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Effects of Simulated Nitrogen Deposition on the Bacterial Community of Urban Green Spaces

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Abstract: Continuing nitrogen (N) deposition has a wide-ranging impact on terrestrial ecosystems. To test the hypothesis that, under N deposition, bacterial communities could suffer a negative impact, and in a relatively short timeframe, an experiment was carried out for a year in an urban area featuring a cover of Bermuda grass (*Cynodon dactylon*) and simulating environmental N deposition. NH_4NO_3 was added as external N source, with four dosages (N0 = 0 kg N ha^{-2} y^{-1}, N1 = 50 kg N ha^{-2} y^{-1}, N2 = 100 kg N ha^{-2} y^{-1}, N3 = 150 kg N ha^{-2} y^{-1}). We analyzed the bacterial community composition after soil DNA extraction through the pyrosequencing of the 16S rRNA gene amplicons. N deposition resulted in soil bacterial community changes at a clear dosage-dependent rate. Soil bacterial diversity and evenness showed a clear trend of time-dependent decline under repeated N application. Ammonium nitrogen enrichment, either directly or in relation to pH decrease, resulted in the main environmental factor related to the shift of taxa proportions within the urban green space soil bacterial community and qualified as a putative important driver of bacterial diversity abatement. Such an impact on soil life induced by N deposition may pose a serious threat to urban soil ecosystem stability and surrounding areas.

Keywords: Nitrogen deposition; bacteria; soil biodiversity; urban; 16S rRNA

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1. Introduction

Increasing nitrogen (N) deposition caused by industrialization and anthropogenic activities has become an ecological problem that attracted worldwide attention [1–3]. The terrestrial ecosystems in recent decades have suffered increases in anthropogenic inputs of pollutants and increases in the deposition of ammonia (NH_3), nitrogen oxides (NO_x), and their reactive products (NH_4^+-N, NO_3^--N, and HNO_3) [4]. Especially in China, atmospheric N deposition has increased by 8 kg N ha^{-1} since the 1980s [5]. Sustained N deposition has a wide-ranging impact on terrestrial ecosystems [6], which can cause soil acidification, reduction in biological functions, and diversity [4,7].

With the fast-growing urbanization, city areas are the most intense zones of human activity. Urbanization can alter the abiotic and biotic soil environment through several means, including atmospheric deposition, urban heat island effect, and invasion of exotic species, which may have contrasting effects on microbial activity and function [8]. High population density, heavy traffic, industrial, and agricultural impacts can cause high N emissions in urban areas [9]. The sources of N falling out on urban are complex.

Some studies found that nitrogen oxides produced in cities can return to urban areas through atmospheric deposition [10], which ultimately became a source of nutrients for soil biota in urban ecosystems [11]. Atmospheric N deposition can change the structure, function, and processes of ecosystems [12,13]. Therefore, sustained N deposition may affect the complexity of the entire ecological environment, including the urban green spaces, which are particularly prone to receive an impact, since those are the areas that allow concentrated water infiltration, being the sole permeable points among a system of paved surfaces and buildings, and being located usually in contexts suffering massive pollution fallout. As an essential part of the city environment, urban green space provides a variety of ecosystem services [14], such as contaminant degradation [15], carbon and nitrogen nutrient cycling [16], and general biochemical cycling [17]. It constitutes an interactive arterial system that helps to improve the quality of life of urban residents [18]. A better understanding of urban green space functionality, in response to external inputs in the urban soil ecosystem, is fundamental to improve urban soil management, and, in particular, nitrogen requirements, and to reduce threats to the environment.

The functioning of a soil depends on a sophisticated, but the coordinated system of interactions among the environment, vegetation, and organisms living in the soil [19–23]. Large in quantity, and extreme in diversity, soil microbes are important participants in the soil ecosystem and play a key role in biogeochemical cycling [24]. They assist the ecological processes of litter decomposition, soil fertility formation and maintenance, and nutrient cycling [25,26]. Soil microorganisms are commonly used to indicate the environmental changes of soil because they are sensitive to the changes in environmental factors (e.g., soil nutrients and pH) [27]. In recent years, with the development of high-throughput sequencing techniques, research methods for the analysis of environmental microorganisms have entered their most advanced stage [28]. Metagenomics is the study of a living community by direct extraction and subsequent reading of DNA sequences from all of the different genomes of organisms hosted in a given space. It can be used to understand the complete structure of microbial communities, revealing their environmental diversity and complexity [29,30]. Studies of the microbial diversity of soil, water, wetlands, and extreme environmental by means of DNA sequences have increased lately [31–34].

Other analyses have confirmed that there are a large number of bacteria that find their critical habitats in soil, and that their activities are strictly related to underground processes [35]. Bacteria that adjust to living in the soil can also affect plant growth and health through their direct and indirect effects in ecological processes in relation to N cycling [36]. Studying the feedback of urban soil bacteria to N deposition is therefore of primary importance to understand the effects of regional environmental changes on ecosystem processes regulated by soil bacteria. Although the structure of bacterial communities entails particular importance for the stability and productivity of urban green spaces, there are still few studies on the effect of anthropogenic N deposition on these habitats.

