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Effect of nitrogen addition on the carbon metabolism of soil microorganisms in a *Calamagrostis angustifolia* wetland of the Sanjiang Plain, northeastern China

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Abstract

Purpose: Soil microorganisms are important mediators of land ecosystem functions and stability. However, carbon sources in different amounts of nitrogen addition are known to affect the function of soil microbial communities. Thus, this study sought to evaluate the effects of nitrogen addition on the carbon utilization capacity of soil microorganisms in the Sanjiang Plain wetland, northeastern China.

Methods: Three nitrogen treatments (CK, 0 kg N ha⁻¹ a⁻¹; N40, 40 kg N ha⁻¹ a⁻¹; and N80 kg N ha⁻¹ a⁻¹) were evaluated in the Honghe National Nature Reserve of the Sanjiang Plain. The carbon metabolism capacity of soil microorganisms in the *C. angustifolia* wetland was investigated after five consecutive year's nitrogen addition treatment using the Bio-Eco technique.

Results: Different amounts of nitrogen addition conditions resulted in significant differences in pH, ammonium nitrogen (NH₄⁺), dissolved organic carbon (DOC), and soil microbial alpha diversity. The average well-color development (AWCD) in the Bio-Eco Plate assay increased gradually with incubation time, and different nitrogen levels significantly affected these AWCD values ($P < 0.05$), with the N40 treatment exhibiting the highest value. Furthermore, the N80 treatment had significantly lower Shannon and Pielou diversity indices ($P < 0.05$). N40 significantly promoted carbohydrate, amino acid, and ester utilization rates by soil microorganisms, whereas N80 significantly inhibited carbohydrate, amino acid, alcohol, amine, and organic acids utilization. Redundancy analysis (RDA) showed that the three treatments had remarkable differences in soil microbial community metabolism, and the cumulative variance contribution was 72.86%. In addition, RDA revealed that the N80 treatment was positively correlated with the TN, SMC, DON, and TOC but negatively correlated with DOC, NH₄⁺, pH, and NO₃⁻.

Conclusion: Long-term nitrogen addition leads to changes in soil microbial community structure and significantly alters the ability of soil microorganisms to utilize carbon sources in the *Calamagrostis angustifolia* wetland.

Keywords: *Calamagrostis angustifolia* wetland, Soil microorganism, Functional diversity, Bio-Eco Plate

Background

Soil nitrogen is one of the important nutrient elements in ecosystems and plays critical roles on ecosystem structure and function. In wetland ecosystems, nitrogen is considered to be one of the mainly limiting nutrient elements for primary productivity, but due to the

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development of agricultural practice and long-term large amounts of nitrogen fertilizers utilization, the amounts of available nitrogen in the soil increase quickly and impact wetland ecosystem structure and function (Vitousek and Howarth 1991; Feng et al. 2015). Therefore, nitrogen-saturated inputs affect a number of ecological processes (Nakaji et al. 2001; Lu et al. 2015), such as soil acidification (Ouyang et al. 2005), affecting apoplastic decomposition (Song et al. 2011), and stimulated CO₂ emissions (Song et al. 2013; Tao et al. 2018), as well as reducing soil microbial diversity and affecting microbial ecological functions (Wang et al. 2018; Zhang et al. 2018), ultimately having an impact on wetland ecosystems.

Wetlands are important terrestrial ecosystems, covering 5 to 8% of the total area of the earth. Wetlands possess the characteristics of both terrestrial and aquatic ecosystems and are therefore highly biodiverse and productive, in addition to providing a wide range of ecosystem services (Wang et al. 2006a). Nitrogen addition affects the structure and function of wetland ecosystems by altering soil environmental conditions such as nitrogen content and soil organic matter, influencing soil microbial activity, as well as the composition and diversity of soil microbial communities (Li et al. 2006; Wang et al. 2006b). Soil microorganisms are major participants in soil nitrogen transformation, and have an important regulatory role in the soil nitrogen cycle (Zhang et al. 2009), and contribute significantly to the stability and function of wetlands. Since microbial response to environmental changes is very sensitive, so soil microbial structure and function is an important indicator for soil quality change (Yan et al. 2010).

Previous studies have shown that the continuous input of nitrogen affects soil microorganisms in different ecosystems. For example, Li et al. (2013) results showed that the soil microbial activity decreased significantly was changed when the ammonium sulfate concentration was higher than 528.5 mg/kg. In contrast, Wu et al. (2017) investigated the effect of nitrogen addition (NH₄NO₃) on soil microorganisms in coastal wetlands and found that soil microbial activity was increased under N addition (3 and 6 g N ha⁻¹ a⁻¹). The reason may be the different forms and amounts of N could affect the type of carbon source used by the soil microbiota. Fang et al. (2014) reported that nitrogen source inputs of ammonium and nitrate nitrogen significantly promoted microbial metabolic activity and utilization of carbon substrates. Long-term nitrogen input experiments conducted by Compton et al. (2004) at Harvard University showed that increased nitrogen decreased microbial biomass and reduced the utilization ability of microbial communities for carbon sources. It is seen that nitrogen input affects grassland ecosystems, forest ecosystems, and wetland ecosystems.

Wetlands play an important role in ecological processes such as controlling greenhouse gas emissions, regulating climate, and maintaining ecosystem balance (Liu 2004). Many scholars have begun to focus on the effects of nitrogen addition on wetland soil microorganisms. Lu et al. (2021) found in the effect of nitrogen addition on soil microbial structure and function in coastal saline wetlands that high N treatment (200 kg N ha⁻¹ a⁻¹) with NH₄NO₃ as a nitrogen source increased nutrients in the soil but reduced soil microbial diversity. Wang et al. (2019) studied the effects of nitrogen addition on temperate marshland soils in northeastern China and found that nitrogen treatments (8 g N m⁻² a⁻¹) with NH₄NO₃ as a nitrogen source for nitrogen input reduced soil pH value and alteration in the microbial community.

The Sanjiang Plain is the largest freshwater marsh region in China. However, due to the extensive and intensive use of the region for agricultural production over the past 50 years, the natural freshwater marshes in this region often received increasing amounts of exogenous N inputs (Zhao 1999; Wang et al. 2010; Zhang et al. 2007). Studies have shown that nitrogen input promotes bacterial growth (Bragazza et al. 2012), enhances methanogenic and anaerobic respiratory bacterial activity (Yavitt et al. 2012), accelerates soil denitrification processes (Francez et al. 2011), and inhibits aerobic methane oxidation (Lozanovska et al. 2016). The previous studies reported that the ratio of NH₄⁺-N/NO₃⁻-N has been reduced from 5 to 2 since the 1970s, so the nitrogen form has been changed (Hu et al. 2019). Our previous study also found that the bacterial and fungal diversities and compositions in the wetlands of the Sanjiang Plain were significantly changed by NH₄NO₃ addition (Sui et al. 2016). However, the function of soil microorganisms in the wetlands of the Sanjiang Plain by adding different nitrogen forms is still unknown. Therefore, we aim to (i) evaluate the variation of soil physicochemistry properties in different amounts NO₃⁻ addition and (ii) clarify the changes of soil microbial carbon metabolism and function diversity in different amounts NO₃⁻ addition.

