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Letter

Hydrological Perturbations Facilitated Phyllosphere Denitrification of an Urban Greening Tree

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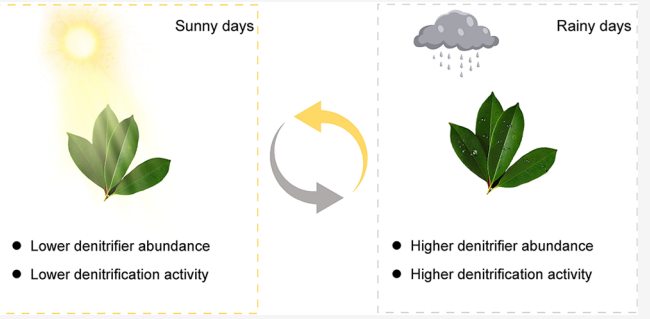
ABSTRACT: In urban ecosystems, nutrient stocks, such as carbon (C), nitrogen (N), and phosphorus (P), are anthropogenically being enriched for material and energy supply. The overloaded nutrients have adverse ecological consequences, such as causing eutrophication of waters and soil in urban areas. Studying the ecophysiology of nutrient-cycling microbes in urban areas is the foundation to explore strategies for removing such excessive nutrients. The phyllosphere is an understudied microbial habitat for examining how the urban microbiome responds to common environmental changes, such as hydrological perturbations. Here, we investigated how successive rainy−sunny cycles within a season affect the genetic potential (gene abundances) for leaf nutrient cycling and particularly the functional potential (enzyme activities) for leaf denitrification of the greening tree *Photinia fraseri*. Of 41 detected C, N, P, and sulfur (S) cycling genes using high-throughput quantitative polymerase chain reaction, rainfalls only significantly ($p < 0.05$) increased the abundances of denitrification marker genes *nirK* and *nirS* and one C-fixation gene on the phyllosphere while having no significant impacts on other nutrient-cycling genes. The *nirK* and *nirS* genes encode nitrite reductases, which catalyze the hallmark step of the denitrification process. Further, a denitrification enzyme activity assay of phyllosphere microbiota showed that, in comparison to sunny weather, rainfalls significantly promoted nitrate reduction (5.48 μmol of NO_3^- g^{-1} h^{-1}; $p < 0.001$) and N_2O production (2.07 nmol of N_2O g^{-1} h^{-1}; $p < 0.05$) rates, respectively. Together, this study revealed that hydrological perturbations can affect tree phyllosphere denitrification. Understating the ecophysiology of urban phyllosphere denitrifying microbes might be important for developing suitable phylloremediation strategies to attenuate urban N inputs.



KEYWORDS: *rainfall, leaf microbiota, nitrate reduction, nitrous oxide, urban ecosystem*

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1. INTRODUCTION

Globally, nutrient stocks, including carbon (C), nitrogen (N), and phosphorus (P), are increasingly being imported into urban ecosystems for food supply and economic development. Nutrients, in other words, are also urbanized in addition to the population. This can accelerate and aggravate the imbalance of resource flows along the urban-to-rural gradient.[1,2] Of these life-relevant elements, N occurs at most valence states and species, and its biogeochemical cycling is highly dynamic in Earth's critical zone, i.e., the life-sustaining surface of our planet.[1] As a key component of Earth's critical zone, the urban ecosystem has received growing concerns for studying N biogeochemistry. To date, studies documenting urban microbial N cycling predominantly focus on soils[3−6] and waters.[7,8] In contrast, the plant phyllosphere is an understudied microbial habitat for evaluating the interactions between residing N-metabolizing microbes and urban environment changes.