In this respect, since (a) nitrogen is the primary nutrient for soil fertilization and (b) life is N-dependent and N-limited in almost all environments, one could not predict a priori whether N deposition would be deleterious for microbial ecology, or it could instead boost its development and diversification, as is the case for crops and weeds. The hypothesis we aimed at testing here was that, in spite of its nature of essential macro-element for nutrition, sustained N additions to a soil could lead to a fast depression of the main ecological indicators for bacterial diversity in soil. To unveil the potential effects of different degrees of N deposition on the bacterial communities of urban green areas, we set up a simulated atmospheric N deposition-controlled experiment. Lawns, planted with *Cynodon dactylon*, which is one of the most popularly used grasses for turf, yards, and recreational greens, were selected as the research object. We analyzed the bacterial diversity and community structure of urban green space undergoing deliberate supplementation of N deposition by Illumina MiSeq 16S NGS metabarcoding. The present study aimed at (1) detecting the dynamics of the urban green space bacterial diversity along an N loading gradient;

and (2) investigating the direct and indirect effects of N deposition on soil microbial communities in urban green spaces.

2. Materials and Methods

2.1. Study Sites and Experimental Design

The study was carried out in a green space experimental field at Guangzhou University in Guangzhou, China (113°23′ E, 23°02′ N). The climate of the area is a typical subtropical monsoon climate, with warm and wet summer, and short and dry winter. The annual average temperature is 21.5 °C, and the average relative humidity is 77%. The hottest month is July, with an average monthly temperature of 28.7 °C. The coldest month is January, and the monthly average temperature is 13.3 °C. The rainy season is from April to September. The N deposition inputs in the four Guangzhou districts (Baiyun, Tianhe, Luogang, and Conghua) average 43.3, 41.2, 35.2, and 30.1 kg N ha^{-1} y^{-1}, respectively [37].

The experiment started in May 2016 and continued until May 2017. A randomized block design was used, with four treatments and five replicate plots of each treatment. Twenty 2 m × 2 m plots were arranged in a 4 × 5 matrix; 50 g of *Cynodon dactylon* seeds were evenly planted in each plot. The distance between any two adjacent plots was 0.5 m. The chemical parameters of the chosen soil at time zero, before the beginning of the trial (means of four replicates ± standard deviation) were the following; pH: 8.28 ± 0.06; ammonium nitrogen (mg kg^{-1}): 1.54 ± 0.10; nitrate nitrogen (mg kg^{-1}): 3.37 ± 0.03; total nitrogen (g kg^{-1}): 0.61 ± 0.06; total carbon (g kg^{-1}): 13.41 ± 0.96. NH_4NO_3 was added as the external N sources once a month, and the final N input corresponded to N0 (0 kg N ha^{-2} y^{-1}), N1 (50 kg N ha^{-2} y^{-1}), N2 (100 kg N ha^{-2} y^{-1}), N3 (150 kg N ha^{-2} y^{-1}). The experimental field was fenced off to prevent disturbance.

2.2. Soil Sampling and Analysis

Three months (July 2016), 6 months (October 2016), and 12 months (May 2017) after the start of the N supplementation treatment, three subsamples were sampled at a 0–5 cm depth of every plot. Subsequently, subsamples were mixed thoroughly and then separated into two portions. One portion of the soil was immediately frozen in liquid nitrogen and then stored at −80 °C for genomic DNA extraction. The remaining portion of soil was passed through a 2 mm sieve and used for soil chemical analysis. A total of 20 samples represented the five replicates of four treatments at every sampling time.

Soil pH (water: soil, 2.5:1) was determined using a pH meter. Nitrate (NO_3^--N) and ammonium (NH_4^+-N,) were extracted from 10 g of soil using 2 M KCl and determined using a San^{++} Continuous Flow Analyzer (Skalar, Breda, The Netherlands). Soil total carbon (TC) was determined by the dichromate digestion method, and total nitrogen (TN) was measured by the Kjeldahl method [38].

2.3. DNA Extraction

Total metagenomic DNA was extracted and purified from samples with a Universal Genomic DNA Kit (CwBio Inc., Beijing, China) according to the manufacturer's instructions. The concentration of total DNA was measured using the Qubit Platform (Life Technologies, CA, USA). The V3–V4 hypervariable region of 16S ribosomal RNA gene was amplified from 30 ng of DNA in triplicate polymerase chain reactions (PCR) with Pyro best DNA polymerase (TaKaRa, Dalian, China) and the following forward and reverse primers, respectively, 5′-ACTCCTACGGGAGGCAGCAG-3′ and 5′-GGACTACHVGGGTWTCTAAT-3′ (Sangon Biotech, Shanghai, China), following the manufacturer's instructions. Barcode sequences were attached to the amplification primers to distinguish the different samples. PCR products were inspected on 2% agarose electrophoresis gel and the respective amplicon libraries generated. Microbial DNA was then sequenced by Honortech (Beijing, China) using the Illumina MiSeq platform. Based on the raw data, pair-end reads were spliced using the principle of 98% overlap of 19 bases using the Connecting Overlapped Pair-End software 36. Barcode and primer sequences were then filtered to obtain the clean

data. Operational Taxonomic Units (OTUs) were individuated as those clustering at shared nucleotide identity equal or higher than 97%. The relative abundance of soil bacteria was calculated according to the species annotation and reads number.