The Bio-Eco Plate method is a technique that allows researchers to identify the different types of carbon sources utilized by soil microorganisms (Garland 1997; Preston et al. 2002). This technique has been widely used in recent years as it provides a simple and rapid means of assessing the function of soil microbial communities (Konopka et al. 1998; Garland and Mills 1991). In this study, the carbon utilization capacity of soil microorganisms was investigated using the Bio-Eco Plate technique based on long-term field simulations of NH₄ addition in the Honghe National Nature Reserve in the Sanjiang Plain. Our findings thus provide important insights into the mechanisms by which wetland ecosystems adapt

to human activities, as well as a scientific basis for the promotion of sustainable development and the creation of more effective wetland ecosystem management strategies.

Results

Effect of different nitrogen levels on soil physicochemical characteristics

Table 1 summarizes the average values of main soil physicochemical properties under different nitrogen application levels. Different nitrogen levels had a significant effect on soil pH, NH₄⁺ content, and DOC (*P* < 0.05). The contents of SMC, DON, and TN showed an increasing trend with the increase of nitrogen addition

concentration, as order CK < N40 < N80; the contents of soil pH and NO₃⁻ showed a decreasing trend with the increase of nitrogen addition concentration, as order CK > N40 > N80; the NH₄⁺, DOC, and TOC contents of each treatment showed a decreasing and then increasing phenomenon with the increase of nitrogen addition concentration.

Effects of simulated nitrogen addition on soil microbial carbon source metabolic activity

As illustrated in Fig. 1, the average well color development (AWCD) of the soil microbial community under different nitrogen addition concentrations increased with culture time. Specifically, this value increased rapidly at

Table 1 Physicochemical properties of wetland soil with different nitrogen addition conditions

Treatment	SMC	pH	NO ₃ ⁻ (mg/kg)	NH ₄ ⁺ (mg/kg)	DOC (mg/kg)	DON (mg/kg)	TN (%)	TOC (%)
CK	0.43 ± 0.06a	4.4 ± 0.18a	23.8 ± 6.34a	21.2 ± 3.45a	3897.9 ± 625.37a	299.7 ± 130.71a	0.57 ± 0.23a	5.34 ± 2.23a
N40	0.48 ± 0.03a	4.3 ± 0.0ab	23.4 ± 0.89a	4.3 ± 0.30b	836.0 ± 135.58b	374.2 ± 17.77a	0.77 ± 0.16a	4.62 ± 0.80a
N80	0.50 ± 0.04a	4.1 ± 0.12b	21.7 ± 1.41a	9.4 ± 2.28b	1575.6 ± 325.29b	444.2 ± 134.93a	0.94 ± 0.29a	5.31 ± 1.44a

Each treatment was performed in triplicate. The data are expressed as the mean ± standard deviation; lowercase letters indicate significant differences (*P* < 0.05)

CK control, N40 40 kg N ha⁻¹ a⁻¹, N80 80 kg N ha⁻¹ a⁻¹, SMC soil moisture contents, DOC dissolved organic carbon, DON dissolved organic nitrogen, TN total nitrogen, TOC total organic carbon

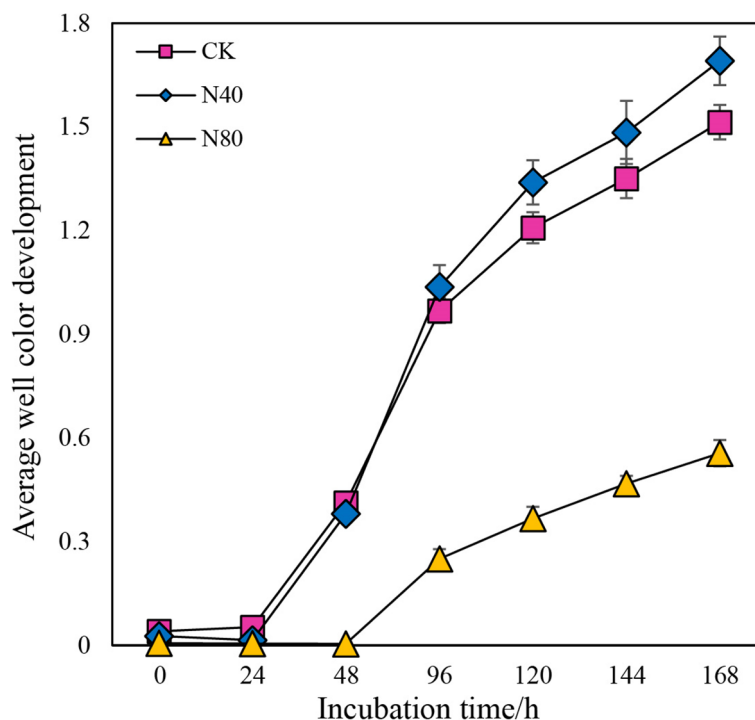


Fig. 1 Average well color development (AWCD) of the soil microbial community under different nitrogen addition concentrations as a function of incubation time

0–96 h, indicating a high microbial metabolic activity at this culture stage. These increases decelerated after 120 h and stabilized thereafter. Therefore, the AWCD values that were incubated for 120 h were selected for subsequent analysis. The AWCD (i.e., and indicator of carbon utilization) of the microbial communities under different nitrogen addition conditions exhibited the following order: N40 > CK > N80. Interestingly, the N40 treatment exhibited the highest carbon metabolic capacity.

Changes in soil microbial functional diversity

To further determine the effect of different nitrogen concentrations on soil microbial carbon source utilization, different diversity indices were used to evaluate the AWCD value at 120 h of culture. As indicated in Table 2, there was no significant difference between the soil microbial functional diversity of the N40 and CK treatments, but there was a significant difference between N80 and CK ($P < 0.05$).

Utilization of different carbon sources by the soil microbial community

Figure 2 illustrates the effects of different nitrogen addition levels on the utilization rates of different types of carbon sources by soil microorganisms. A total of 31 carbon sources were evaluated with the Bio-Eco Plate assay, which in turn were divided into six categories: carbohydrates (7), amino acids (6), alcohols (3), esters (4), amines (3), and organic acids (8). As shown in Fig. 2, N40 significantly promoted carbohydrate, amino acid, and ester utilization rates by soil microorganisms ($P < 0.05$), whereas N80 inhibited carbohydrate, amino acid, alcohol, amine, and organic acids utilization ($P < 0.05$).

As shown in Fig. 3, the metabolic fingerprint of the N40-treated soil was constituted by eight kinds of carbon sources with AWCD > 2.0, including α -D-lactose, β -methyl-D-glucoside, D-cellobiose, L-serine, D-mannitol, N-acetyl-D-glucosamine, D-galacturonic acid, and D-glucosaminic acid. Among these, the AWCD of D-mannitol was the highest, reaching a value of 3.42. The AWCD values of the N80 treatment were all below 2. Among these, tween 40 had the highest AWCD value

but reached only a value of 1.67. Overall, the compounds within each group did not always behave consistently. The utilization intensity of D-xylose, *i*-erythritol, phenylethyl-amine, itaconic acid, and D-malic acid by soil microorganisms decreased with the increase of nitrogen concentration, as follows: CK > N40 > N80; the utilization intensity of tween 40 and 4-hydroxy benzoic acid increased with the increase of nitrogen concentration, as follows: N80 > N40 > CK; the γ -hydroxybutyric acid showed N80 > CK > N40; and the utilization intensity of other carbon sources by soil microorganisms showed a trend of increasing and then decreasing with the increase of N concentration, as follows: N40 > CK > N80.

