establish that its absolute abundance increased; it may equally reflect a decline in other members of the *nirS* library, including *Rhodanobacter*. The same caveat applies to every treatment-associated taxon identified by LEfSe. LEfSe assigned *Burkholderiales* biomarkers to different treatments in different seasons, so the replacement was not a fixed property of one N source. *Herbaspirillum* and *Cupriavidus* occupy a broader redox and substrate range than acid-specialist *Rhodanobacter*, and several strains carry *nirS* [18]. A modest rise in pH under fertilized treatments, together with straw-derived C, is a plausible local explanation for the A phase accounting for a larger proportion of *nirS* reads of *Burkholderiales* under reduced ammonium. The winter recurrence of the same genera under CK and A2 suggests that frost selects a second, overlapping set of *Burkholderiales* rather than simply reversing the spring pattern. LEfSe assignments are descriptive. A taxon associated with A2 in spring and with a different treatment in winter is not evidence that the genus responded mechanistically to that fertilizer.

4.4. Soil C, N and Enzymes as Seasonal Correlates of Community Structure

CK remained more acidic than the fertilized treatments except in winter. A2 lowered TN and spring NO_3^- -N. B1 raised TN, available K and winter NO_3^- -N. NH_4^+ -N peaked in summer and fell to its lowest values in winter, whereas NO_3^- -N accumulated under frost, especially with full-rate KNO_3 . Invertase activity was highest in autumn. These

chemical trajectories match the expected behaviour of a straw-amended soil that is first warmed, then cooled: ammonification and nitrification are favoured at 15–20 °C, while nitrate left unused at −15 °C remains in the extractable pool [11,12]. NO_3^- -N was lowest after the 5 °C phase, particularly under A2 and B2 (12.04 ± 0.58 and 11.80 ± 0.60 mg kg^{−1}), and highest after the −15 °C phase, when B1 reached 76.01 ± 0.89 mg kg^{−1} and the other treatments ranged from 43.73 to 57.74 mg kg^{−1} (Table 3). This seasonal ranking should not be read as nitrification during the frozen phase. At −15 °C, most soil water would have been frozen, substrate diffusion would have been restricted, and autotrophic nitrification is not expected to proceed at a measurable rate. Columns were removed from the incubator and thawed at 4 °C before KCl extraction, so the winter values describe the post-thaw state rather than the soil at −15 °C. Nitrate already present at the end of the 15 °C phase, nitrate released from cells or organic N during freezing, and nitrate produced after thaw cannot be separated with the present sampling. The high post-thaw NO_3^- -N under B1 is therefore reported as an observation on thawed soil. It is consistent with the KNO_3 addition, and it is not evidence that nitrification continued at −15 °C.

RDA linked community structure to invertase and to N-related variables (TN, NH_4^+ -N, NO_3^- -N, available N and urease) in summer, autumn and winter, but the constrained model was not significant in spring ($p = 0.592$). The spring community therefore changed among treatments without a detectable linear mapping onto the measured chemistry. pH, SOC and inorganic N are frequently reported as drivers of *nirS* composition [7,9,10,27,29]. The present data do not contradict that view; they show that the strength of the coupling is itself seasonal. In spring, unmeasured factors—labile C pulses from the straw bag, local anoxia around the residue, or physiological lag after the 5 °C shift—may have outweighed the bulk properties included in the RDA.

pH of the composite at packing was 5.23. After incubation, CK remained more acidic than the fertilized treatments following the 5 °C phase (5.49 versus 5.82–6.18), the 20 °C phase (5.68 versus 6.12–6.37), and the 15 °C phase (5.92 versus 6.01–6.22). No treatment difference was detected after the −15 °C phase (Table 3). The higher pH under A1/A2 and B1/B2 is not the acidification often reported for ammonium sulfate alone; straw decomposition and nitrate addition are confounded with the N source, and pH was not manipulated separately. *Rhodanobacter* accounted for most *nirS* reads in these still-acid samples, which fits reports of this genus from acidic, nitrate-bearing soils [17]. The correspondence is only an association. The RDA model was not significant after the 5 °C phase, when the pH contrast was already present, so pH does not account for the spring-phase separation of A2.

4.5. Methodological Limits and Implications for N Management

Two caveats follow directly from the design. First, denitrification potential and N_2O were not measured. Compositional change cannot be translated into N loss [25]. Claims that high straw or high N “increase the possibility of N loss” are not warranted by these data. Second, *nirS* is one functional marker for nitrite-reducing denitrifiers. *nirK*-type denitrifiers and *nosZ*-carrying organisms involved in N_2O reduction were not characterized. Because the denitrification pathway is modular, changes in the *nirS*-type community cannot be read as changes in the denitrifying community or in N_2O reduction [21,30]. *Rhodanobacter* itself often carries *nirK* rather than *nirS* [17], so the numerical dominance of this genus in a *nirS* library should be read as the recoverable *nirS*-bearing fraction of a *Rhodanobacter*-rich soil, not as proof that every *Rhodanobacter* cell in the microcosm uses *nirS*. Broader primer sets or metagenomes would be needed to test whether *nirK* assemblages followed the same seasonal path. The strongest claim supported here is that N form, N rate, and the sequential thermal regime reorganized *nirS*-type community structure and diversity.