2.4. Statistical Analysis

Alpha diversity was determined based on the Chao1, and by Shannon–Wiener indices. Chao1 Index is based on the number of OTUs with an individual sequence called "singletons" and the number of OTUs containing a pair of sequences is called "doubletons". The Chao1 index being more sensitive to rare species in the community [39]. The Chao1 were calculated by using the following equations:

$$\text{Chao1} = S_{OTU} + \frac{{F_1}^2}{2F_2} \quad (1)$$

where S_{otu}, F_1 and F_2 represent the number of observed species, singletons, and doubletons

Shannon index uses the number of sequences in each OTU and the total number of sequences in the community for calculation. The Shannon Index estimate is given by:

$$\text{Shannon index} = -\sum_{i=1}^{s} p_i \ln p_i \quad (2)$$

where P_i is the proportion (n/N) of individuals of one particular species found (n) divided by the total number of individuals found.

Soil properties, bacterial abundance, and alpha diversity index data were analyzed by one-way analysis of variance (ANOVA) to determine significant differences among the treatments. Associations between bacterial community composition and a single environmental variable (N deposition) were identified by preliminary verification of variance homogeneity and application of the Anderson's PERMDISP2 procedure, (http://scikit-bio.org/docs/0.5.4/generated/generated/skbio.stats.distance.permdisp.html), which visualizes the distances of each sample to the group centroid in a PCoA and provides a *p*-value for the significance of the grouping

The Pearson correlation coefficient was used to test the relationships between soil properties and alpha diversity indexes. The Spearman's rank correlation coefficient was utilized to assess the correlations between soil properties and bacterial main phyla, as in those cases, some of the data did not comply with distribution normality, calling for a non-parametric method. The correlation between soil properties and bacterial community structure was further investigated by the Mantel test. Pearson and Spearman's correlation tests were performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). ANOVA, PERMDISP2, and Mantel tests were performed using R software (Version 3.6.1). PERMDISP2 is implemented in R using the vegan betadisper function (version 2.4-2, License: GPL-2).

3. Results

3.1. Effects of N Deposition on Soil Properties

Inorganic nitrogen dosage markedly affected most of the measured soil properties, such as pH, NH_4^+-N, NO_3^--N, total nitrogen (TN), and total carbon (TC) contents (Table 1). After 12 months of N deposition, soil pH under N3 treatment was significantly lower than that of the N0 ($p < 0.01$) and N1 ($p < 0.05$) plots. Moreover, the pH under the N2 treatment was significantly lower than that of the N0 control plot ($p < 0.05$). The contents of NH_4^+-N and NO_3^--N under the N2 and N3 treatments were significantly higher than those of the N0 and N1 treatments ($p < 0.01$). While total nitrogen (TN) and total carbon (TC) did not show significant differences ($p > 0.05$) within the N gradient of the same time point, changes were nevertheless occurring in a time-dependent fashion with a steady decrease of TC and an increase of TN.

Table 1. Effects of N deposition on soil properties.

Time	Treatment	pH	NH_4^+-N (mg kg^{-1})	NO_3^--N (mg kg^{-1})	TN (g kg^{-1})	TC (g kg^{-1})
3 months	N0	7.89 ± 0.18 ^{a}	2.95 ± 0.54 ^{a}	27.712 ± 18.84 ^{a}	0.78 ± 0.09 ^{a}	12.77 ± 1.39 ^{a}
	N1	7.77 ± 0.29 ^{a}	3.50 ± 0.40 ^{a}	11.05 ± 5.37 ^{a}	0.82 ± 0.14 ^{a}	13.96 ± 1.23 ^{a}
	N2	7.58 ± 0.29 ^{a}	2.23 ± 0.57 ^{a}	42.04 ± 15.71 ^{a}	0.72 ± 0.07 ^{a}	12.93 ± 1.14 ^{a}
	N3	7.50 ± 0.43 ^{a}	3.03 ± 0.24 ^{a}	19.68 ± 5.79 ^{a}	0.67 ± 0.05 ^{a}	12.14 ± 1.25a
	p *	0.81	0.31	0.40	0.66	0.78
6 months	N0	7.92 ± 0.13 ^{a}	7.49 ± 0.12 ^{b}	0.59 ± 0.03 ^{d}	0.64 ± 0.12 ^{a}	10.88 ± 0.45 ^{a}
	N1	7.22 ± 0.45 ^{a,b}	15.40 ± 6.28 ^{b}	4.22 ± 0.32 ^{c}	0.78 ± 0.18 ^{a}	12.61 ± 1.66 ^{a}
	N2	6.56 ± 0.23 ^{b}	31.61 ± 3.80 ^{b}	9.29 ± 1.04 ^{b}	0.82 ± 0.10 ^{a}	11.34 ± 0.84 ^{a}
	N3	6.23 ± 0.47 ^{b}	75.26 ± 17.67 ^{a}	15.29 ± 1.81 ^{a}	0.94 ± 0.09 ^{a}	10.76 ± 0.41 ^{a}
	p *	<0.05	<0.01	<0.01	0.45	0.54
12 months	N0	7.79 ± 0.08 ^{a}	19.34 ± 0.74 ^{b}	1.48 ± 0.38 ^{b}	0.82 ± 0.08 ^{b}	8.78 ± 0.81 ^{a}
	N1	7.08 ± 0.47 ^{a,b}	37.34 ± 9.41 ^{b}	6.49 ± 0.99 ^{b}	0.88 ± 0.07 ^{b}	8.90 ± 0.74 ^{a}
	N2	6.23 ± 0.47 ^{b,c}	78.94 ± 11.71 ^{a}	17.13 ± 2.45 ^{a}	1.12 ± 0.23 ^{a,b}	8.66 ± 1.31 ^{a}
	N3	5.73 ± 0.47 ^{c}	108.69 ± 18.90 ^{a}	23.18 ± 4.13 ^{a}	1.33 ± 0.12 ^{a}	8.60 ± 0.34 ^{a}
	p *	<0.05	<0.01	<0.01	0.08	0.99