As illustrated in Fig. 4, the results of the metabolic activity heat map of the soil microbial community can be divided into four groups. In group I, the AWCD values of D-galactonic acid- γ -lactone, phenylethyl-amine, D,L- α -glycerol phosphate, and glycyl-L-glutamic acid were not significantly different between the three treatments; in group II, the AWCD values of D-galacturonic acid, N-acetyl-D-glucosamine, D-mannitol, D-cellobiose, α -D-lactose, and β -methyl-D-glucoside in N80 were significantly lower than in CK and N40; in group III, putrescine, L-Serine, D-malic acid, and α -D-glucose-1-phosphate were significantly higher in CK and N40 than in N80; and in group IV, the AWCD values of D-glucosaminic acid in N80 were significantly lower than in CK and N40. These results demonstrated that different nitrogen concentrations had significantly different effects on the activity and carbon source utilization preference of the soil microbiota.

Factors influencing soil microbial carbon source utilization patterns under different nitrogen addition treatments

Table 3 summarizes the correlation coefficients of the main components of the 31 carbon sources. As shown in Table 3, a total of 21 carbon sources constitute the first principal component (PCA1), including five carbohydrates, four amino acids, two esters, three alcohols, three amines, and three organic acids. Among them, D-cellobiose was the carbon source most related to PC1 with a 0.989 load value, followed by β -methyl-D-glucoside (0.987) and α -D-glucose-1-phosphate (0.979). Therefore, D-cellobiose, β -methyl-D-glucoside, and α -D-glucose-1-phosphate had a major effect on PCA1. Additionally, seven kinds of carbon sources constituted the second principal component (PC2), including two carbohydrates, two amino acids, one ester, and two organic acids. Among them, D-xylose is the most relevant carbon source for PC2 (loading value of -0.841), followed by L-phenylalanine (0.805) and glycogen (0.785). Collectively, nitrogen addition makes the carbon source metabolic activity of soil microorganisms most correlated

Table 2 Soil microbial functional diversity index under different nitrogen addition concentrations

Treatments	Shannon index	Simpson index	Pielou index
CK	3.01 \pm 0.04a	0.94 \pm 0.0a	0.92 \pm 0.01a
N40	3.02 \pm 0.03a	0.94 \pm 0.0a	0.91 \pm 0.01a
N80	2.45 \pm 0.09b	0.90 \pm 0.01b	0.87 \pm 0.02b

Each treatment was performed in triplicate. The data are expressed as the mean \pm standard deviation; lowercase letters indicate significant differences ($P < 0.05$)

CK control, N40 40 kg N ha⁻¹ a⁻¹, N80 80 kg N ha⁻¹ a⁻¹

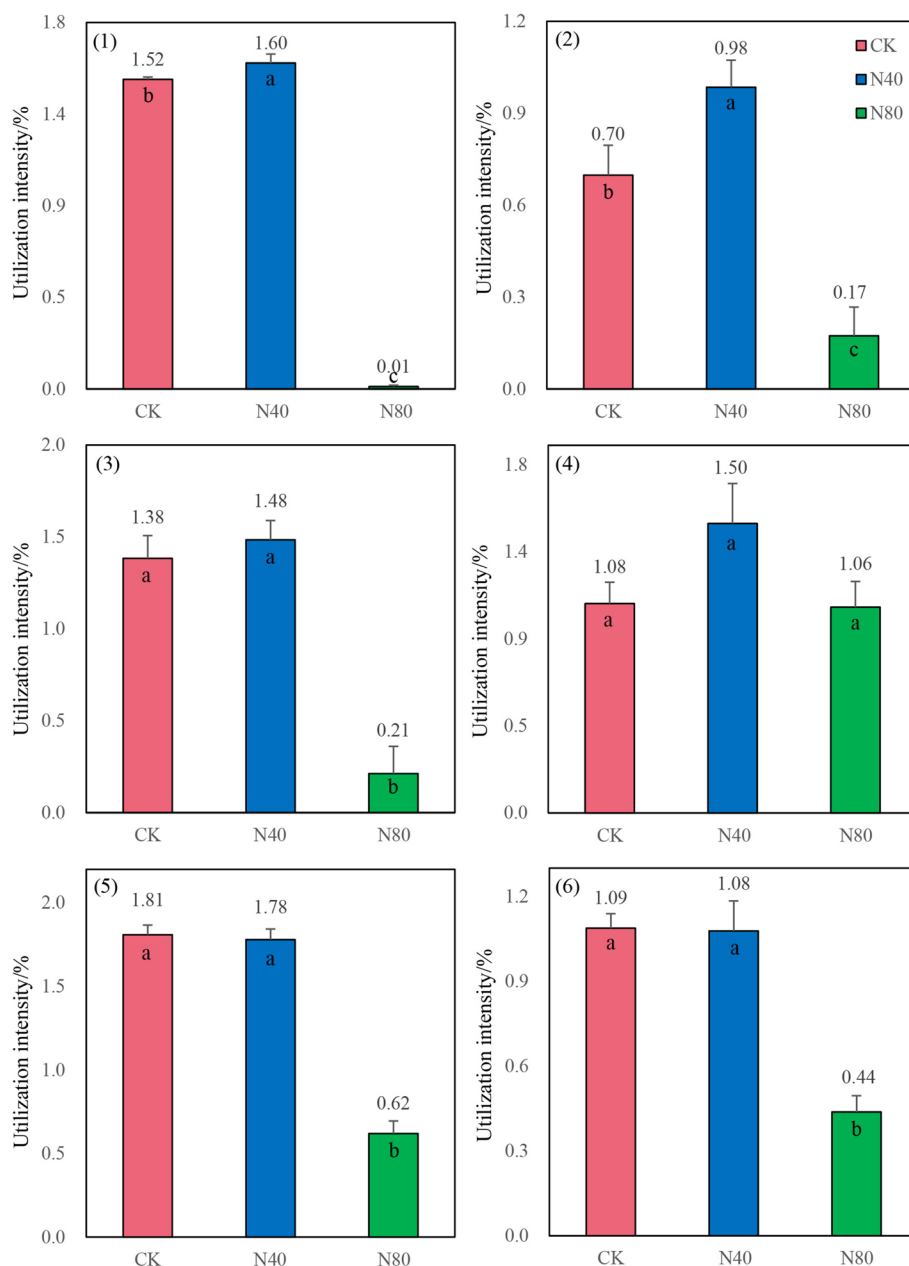


Fig. 2 Utilization of different carbon sources by soil microbial communities under different nitrogen addition concentrations. 1 Carbohydrate, 2 amino acids, 3 alcohols, 4 esters, 5 amine, 6 acids; lowercase letters indicate significant differences ($P < 0.05$). CK, control; N40, 40 kg N ha⁻¹ a⁻¹; N80, 80 kg N ha⁻¹ a⁻¹

with D-cellobiose and D-xylose in the *Calamagrostis angustifolia* wetland of the Sanjiang Plain.