The phyllosphere, the aboveground portions of plants, has been estimated to constitute around 60% of the Earth's biomass,[9] with the total area of leaf surfaces (both upper and lower sides) over 10^9 km^2 across the globe.[10,11] The external surface and internal tissues of the leaf are populated by diverse microbes, occupying epiphytic and endophytic niches, respectively.[11] The phyllosphere is an ecologically open milieu, and the inhabiting microbes are at the interface between the plant and the atmosphere.[12] In addition to being tightly linked to the productivity and fitness of the host plants,[13] the phyllosphere microbiota can influence atmospheric chemistry, for example, through microbial N fixation.[14] Given that N makes up the majority of Earth's atmosphere, the interplay between phyllosphere N-metabolizing microbes and the extant atmosphere should not be overlooked in biogeochemical

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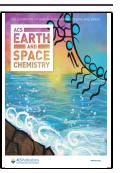

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studies. Existing literature investigating phyllosphere microbial N-cycling processes mainly focus on N fixation[15−18] and aerobic nitrification.[19,20] In contrast, phyllosphere denitrification receives much less attention and remains largely unexplored in terms of its ecological significance in N biogeochemistry.[21] Denitrification describes the process of anaerobic respiration of nitrate/nitrite to nitrogenous gases, including complete ($NO_3^-/NO_2^-/\rightarrow NO \rightarrow N_2O \rightarrow N_2$) and incomplete ($NO_3^-/NO_2^-/\rightarrow NO \rightarrow N_2O$) denitrification, with the potent greenhouse gas N_2O as an intermediate or an end product, respectively.[22,23] Because urban areas receive large N inputs,[24] including nitrate and other nitrogen oxides (NO_x), via wet/dry deposition,[25] complete denitrification can help to remove excessive N from urban ecosystems and release it into the atmosphere as harmless N_2. Unlike urban waterbodies and soils, which have been well-studied for the denitrification process and associated microbes over the past 2 decades, denitrification in the urban plant phyllosphere has scarcely been explored. Digging into the ecophysiology of phyllosphere denitrifiers, particularly the epiphytes that colonize the surface of plants and can directly interact with the surroundings, could aid in developing "phylloremediation"[26,27] strategies for curbing overloaded N in urban settings.

Precipitation (e.g., rainfall and snow) is the primary hydrological perturbation for terrestrial trees and shrubs, particularly to upper and exterior layers of the canopy. In the context of global change, the interplay between the atmospheric water vapor content and temperature have intensified daily rainfall extremes over global land areas.[28−30] However, it is not well-understood how phyllosphere nutrient-cycling microbes and particularly the denitrifiers and denitrifying function respond to rainfall events in urban areas. Notably, next to enhancing the mobility of substates, rainfall may create more anoxic microsites and lower the redox potential of the leaf surfaces. This is expected to be favorable for the denitrification process. Hence, the present study aims to determine how rainfalls affect the genetic (gene abundances) and functional (enzyme activities) potential of leaf epiphytes for denitrification. To this end, we sampled leaves of *Photinia fraseri*, a typical urban greening plant in China, over successive rainy−sunny cycles in a summer−rainy season. High-throughput quantitative polymerase chain reaction (HT-qPCR) was employed to quantify genes associated with C, N, P, and S (CNPS) cycling, including denitrification. A denitrification enzyme activity assay was conducted to evaluate activities of denitrification-related enzymes. We anticipate the findings would elucidate the role of phyllosphere microbes in the N removal from the urban ecosystems and, therefore, shed light on new strategies confronting climate change.

2. MATERIALS AND METHODS

2.1. Leaf Sampling. In summer 2021, leaves of the evergreen tree *P. fraseri* were collected from a suburb park (29° 45′ N, 121° 53′ E) in Ningbo, China. Intact green leaves with nearly identical size from three adjacent *P. fraseri* (around 3 m high) (Figure 1a) were sampled at 1.5 m height above ground using sterile scissors and transferred to sterile bags (Nasco, Whirl-Pak, Madison, WI, U.S.A.) for storage. We selected leaves from the exterior layer because they are readily exposed to rains. For each individual *P. fraseri* tree, sampled leaves were mixed. Within 15 min after sampling, leaves were used for phyllosphere microbial DNA extraction and denitrification activity assay in the lab as described later, with the remaining oven-dried for moisture and elemental analysis (Table S1 of the Supporting Information). In total, we sampled leaves over four successive rainy−sunny cycles from June to August when rain took place weekly, generating four sampling time points for rainy and sunny days, respectively. The time interval between each rainy−sunny cycle that we selected was 2 weeks. For each hydrological cycle, leaves were collected 2 days before rainfalls (sunny weather) and on the second day during rainfalls (with 0.058 mM nitrate and 0.027 mM ammonium on average). The rainy season had an average temperature of 27.2 °C and a mean precipitation of 341.1 mm. During this period, the greening tree *P. fraseri* received no fertilizers and pesticides.