Data are presented as mean and standard errors (in brackets of the five replicates; different letters indicate significant differences between treatments *: *p*-values are based on ANOVA.

3.2. Effects of N Deposition on Soil Bacterial Richness and Diversity

With the increase of N deposition in time and dosages, ecological parameters, such as richness and diversity of soil bacteria, decreased (Figures 1 and 2). At 6 months, soil bacterial richness and diversity were lower than those at 3 months, but the difference among the four dosages was not significant. After 12 months of N treatment, richness and diversity showed an ordered array N3 < N2 < N1 < N0, and there was a significant difference between N3 and N2 treatments ($p < 0.05$), and a highly significant difference between N0 and N1 treatments ($p < 0.01$).

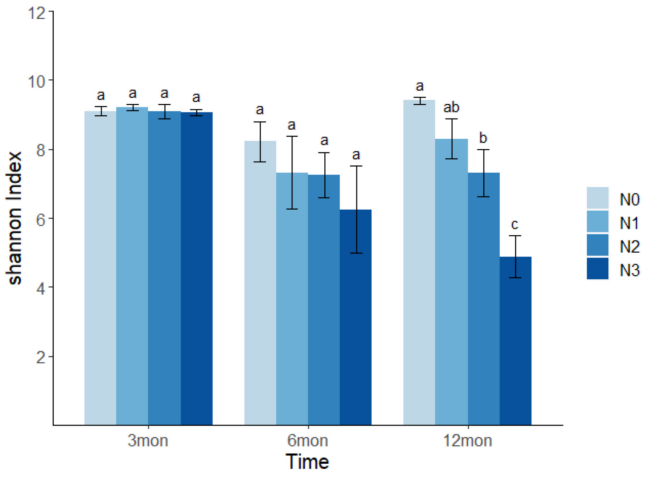

Figure 1. Effects of N deposition on soil bacterial Shannon diversity index. Different letters indicate significant differences among treatments within the same sampling time ($p < 0.05$, ANOVA).

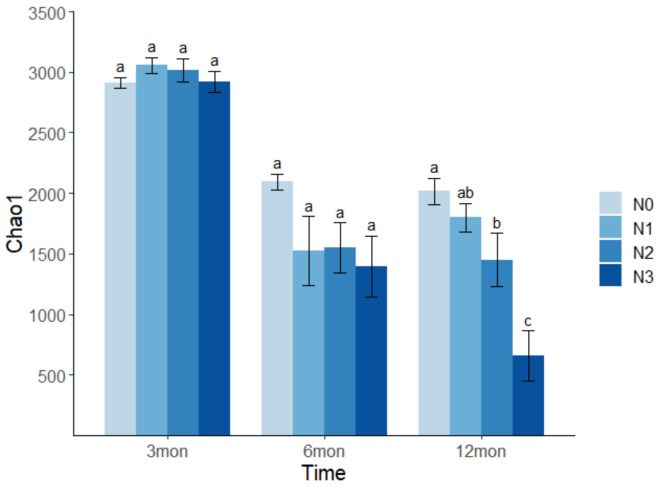

Figure 2. Effects of N deposition on soil bacterial chao1 index. Different letters indicate significant differences among treatments within the same sampling time ($p < 0.05$, ANOVA).

3.3. Effects of N Deposition on Soil Bacterial Community Composition

Upon reads processing ad filtering the actual sequences that were obtained in the bacterial community analysis of three sampling times were 530,447; 960,673; and 522,682; respectively. Classified OTUs belonged to 51 phyla among all samples. As common, due to our still partial knowledge of global microbial diversity, available reference databases do not allow an unambiguous assignment up to the genus or species level for a substantial number of the obtained sequence reads. Therefore, the most reliable annotation is to date prudentially assumed at relatively high taxonomy ranks. For this reason, we will present to the majority of results sticking to the phylum level, and we will later comment the data at finer resolution (from order to genus level) for those cases in which the assignment was backed up by a robust degree of sequence identity.

The bacterial community composition at phylum level (relative abundance >1%) is shown in Figure 3. The dominant phyla (i.e., the ones showing highest values in relative sequence abundance) in all samples were Proteobacteria and Acidobacteria. Besides, the subdominant phyla in all samples were Chloroflexi, Bacteroidetes, Nitrospirae, Acidobacteria, Gemmatimonadetes, Verrucomicrobia, Planctomycetes, Firmicutes, Actinobacteria, Parcubacteria, and Latescibacteria. These phyla represented more than 88% of the sequences in all the samples. After 12 months of N treatment, the relative abundance of Proteobacteria significantly differed among different treatments ($p < 0.01$, Figure S1 in the Supplementary Material), specifically increasing following N enrichment. The percentage of Acidobacteria was low in the N3 treatment but did not differ significantly among the treatments (Figure S1).