The microorganisms in the soil samples containing varying nitrogen levels were cultured for 120 h. The results of the redundancy analysis (RDA) showed that the variance contributions of RDA1 and RDA2 were 55.36% and 17.5%, respectively, and the cumulative variance contribution was 72.86%. As illustrated in Fig. 5, Soil microbial

Biolog-substrate utilization patterns were separated with the alteration of nitrogen addition. Among them, the total N80 treatment clustered farthest from the total CK and N40 treatments, whereas the total CK and N40 treatments clustered closer to each other. Therefore, we concluded that the N80 treatment significantly changes the carbon source utilization capacity of soil microorganisms. In addition, the N80 treatment was positively

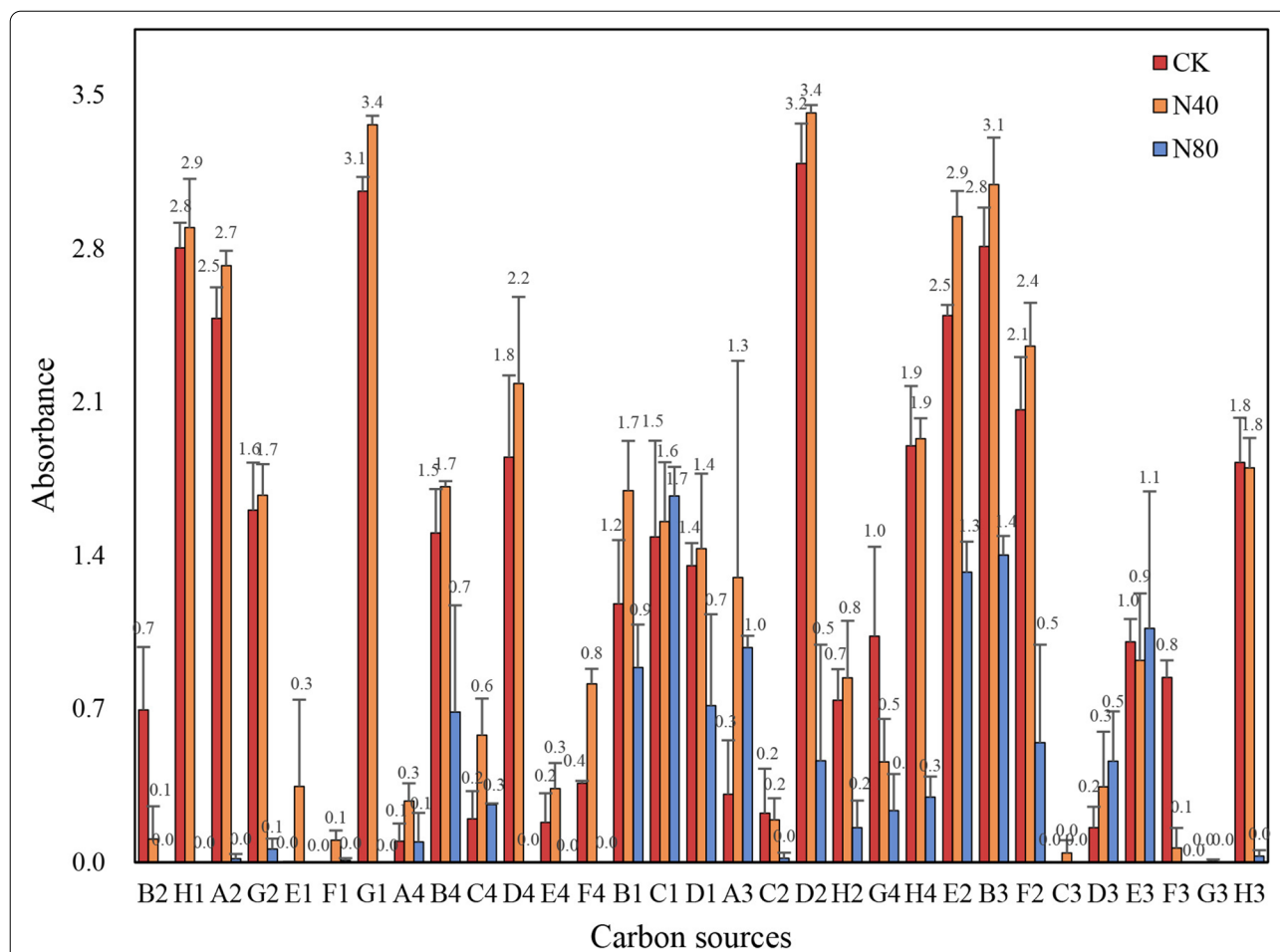


Fig. 3 Carbon physiological spectrum and metabolic fingerprint of soil microbial community in the *Calamagrostis angustifolia* wetland of the Sanjiang Plain. Carbohydrate, B2-D-xylose, H1- α -D-lactose, A2- β -methyl-D-glucoside, G2- α -D-glucose-1-phosphate, E1- α -cyclodextrin, F1-glycogen, G1-D-cellobiose; amino acids, A4-L-arginine, B4-L-asparagine, C4-L-phenylalanine, D4-L-serine, E4-L-threonine, F4-glycyl-L-glutamic acid; esters, B1-pyruvic acid methyl ester, C1-tween 40, D1-tween 80, A3-D-galactonic acid- γ -lactone; alcohols, C2-*i*-erythritol, D2-D-mannitol, H2-D, L- α -glycerol phosphate; amine, G4-phenylethyl-amine, H4-putrescine, E2-N-acetyl-d-glucosamine; acids, B3-D-galacturonic acid, F2-D-glucosaminic acid, C3-2-hydroxy benzoic acid, D3-4-hydroxy benzoic acid, E3- γ -hydroxybutyric acid, F3-itaconic acid, G3- α -ketobutyric acid, H3-D-malic acid