Within those limits, the practical implication is temporal. Under a single straw rate, full-rate ammonium sulfate reduced *nirS* diversity only in spring; reduced ammonium reorganized dominant genera at the same temperature; nitrate fertilizers left their strongest chemical imprint as winter NO_3^- -N under Full-rate potassium nitrate (B1). Management that aims to limit denitrifying N loss in cold-temperate farmland should therefore be timed to the periods when the assemblage is most plastic-around spring thaw and, for nitrate, during the frozen interval-rather than applied as a season-blind change in fertilizer product. Field measurements of process rates at those two windows are the necessary next step. The *nirS* gene copies were not quantified. All taxonomic contrasts are therefore relative. An increase in the proportion of *nirS* reads assigned to *Rhodanobacter*, *Herbaspirillum* or any other taxon cannot be translated into a change in absolute abundance or into a change in nitrite-reduction capacity. Interpretation of N transformations is limited by the missing texture, CEC and field bulk density. Denitrification potential and N_2O were not measured, so these compositional shifts cannot be read as a change in denitrification rate or N loss. The results apply to this packed composite at 60% of its measured water-holding capacity, not to a named soil type of the Sanjiang Plain. Because the three sites were composited before incubation, the experiment does not describe differences among Jiamusi farmland soils, nor does it estimate how representative the composite is of the Sanjiang Plain. The results apply to this packed composite only. The agronomic reading is limited by residue placement. Field straw return usually incorporates chopped straw into the cultivated layer. Here the straw remained inside a mesh bag at one depth, so soil–straw contact was smaller, and decomposition was localized. Patterns in the *nirS*-type community and in soil N should not be transferred directly to broadcast or mixed straw return.

5. Conclusions

Phase and N treatment jointly structured the *nirS*-type denitrifying bacterial community in straw-amended composite soil from Jiamusi. Richness of the DNA-detected library was lowest after the 5 °C phase and was further reduced under full-rate ammonium sulfate (A1). Shannon and Simpson diversities differed among treatments only after that phase, when A1 was lower than CK, A2, B1 and B2. Community composition differed among treatments after the 5 °C phase ($p = 0.042$), the 15 °C phase ($p = 0.001$) and the −15 °C phase ($p = 0.001$), but not after the 20 °C phase ($p = 0.060$). Higher richness after the −15 °C phase refers to DNA-detected membership, not to active denitrifier diversity. A2 was separated from the other treatments after the 5 °C phase, coincident with a higher relative abundance of *Herbaspirillum* and related *Burkholderiales* and a lower relative abundance of *Rhodanobacter*. The number of ASVs shared by all five treatments increased from the 5 °C phase to the −15 °C phase. LEfSe treatment-associated taxa were phase-specific: *Burkholderiales*-affiliated taxa recurred across phases but were assigned to different treatments, and *Actinomycetota* were discriminative only after the −15 °C phase. These calls are descriptive ($n = 3$) and are not confirmed indicators.

Soil chemistry and potential enzyme activities showed significant phase $\times$ treatment interactions. Straw without mineral N (CK) remained more acidic than the fertilized treatments after the 5, 20 and 15 °C phases. A2 was associated with lower TN and with lower NO_3^- -N after the 5 °C phase. Full-rate potassium nitrate (B1) was associated with higher TN and higher winter NO_3^- -N; the higher available K under B1 includes K added as KNO_3 . Invertase and N-related variables covaried with community structure in the RDA after the 20, 15 and −15 °C phases, but the constrained model was not significant after the 5 °C phase. These are concurrent associations, not demonstrated drivers.

Under a common straw addition, N form and rate did not produce a uniform shift in the *nirS*-type assemblage. Full-rate ammonium sulfate was associated with lower *nirS*

evenness only after the 5 °C phase, whereas reduced ammonium and nitrate additions were associated with phase-dependent shifts in relative abundance. Because only *nirS* was sequenced, the data describe the *nirS*-type denitrifying bacterial community. They do not describe *nirK*- or *nosZ*-type organisms. Denitrification potential, N₂O, *nirS* gene copies and transcripts were not measured, so the data cannot be read as a change in denitrification rate, population size or N loss. Temperature cannot be separated from incubation duration, straw-decomposition stage and preceding thermal history. These patterns were observed in one composite soil under sequential incubation and should not be read as a regional response of Jiamusi farmland. A season-blind choice of N product was not tested against timed application. Denitrification potential, N₂O, *nirS* gene copies and transcripts were not measured, so the data cannot be read as a change in denitrification rate, population size or N loss.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d18100638/s1>, Table S1: Repeated-measures ANOVA of soil properties and potential enzyme activities; Table S2 Repeated-measures ANOVA of α -diversity indices of the *nirS*-type community; Figure S1. Design of the 25 columns. Each treatment had five columns at day 0. Three columns (filled) were subsampled at 60, 120, 180 and 240 d and remained in the incubation throughout. Two columns (open) were never sampled. No column was removed at a sampling date.

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