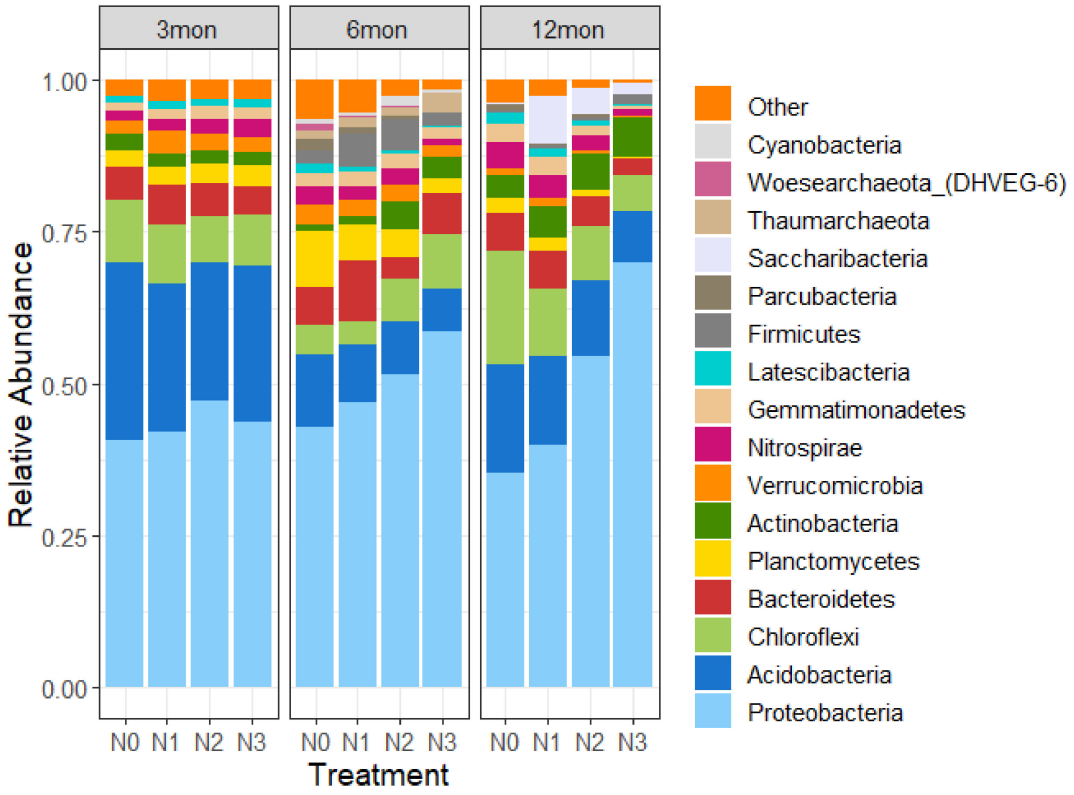

Figure 3. Effects of N deposition on soil bacterial relative abundance. The groups accounting for ≥1% are shown while those >1% are integrated into "other".

The variations in soil bacterial communities caused by N deposition were evaluated by PERMDISP2. Differences in the overall bacterial composition were expressed based on the Bray–Curtis distance. Results are shown in Figure 4. After 3 months (July 2016) of N deposition, different N treatments did not separate, which indicated that at such early time, the bacterial community composition was still similar among all treatments (Figure 4). Subsequently, starting from the second time point (October 2016), a significant difference arose between communities in relation to the N level received. After 12 months (May 2017) of N deposition, the communities separate clearly between N3 and N0 treatments (Figure 4), and the bacterial community composition of N3 treatment resulted profoundly changed. Such significance attains its highest value in the last sampling at 12 months (May 2017). An N deposition rate of N3 or higher was required for a significant shift to occur.