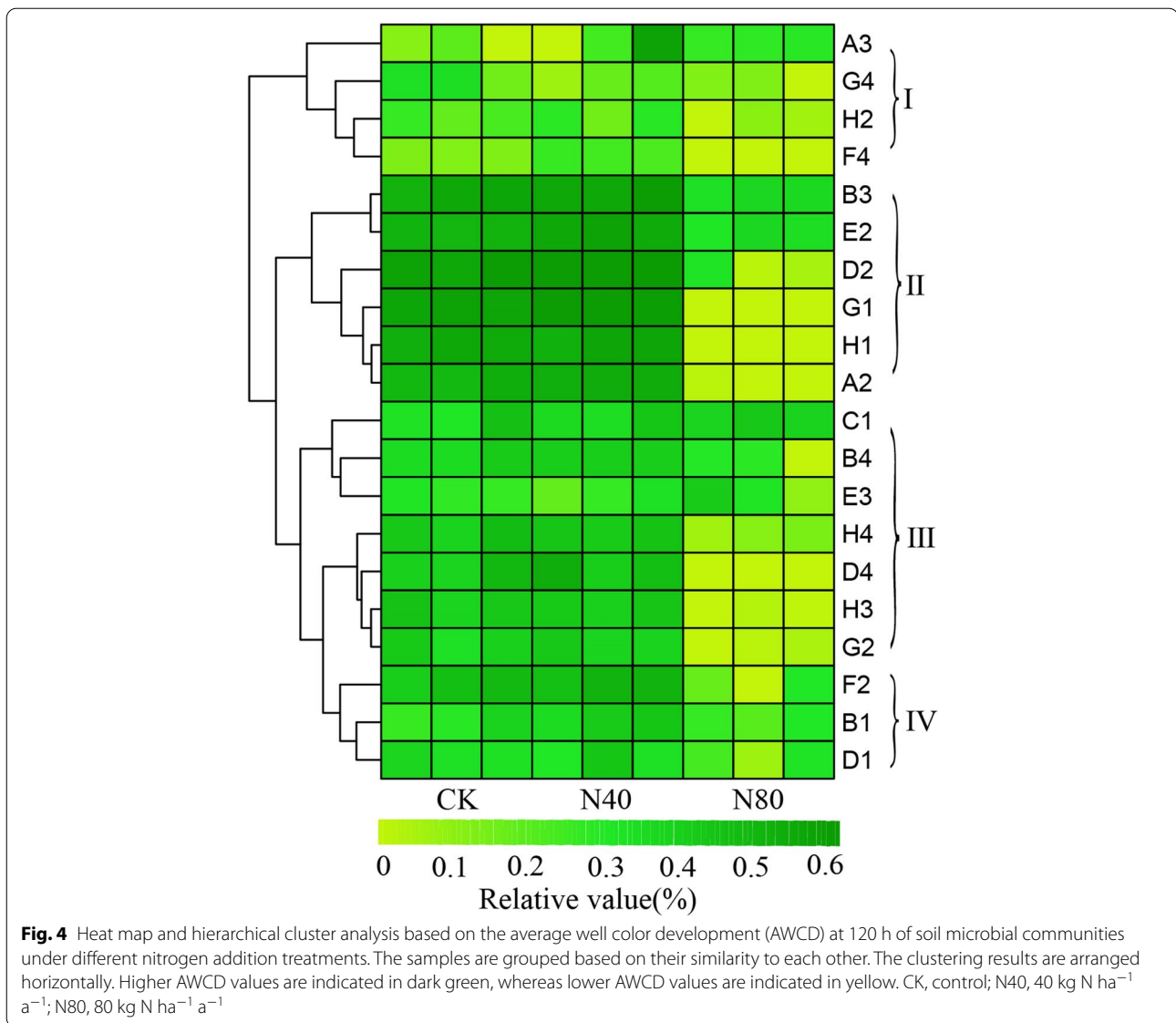
correlated with the TN, SMC, DON, and TOC but negatively correlated with DOC, NH_4^+ , pH, and NO_3^- .

Table 4 presents the relationships between different soil microbial functional diversity indices and soil physicochemical properties in the Sanjiang Plain. The AWCD value was highly positively correlated with pH ($R^2 = 0.69$, $P < 0.01$). Furthermore, the Shannon-Wiener index (H), Simpson index (D), and Pielou index were highly correlated with pH, with R^2 values reaching 0.68, 0.70, and 0.74, respectively.

Discussion

Different soil conditions can strongly affect soil microbial composition and diversity, and therefore, shifts in the functions of the soil microbiota can be used as

relevant ecological indicators. Long-term nitrogen input ultimately affects the structure and function of wetland ecosystems, and therefore, assessing the impact of long-term nitrogen addition on soil microbial function is critical to gain a comprehensive insight into wetland ecosystem dynamics. In this study, the AWCD value of the soil microbial community increased with culture time, and the different experimental treatments exhibited the following order: N40 > CK > N80 (Fig. 1). Different times of N addition and different nitrogen forms had significant differences in soil microbial carbon metabolism. For example, Yuan et al. (2012) found that the AWCD of N treatments ($\text{CO}(\text{NH}_2)_2$) in a Chinese fir plantation after seven consecutive year N addition showed nitrogen treatment (60 kg N ha^{-1}



a^{-1}) significantly increased the AWCD, but nitrogen treatment ($120 \text{ kg N ha}^{-1} \text{ a}^{-1}$) significantly decreased the AWCD value. However, Yu et al. (2013) found that treatment with applied NH_4NO_3 ($150 \text{ kg N ha}^{-1} \text{ a}^{-1}$) promoted AWCD in shrub, but nitrogen treatment ($50 \text{ kg N ha}^{-1} \text{ a}^{-1}$) inhibited AWCD in shrub after 1 month of N addition. Sui et al. (2016) conducted simulating nitrogen addition (NH_4NO_3), and four consecutive years on the functional diversity of soil microorganisms in the *Calamagrostis angustifolia* wetland of the Sanjiang Plain showed that after AWCD, values increased with increasing nitrogen concentration, showing HN ($8 \text{ g N ha}^{-1} \text{ a}^{-1}$) > LN ($4 \text{ g N ha}^{-1} \text{ a}^{-1}$) > CK. The differences in the results of these studies may be related to differences in the form and the concentration of N

addition. In soils with short-term nitrogen addition, nitrogen application helps to alleviate nitrogen limitation and improve soil available nitrogen content, thereby promoting the functional activity of soil microbial carbon metabolism (Liu et al. 2010). However, long-term high nitrogen addition can result soil acidification and reduced organic carbon content, affect the effectiveness of heterotrophic microbial communities on substrate utilization, and reduce soil microbial productivity (Deforest 2004).

Our findings indicated that higher nitrogen addition substantially decreased soil microbial activity, which also coincided with higher soil moisture. In soils with low nitrogen content, nitrogen application helps to alleviate nitrogen limitation and improve soil

Table 3 Correlation coefficients of major components in 31 kinds of carbon sources

Category	Biolog EcoPlate well	Carbon source types	PC1	PC2
Carbohydrate	B2	D-xylose	0.383	-0.841
	H1	α -D-lactose	0.969	-0.205
	A2	β -methyl-D-glucoside	0.987	-0.117
	G2	α -D-glucose-1-phosphate	0.979	-0.092
	E1	α -cyclodextrin	0.434	0.719
	F1	Glycogen	0.549	0.785
	G1	D-cellobiose	0.989	-0.119
	Amino acids	A4	L-arginine	0.463
B4		L-asparagine	0.809	0.018
C4		L-phenylalanine	0.469	0.805
D4		L-serine	0.96	0.09
E4		L-threonine	0.746	-0.119
F4		Glycyl-L-glutamic acid	0.917	0.343
Esters		B1	Pyruvic acid methyl ester	0.699
	C1	Tween 40	-0.226	0.262
	D1	Tween 80	0.723	-0.213
	A3	D-galactonic acid- γ -lactone	-0.072	0.088
Alcohols	C2	<i>i</i> -erythritol	0.575	-0.178
	D2	D-mannitol	0.968	-0.113
	H2	D, L- α -glycerol phosphate	0.852	0.04
Amine	G4	Phenylethyl-amine	0.408	-0.825
	H4	Putrescine	0.963	-0.103
	E2	N-acetyl-D-glucosamine	0.976	0.036
Acids	B3	D-galacturonic acid	0.956	-0.063
	F2	D-glucosaminic acid	0.926	-0.073
	C3	2-hydroxy benzoic acid	0.381	0.697
	D3	4-hydroxy benzoic acid	-0.396	0.089
	E3	γ -hydroxybutyric acid	-0.169	-0.261
	F3	Itaconic acid	0.392	-0.772
	G3	α -ketobutyric acid	0.334	0.116
	H3	D-malic acid	0.966	-0.181

PC1 principal component 1, PC2 principal component 2

available nitrogen content, thereby promoting the functional activity of soil microbial carbon metabolism (Liu et al. 2010). Therefore, our study is consistent with the results of Sui et al. (2016) and Wu et al. (2017) that low concentration of nitrogen addition promotes the functional activity of carbon metabolism in soil microorganisms. However, long-term high concentration of nitrogen addition can affect the effectiveness of heterotrophic microbial communities on substrate utilization and reduce soil microbial productivity (Deforest 2004); Compton et al. (2004) conducted a long-term nitrogen input experiment in Harvard Forest, and the

study showed that high nitrogen addition will lead to the decrease of soil microbial carbon biomass, which reduces the utilization rate of soil microorganisms for substrates. In addition, excessive nitrogen additions can lead to soil acidification (Table 1), and lower soil pH leads to changes in microbial biomass and microbial communities (Li et al. 2019). This may be the reason that the high concentration of nitrogen addition decreased the soil microbial AWCD value. However, Frey et al. (2004) carried out nitrogen addition experiments in Harvard Forest, and their studies have shown that the utilization of substrates by soil microorganisms in broad-leaved forests and mixed forests is not significantly related to nitrogen increase. So, the changes of soil microbial carbon metabolism under different nitrogen addition conditions may be related to ecosystem types, nitrogen application time, study period, nitrogen application amount and nitrogen application form, etc. Therefore, the effect mechanism of nitrogen addition on soil microbial metabolic activity still needs to be further studied.

The Shannon diversity index, Simpson index, and Pielou index are composite indicators of the richness and evenness of microbial species (He et al. 2013a). Our findings indicated that different levels of nitrogen addition significantly changed the alpha functional of the soil microbial community (Table 2). Specifically, the Shannon-Wiener, Simpson, and Pielou indices of the soil microorganisms were significantly higher in the CK and N40 treatments compared to N80. These findings were consistent with those of Sui et al. (2016), whose study demonstrated that the Shannon and Simpson indices increased with the low concentration of nitrogen addition ($4 \text{ g N ha}^{-1} \text{ a}^{-1}$) and decreased with the high concentration of nitrogen addition ($8 \text{ g N ha}^{-1} \text{ a}^{-1}$) in wetland soils, and the indices differed significantly between treatments. This may be because moderate nitrogen application favors the growth of soil microorganisms, whereas high nitrogen content promotes the proliferation of some microbial populations while suppressing others, resulting in a decrease in microbial community diversity indices.

The heat map shows that different nitrogen concentrations had different effects on the carbon source utilization patterns of the microbes in the soil samples. Combining Fig. 2 and Fig. 3, it can be seen that N40 promoted the utilization of all carbon sources in carbohydrates, amino acids, and esters by soil microorganisms. N80 inhibited the utilization of all carbon sources in carbohydrates, amino acids, alcohols, amines, and organic acids, except for 4-hydroxy benzoic acid and γ -hydroxybutyric acid in organic acids. In addition, the most relevant D-cellobiose and D-xylose for PC1 and PC2 are both derived from carbohydrates, which exactly correspond to the results

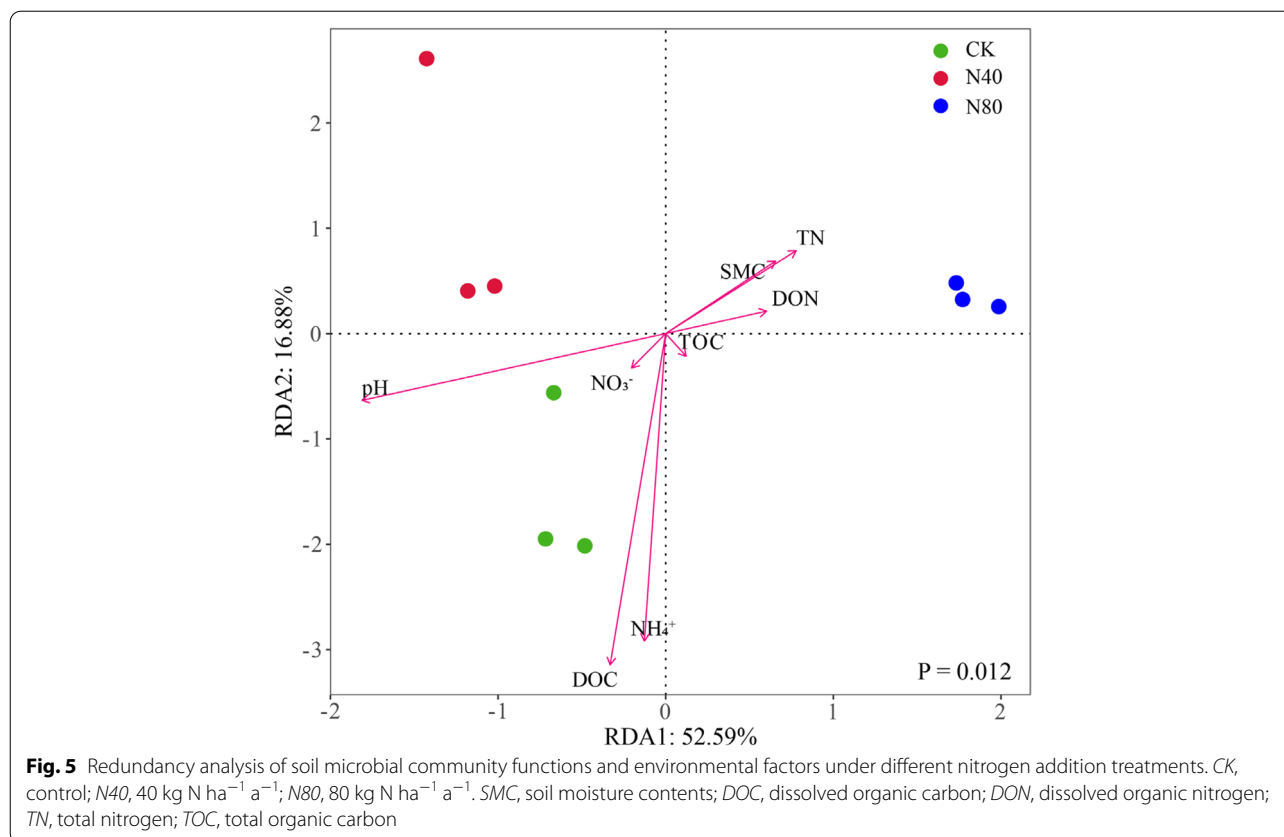


Table 4 Correlation between soil environmental factors and soil microbial functional diversity under different nitrogen addition treatments

	SMC	pH	NO ₃ ⁻	NH ₄ ⁺	DOC	DON	TN	TOC
AWCD	-0.437	0.69*	0.17	0.08	0.14	-0.42	-0.48	-0.2
Shannon	-0.412	0.68*	0.18	0.19	0.23	-0.46	-0.49	-0.13
Simpson	-0.445	0.70*	0.15	0.21	0.23	-0.5	-0.52	-0.17
Pielou	-0.574	0.74*	-0.06	0.31	0.33	-0.65	-0.64	-0.26

SMC soil moisture contents, DOC dissolved organic carbon, DON dissolved organic nitrogen, TN total nitrogen, TOC total organic carbon, AWCD average well color development

*significant correlation at the 0.05 level (two-tailed)

in Fig. 2 and Fig. 3. Frey et al. (2004) also evaluated the effect of simulated nitrogen settlement on the soil microbial community of a hardwood forest and found that the carboxylic acids and carbohydrates were significantly higher in the low nitrogen plots, whereas the citric acid and malonic acid were significantly higher in the high nitrogen plots. Chakraborty et al. (2011) reported that the application of nitrogen fertilizer reduced the ability of soil microbial groups to decompose organic acids and amines. Zhu et al. (2014) found that anaerobic bacteria increased the use of amino acids and decreased the use of organic acids and carbohydrates after nitrogen

application. Furthermore, gram-negative bacteria increased the use of carboxylic acids and decreased the use of amino acids and polymeric carbon sources. In contrast, yeast increased the use of carboxylic acids and polymeric carbon sources. These findings demonstrate that the differences in the carbon source utilization patterns of soil microorganisms may be an adaptation to soil environmental changes.