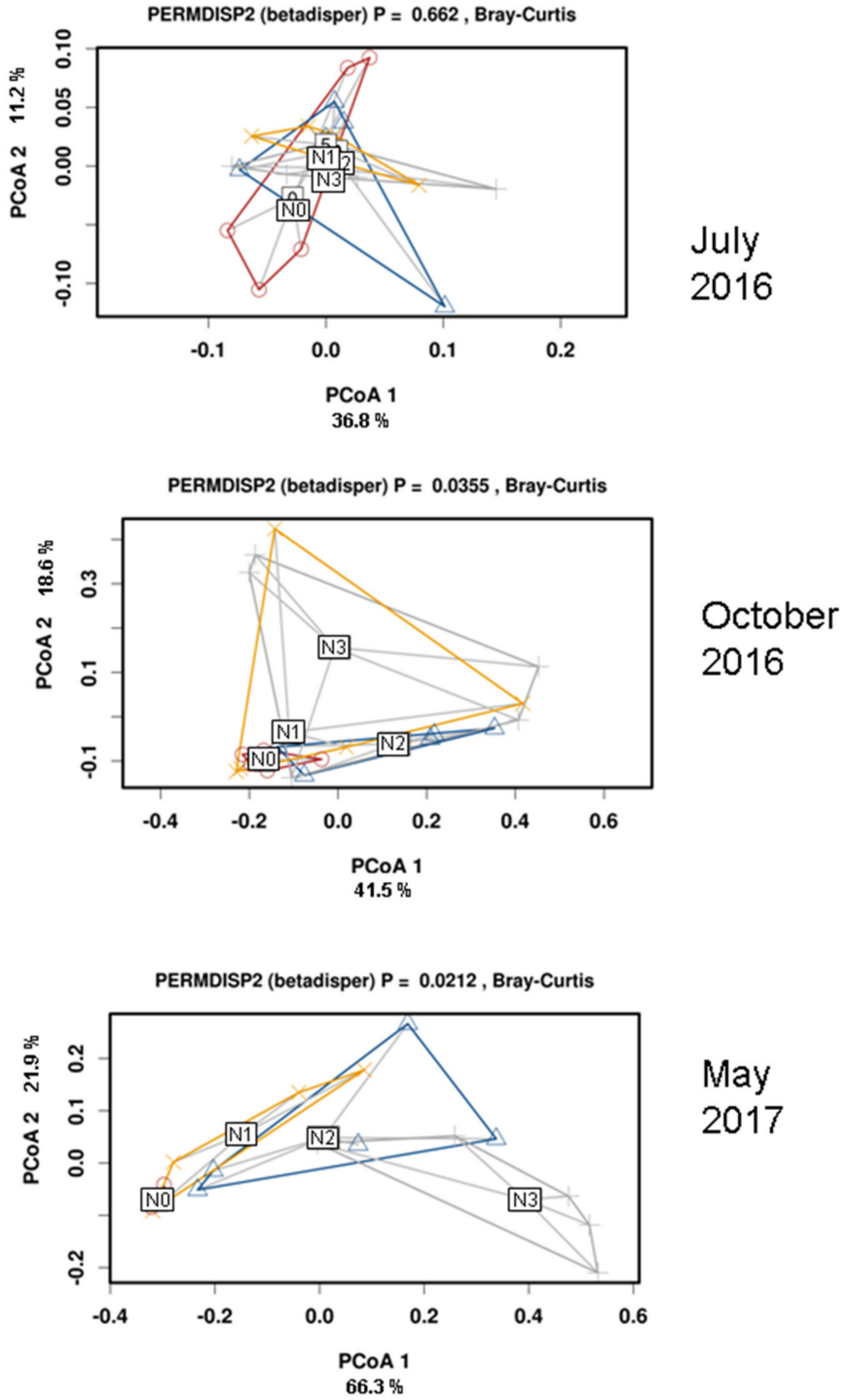

Figure 4. Differences in bacterial community composition among N treatments across different sampling times. PERMDISP2 visualizes the distances of each sample to the group centroid in a principal coordinate analysis (PCoA) and provides a *p*-value for the significance of the treatments.

3.4. Effects of Soil Chemical Properties on the Soil Bacterial Diversity and Community Composition

The correlations of bacterial diversity indices and main phyla with soil properties are shown in Figure 5. Values of the Chao1 index were positively correlated with soil pH ($p < 0.001$) and TC contents ($p < 0.001$) and were significantly negatively correlated with NH_4^+-N ($p < 0.001$), and TN contents ($p < 0.05$). Values of the Shannon index were positively correlated with soil pH ($p < 0.001$) and TC contents ($p < 0.05$) and were signifi-

cantly negatively correlated with NH_4^+-N ($p < 0.001$). The majority of the predominant phyla showed a significant correlation with certain soil chemical properties, whereas only the phyla. The relative abundances of Proteobacteria were negatively correlated with soil pH. Actinobacteria and Firmicutes were also negatively correlated with soil pH, whereas they showed a positive correlation with the NH_4^+. The abundances of Acidobacteria, Planctomycetes, Latescibacteria, Verrucomicrobia, and Parcubacteria presented a positive relationship with pH, while they showed a negative relationship with NH_4^+-N. Nitrospirae and Gemmatimonadetes did not exhibit a significant correlation with any of the soil parameters.