In this study, KNO₃ was the main nitrogen source in this experiment; therefore, the NO₃⁻/NH₄⁺ increased in the soil, so this may reduce the soil microbial activity and used the ability of some carbon substrate. Soil carbon

concentration also affected significantly on soil microbial function and diversity. Fang et al. (2014) reported that high nitrogen concentration significantly increases DOC content in soil by changing the metabolic activity of microorganisms and the way of utilizing carbon substrate, and nitrogen has no effect on it. This reason may be the soil organic carbon is an important nutrient on soil microbial function, and carbon metabolism activity and the variation of soil DOC concentration would directly affect the soil microbial composition and diversity and therefore change the soil microbial function (He et al. 2013b). The soil carbon metabolism types of N80 treatment were positively correlated with the TN, SMC, DON, and TOC but significantly negatively correlated with DOC and NH_4^+ , pH, and NO_3^- (Fig. 5). The increasing N addition concentration also significantly reduced soil pH and NH_4^+ content (Table 1). The reason may be adding nitrogen will lead to reduce the soil pH and affect the soil carbon metabolism ability. Liu et al. (2010) found that long-term high nitrogen addition would lead to soil acidification, affecting the soil microbial metabolism change and activity decline. Sui et al. (2021) reported that soil pH and total nitrogen had a significant effect on soil bacterial community structure. Fierer and Jackson (2006) demonstrated that soil pH significantly affected the composition of the bacterial community. Furthermore, Diao et al. (2019) found that soil pH was the main factor affecting microbial carbon source utilization under different nitrogen levels. Additionally, our redundancy analyses showed that there was a distinct separation between the different nitrogen treatments tested herein, while the effect of the N80 treatment was significantly greater than that of the N40. It is possible that a nonlinear threshold is crossed somewhere between the N40 and N80 treatments, which in turn allows the N80 treatment to have a greater effect on the composition, diversity, and carbon metabolism patterns of the microbial community than the N40 treatment. With the development of agriculture and industry in the future, it can be predicted that the amount of nitrogen input in the Sanjiang Plain will continue to increase, which will affect the structure and function of soil microorganisms. Carbon is the most important component in nature that can be utilized by soil microorganisms, and it is also the most consumed and utilized nutrient substrate. A large amount of nitrogen input will increase the amount of litter, change the quantity and quality of soil organic matter (Grandy et al. 2009; Liu et al. 2010), and also directly change the quality of soil microbial decomposition substrates (C:N:P ratio), quantity, and soil environment. Therefore, nitrogen addition affects soil nutrient availability, causing changes in microbial utilization of carbon sources and ultimately

altering the structure and function of microbial communities (Chakraborty et al. 2011).

In summary, the simulated nitrogen addition treatments had a significant effect on the soil microbial communities of Sanjiang Plain, demonstrating that increases in nitrogen addition rates will invariably change the physicochemical properties of the wetland environment. Nevertheless, the Biolog analysis method can only reflect changes in microbial functions based on carbon source utilization patterns and thus cannot fully illustrate the functional diversity of soil microorganisms. Therefore, this approach must be combined with high-throughput sequencing technology, molecular biology, and phospholipid fatty acid analysis to better characterize the variations of microbial function diversity.

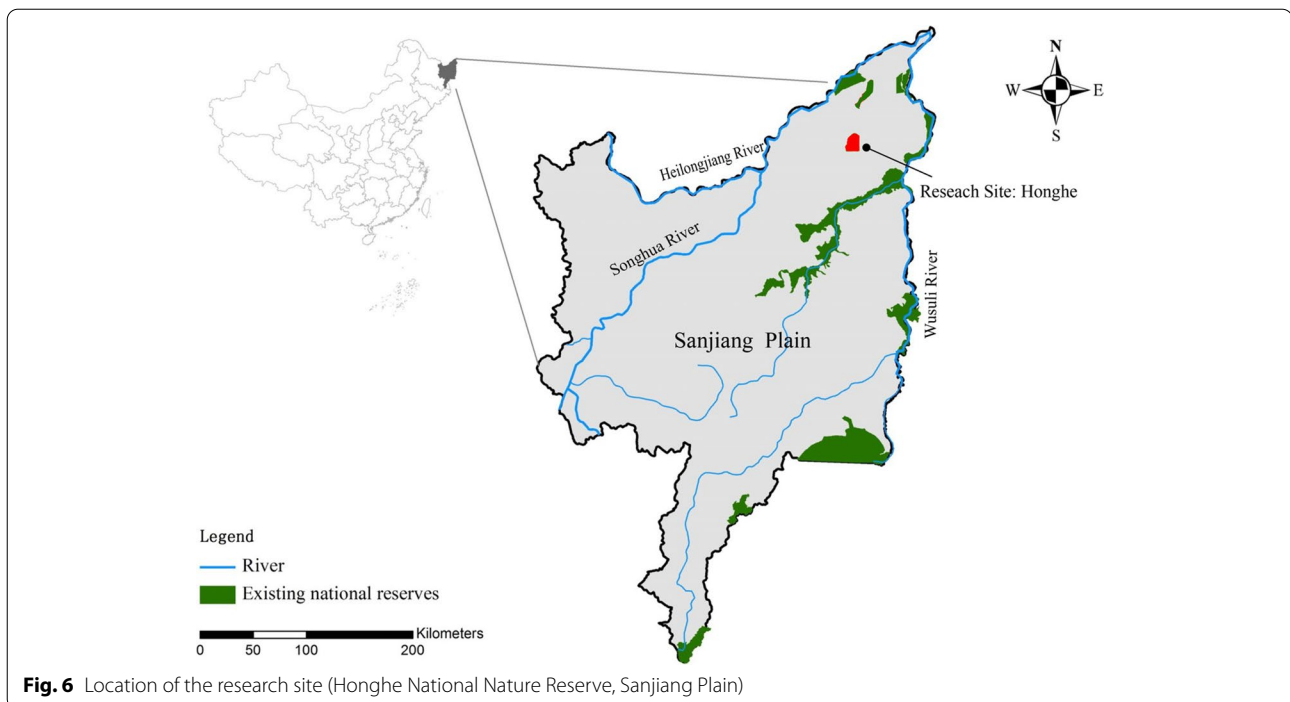
Conclusions

The carbon source utilization capacity of the soil microorganisms in the *Calamagrostis angustifolia* wetland of the Sanjiang Plain changed significantly under different nitrogen addition conditions. Particularly, N80 treatment significantly reduced the Shannon, Simpson, and Pielou diversity indices of soil microorganisms and significantly changes the carbon source utilization capacity of soil microorganisms. However, nitrogen addition affects the aforementioned parameters, thus affecting the carbon source utilization capacity of soil microorganisms. Therefore, once nitrogen addition rates exceed a certain threshold in the future, the stability of the temperate wetland ecosystem will be inevitably compromised if no prevention strategies are implemented.