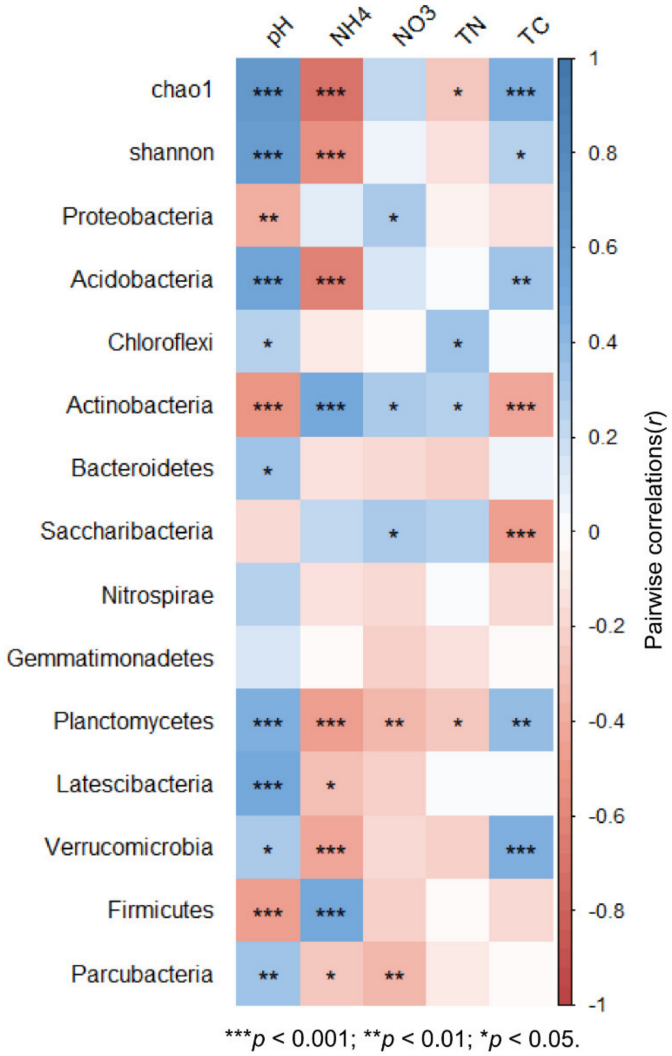

Figure 5. Pairwise correlations of bacterial diversity indices and abundant phyla (relative abundance >1%) with soil properties. The Pearson correlation coefficient was used to test the relationships between soil properties and alpha diversity indexes. Spearman correlation coefficients were utilized to assess the correlations between soil properties and bacterial main phyla.

The Mantel test explored the correlation between bacterial communities and soil properties (Table 2). The correlation coefficients, in decreasing order of absolute values, were, for positive correlations: pH, NH_4^+-N, and TN; while for negative correlations: TC, and NO_3^--N. Two of the most important factors, pH ($p < 0.05$) and NH_4^+-N ($p < 0.05$), were closely correlated with bacterial community composition. The non-opposite trend for pH and ammonium appearing from Table 2 is, in our view, due to the fact that, although ecological diversity indexes are abated by increasing ammonium, and decreasing pH, a number of numerically relevant phyla are enhanced. Thus, the overall value of community

structure is not as impaired by such substitutions in relative abundance as the Simpson and evenness parameters are.

Table 2. Correlations among the overall bacterial community structure and soil proprieties according to the Mantel test.

Soil Properties	*r*	*p*
pH	0.2273	0.004
NH_4^+-N	0.1799	0.037
NO_3^--N	−0.1457	0.986
TN	0.0066	0.401
TC	−0.0815	0.848

It must be observed however that in Table 2, while for the pH parameter, the *p* value is very significant, for the ammonium nitrogen, significance is borderline, and the *r* value is very low.

4. Discussion

4.1. Effects of Soil Chemical Properties on the Soil Bacterial Diversity and Community Composition

To clarify the global interactions between urban soils and their inhabiting microbes, we deemed necessary to understand the specific ones occurring between urban green space soil and hosted bacteria, in order to seize the effects of N enrichment on the whole urban ecosystem functioning. Sequencing analyses revealed that Proteobacteria and Acidobacteria were the dominant phyla in all samples. When considering the time and dose-related patterns, Proteobacteria specifically increased following N enrichment, while Acidobacteria, Chloroflexi, Bacteroidetes, and Nitrospirae exhibited the opposite trend. Therefore, the response of the bacterial community to N deposition appears to vary substantially among different phyla.

The deposition of N as a nutrient may trigger both direct and indirect effects on microbial communities and diversity [40,41]. These can affect the soil nutrient pool, along with environmental factors changes. N deposition could affect soil bacteria by changing soil inorganic N content. In addition to soil N content, pH is one of the critical factors affecting soil microbial diversity [42,43], because N could indirectly affect soil bacterial community by soil acidification [44,45]. It is, in fact, widely accepted that soil acidification can directly influence soil bacterial community composition [43,46]. In this sense, most of the prior studies indicated that soil pH was more important than nutrients in shaping bacterial community structure [47–50].

In our experiments, we observed that the response of urban green space bacteria to N enrichment appears related mainly to NH_4^+-N and to its effects on soil pH (Figure 5). The soil dissolved inorganic N (NH_4^+-N and NO_3^--N were generally correlated with the N treatment gradient, which was accompanied by a corresponding decrease in soil pH. The reason for pH decreases as a consequence of ammonium-based fertilization is mainly to be seen in the fact that when ammonium undergoes nitrification it leaves net H^+ ions into the circulating solution. Bacterial sensitivity to acidic conditions is widespread and mainly due to the scarce attitude to maintain neutrality within the cytoplasm, to avoid amino acid charged groups depolarization.

From the statistical point of view, significant differences resulted in soil pH, NH_4^+-N and NO_3^--N among different N treatments after 12 months of experimentation (Table 1). Considering the different forms of N, both NH_4^+-N and NO_3^--N can cause soil acidification [51]. Our data showed that soil bacterial community composition was closely related to the soil chemical parameters. In fact, some of the parameters did correlate and they overlapped in their explanatory power under N enrichment. The correlations of phylum-level abundances with soil pH and NH_4^+-N were observed for most of the dominant phyla (Figure 5). In particular, our results suggest that the bacterial community structure has