Methods

Study area

The experimental site was located in the Honghe National Nature Reserve of Sanjiang Plain ($47^\circ 42' 18''$ – $47^\circ 52' 07''$ N, $133^\circ 34' 38''$ – $133^\circ 46' 29''$ E) (Fig. 6). This region has a total area of 2.18×10^4 ha, of which the wetland area constitutes approximately 1.1×10^4 ha, accounting for more than half of the total area of the reserve. The reserve exhibits a temperate humid/semi humid monsoon climate with long winters, severe cold and snow, and short spring and autumn seasons. The average annual temperature (MAT) of this region is 1.9°C , the average annual evaporation is 1166 mm, and the average annual precipitation (MAP) is 585 mm, with precipitation mostly concentrated between July and September (Qu et al. 2015). The study site exhibits primarily bleached stagnant soil and fibrous organic soil, its main vegetation types are meadows and swamps, and the dominant plants are *Calamagrostis angustifolia*, *Glyceria spiculose*, *Carex lasiocarpa*, and *Carex pseudocuraica*.



Plot setting and sample collection

To investigate the nitrogen addition concentration on soil microbial function in Sanjiang Plain, sample plots were established in May 2016, and nine plots (5 m × 30 m) were randomly established in the *Calamagrostis angustifolia* wetland experiment station, which were treated with three nitrogen concentration levels. The nitrogen addition treatments included control (CK), 0 kg N ha⁻¹ a⁻¹; N40, 40 kg N ha⁻¹ a⁻¹; and N80, 80 kg N ha⁻¹ a⁻¹ treatments. Each treatment was performed in triplicate and randomly assigned. Nitrogen addition concentrations were set based on the amount of local agricultural fertilizer utilization. Applying large doses of N in the short term can effectively mimic long-term small-dose N concentration inputs (Dise and Stevens 2005). Nitrogen additions greater than the exogenous N input to the wetland ecosystem were used to study the response of the ecosystem to a possible future high N saturation state, and different N additions were set to observe the effects of N input over the next 50 years. The nitrogen source KNO₃ was dissolved in water and sprayed uniformly with a sprinkler during the growing season which is mainly associated with agricultural fertilization (Sun et al. 2007), in May each year. The control plots were sprayed with an equal amount of water.

Sampling was conducted in October 2020 within each sample plot in the *Calamagrostis angustifolia* wetland. Five points (the center point of the diagonal line and the sample point on the diagonal line at an equal distance

from the center sample point) were selected using the diagonal 5-point sampling method in each of the three treatment sample plots, and the soil was collected from the surface soil layer (0–20 cm) with a 4-cm diameter soil auger. After removing plant debris and other impurities in the collected soil samples, the samples were pooled, stored in a 4 °C cooler, and transported to the laboratory. One part of the sample was used for the Bio-Eco Plate experiments, whereas the other part was naturally dried and used for soil physicochemical analyses.

Determination of soil physicochemical factors

Soil pH was determined via the leaching and potentiometric method with a ratio of 2.5:1; soil moisture was determined via the drying method and determined gravimetrically by oven drying at 105 °C for 24 h; soil total nitrogen (TN) was determined with an elemental analyzer; NO₃⁻-N was determined via the phenol disulfonic acid colorimetric method; NH₄⁺-N was determined via the potassium chloride leaching-indophenol blue colorimetric method; dissolved organic carbon (DOC) and dissolved organic nitrogen (DON) were determined via the K₂SO₄ leaching method with a soil-liquid ratio of 1:5, extracted for 30 min, centrifuged and filtered, and then measured by TOC analyzer (Jones and Willett 2005); total organic carbon (TOC) was determined with a TOC analyzer; and NH₄⁺-N and NO₃⁻-N were determined by the Elemental Analyzer (Flash EA 1112 N, Thermo Fisher, Waltham, MA, USA) (Murphy et al. 2000).

Determination of the functional diversity of the soil microbial community

The Bio-Eco Plate incubation method was used to determine the ability of soil microbial communities to utilize 31 different kinds of carbon sources (Qu et al. 2015). The Bio-Eco Plate has 96 microtiter wells, 1 replicate for every 32 wells, and 3 replicates in total. The first well is used as a control without carbon source, while the other wells contain different carbon sources and tetrazolium salt dyes. The microorganisms use the carbon source to respire and cause the dye tetrazolium in the microtiter wells to change color by redox reaction (Xi and Hu 2003). A portion of the soil sample was activated in a thermostat at 25 °C for 24 h. Afterward, 10 g of fresh soil was weighed into a 200 mL triangular flask, to which 90 mL of 0.85% sterile NaCl solution was added. The mixture was then shaken at room temperature for 30 min at 200 r/min. The plates were continuously incubated at 25 °C for 168 h. During the incubation period, the absorbance was measured at 590 nm at 24 h intervals, and the absorbance values were recorded (Feng et al. 2021).

Statistical analysis

AWCD was calculated as follows (Velasco et al. 2009; Jin et al. 2014; Liao et al. 2013):

$$AWCD = \sum (C_i - R) / 31 \quad (1)$$

where C_i is the 590 nm absorbance value of the well containing a carbon source, R is the absorbance value of the control well, and 31 is the number of holes in the ECO plate. If $C_i - R \leq 0$, the value was recorded as 0.

The functional diversity of the soil microbial communities was calculated using 120-h cultivation data. The soil diversity indices were calculated as follows (Pielou 1975; Whittaker 1972):

$$\text{Shannon-Wiener diversity index : } H = P_i \ln P_i \quad (2)$$

$$\text{Simpson diversity index : } D = 1 - \sum (P_i)^2 \quad (3)$$

$$\text{Pielou diversity index : } J = H / \ln S \quad (4)$$

where P_i is the ratio of the i^{th} relative absorbance value to the sum of the relative absorbance values of all samples.

All data were analyzed before processing using Excel 2010. One-way ANOVA was performed using the SPSS 25.0 software with the test level set at 0.05. Scatter plots with trend lines were generated using Excel 2010, and histograms were generated using SigmaPlot 10.0. Diversity index analysis, heat map, and redundancy analysis (RDA) were performed with R (Vegan package).

Abbreviations

CK: Control; N40: 40 kg N ha⁻¹ a⁻¹; N80: 80 kg N ha⁻¹ a⁻¹; AWCD: The average well-color development; RDA: Redundancy analysis; SMC: Soil moisture contents; DOC: Dissolved organic carbon; DON: Dissolved organic nitrogen; TN: Total nitrogen; TOC: Total organic carbon.

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Authors' contributions

XW and XS performed the experiments, analyzed the data, and wrote this manuscript. YL designed this experiment and revised the manuscript. LY helped to analyze the data, and RZ helped to do the experiment and took the soil samples. The author(s) read and approved the final manuscript.

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Availability of data and materials

The original data is recorded in an Excel file named "Data record sheet" and has been attached to this article.

Declarations

Ethics approval and consent to participate

The study did not violate ethics, and all participants agreed to publish the paper.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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