a significant relationship with soil pH and NH_4^+-N (Table 2). Soil mineral N availability was supposedly also tightly connected to the bacterial community structure as other studies reported [50,52]. Besides, Nie et al. pointed out that ammonium nitrogen content was a dominant predictor of bacterial community composition in acidic soil with exogenous nitrogen enrichment [53]. An alternative hypothesis underlying the response of dominant phyla to the N addition is the functional classification model. Proteobacteria and Actinobacteria, that have fast growth rates, were more likely to increase in nutrient-rich conditions, while Acidobacteria and Chloroflexi that have slower growth rates, would likely decline [35,54]. In general, Acidobacteria are adapted to low pH conditions [55]. However, after 12 months of added N, Proteobacteria were enriched under high N treatment, while interestingly, Acidobacteria declined. N2 and N3 treatments were shown to lower pH but, in comparison to the N0 treatment, Acidobacteria did not increase (Figure 3). The reasons for this behavior could be sought in the fact that effects of pH on the different groups of Acidobacteria can be very different [56]. In this sense, as confirmed by our study, pH might not necessarily be the main driver of changes for the Acidobacteria phyla as a whole. Moreover, correlations between soil pH and bacterial communities following N deposition do not necessarily demonstrate nor imply their direct causative relation. In comparison, soil ammonium nitrogen was an important environmental factor that appeared to explain many of the changes in the soil bacterial community structure observed in the present study. Specifically, N deposition directly affected urban green space soil bacterial communities in relation to the increase in soil ammonium.

However, we noticed that also the ammonium in the N0 plot increased along time. Atmospheric N deposition is closely related to precipitation [57]. Previous studies have shown Guangzhou's inorganic N (NH_4^+-N and NO_3^--N) coming as precipitation displays a seasonal fluctuation, and NH_4^+-N was the primary form of N deposition in Guangzhou [58]. In 2011, the NH_4^+-N content in precipitation was 13.876 kg ha^{-1}, and NO_3^--N was6.562 kg ha^{-1}. Especially in summer, NH_4^+-N in precipitation reach 951.44 mg.m^{-2} 9.514 kg ha^{-1}, while NO_3^--N was 4.484 kg ha^{-1} [59]. Since our experiments were set in open air, and were therefore subjected to natural N deposition, this background can explain the observed rise of N also in the control plot. As regards the drop of total carbon that occurs in time, this can be seen as a consequence of the increase of N which is therefore no longer a limiting factor for carbon consumption and assimilation by soil biota, thus lowering the C/N ratio and pushing more carbon towards its mineralization and volatilization as CO_2.

4.2. Temporal Trends and Potential Consequences of N Deposition

The present study focused on the delicate areas of the urban greens in the Chinese atmospheric fallout is consistent with previous ones that showed the decline of soil bacterial biodiversity under N enrichment [40,59–61]. Considering the three observation periods separately, the short-term (3 months) N deposition did not change the composition of soil bacterial diversity and communities (Figures 1, 2 and 4). The response of soil bacterial alpha diversity and community composition to N deposition was gradually confirmed after N addition for 6 months and 12 months (Figures 1, 2 and 4). Results indicate that high deposition (100 and 150 kg N ha^{-2} y^{-1}) caused the critical changes in soil bacterial of urban green space. Experiments that use a high rate of N deposition may be useful for assessing the long-term effects of chronic low rates of N deposition [62]. The results of this study indicate that the higher deposition or accumulation of N in the urban green space deeply affects patterns of bacterial diversity and community structure. The bacterial response is affected by the amount of added N as well as by the duration of the treatment. Overall, nitrogen (N) addition had a negative impact on bacterial richness and diversity in the urban green space in both dose- and time-dependent fashions.

At finer taxonomy level, it is worth reporting that one of the genera that markedly increased, as N was added, was *Mizugakiibacter*, a genus known to be a denitrified, which is expected as a consequence of the added nitrate and the oxidation of ammonium. On the opposite side, the main decline regarded the *Nitrosomonadales* order, which is consistent with

the fact that nitrifiers are made superfluous by our addition of N forms, which are already oxidized, as nitrate is. We must not underestimate that N deposition causes changes in the soil environment because it does change bacterial composition at finer taxonomic levels other than phylum, and most of all that it heavily impacts on overall taxa diversity and richness. If N deposition keeps increasing in the future, the decline of bacterial diversity may become dramatically worse. Soil microbial diversity plays a central role in ecosystem processes by driving the Earth surface's biogeochemical cycles [63,64], and therefore any changes in soil bacteria by N deposition might affect not only urban ecosystem processes and functions, but eventually the global ones.

5. Conclusions

High N deposition rate resulted in significant changes in bacterial community composition and showed a major loss of bacterial diversity in soil. The different responses of major phyla to N enrichment appear to be the main reason for the change in the overall bacterial community composition. pH and ammonium resulted, themselves, correlated and overlapped in their explanatory power under N deposition. Apparently, N deposition directly affected bacterial community composition by increasing soil ammonium content. The changes in bacterial community composition and diversity were tightly connected with the amounts of added N, as well as with the duration of the treatment. The decrease in biodiversity induced by N deposition may pose a serious threat to urban soil ecosystem stability, which emphasizes the necessity of thorough and concerted studies to prompt adequate policies to counteract these globally increasing threats.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-3417/11/3/918/s1, Figure S1: Effects of N deposition on the relative abundance of dominant phyla after 12 months. Different letters indicate significant differences among treatments within the same sampling time ($p < 0.05$, ANOVA).

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