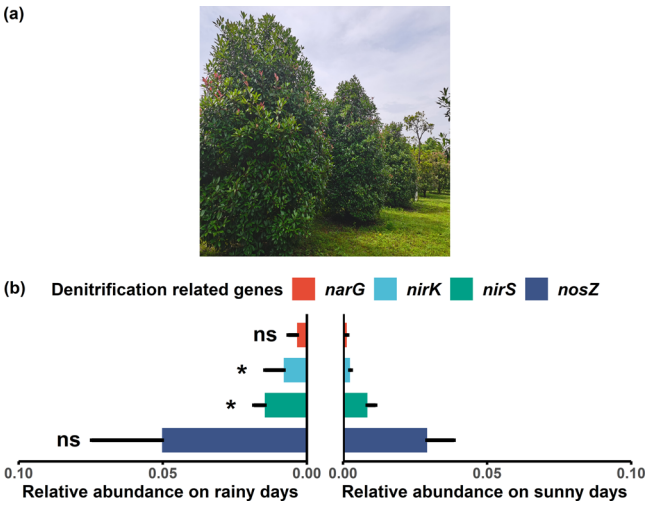

Figure 1. (a) Greening tree *P. fraseri* investigated in this study, photographed by Yi-Fang Zhang. (b) Mean (±standard deviation; n = 12) relative abundances of denitrification-related functional genes on the phyllosphere of leaves sampled on rainy or sunny days. An asterisk denotes a significant statistical difference between treatments (*, p < 0.05), and ns indicates that the statistical difference is not significant.

2.2. DNA Extraction and HT-qPCR. For each individual *P. fraseri* tree, the microbes from leaf surfaces were collected by mixing around 7 g (11−12 pieces) of leaves with 200 mL of 0.01 M autoclaved phosphate-buffered saline (PBS) solution, then sonicating for 5 min, and shaking at 160 rpm for 1.5 h. Afterward, the samples were sterile-filtered through a 0.22 μm cellulose membrane.[31] The filtrate was discarded, and the membrane with the retained microbial community was stored at −20 °C. The DNA was extracted within 3 days using the FastDNA Spin Kit for soil (MP Biomedicals, Santa Ana, CA, U.S.A.) following the instructions of the manufacturer. The purity and concentration of the DNA samples were checked using an ultraviolet−visible spectroscopy spectrophotometer ND-2000 (NanoDrop, Thermo Scientific, Waltham, MA, U.S.A.). The DNA samples were then kept at −20 °C until HT-qPCR analysis.

A HT-qPCR-based chip, namely, quantitative microbial element cycling (QMEC),[32] was employed to quantify the abundance of functional genes involved in microbial C, N, P, and S cycling. QMEC comprises 71 primer pairs for amplifying and quantifying bacterial functional genes, including 36 C-cycling genes, 22 N-cycling genes, 8 P-cycling genes, and 5 S-cycling genes, as well as one primer pair for amplifying and quantifying the bacterial 16S rRNA gene (see Table S2 of the Supporting Information for details of all primers). For *nosZ* genes encoding N_2O reductase, next to the classic clade I *nosZ*,

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we added the atypical clade II *nosZ*[22,33] into the HT-qPCR analysis. The QMEC method allows for the parallel quantification of 72 DNA samples by a Smart-Chip PCR platform (WaferGen Biosystems, Fremont, CA, U.S.A.). The thermal profile of amplification was 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. Results with a threshold cycle (C_T) less than 33 were used for further analysis. Relative copy numbers of the functional gene and 16S rRNA gene as well as their ratio, which represents the normalized relative abundance of the functional gene, were calculated as described in ref 32.

2.3. Microcosm Incubation. To examine the impacts of hydrological perturbations on the denitrification function of epiphytes, we conducted a denitrification enzyme activity assay with fresh leaves sampled at each rainy/sunny time point. For each individual *P. fraseri* tree, leaves (around 7 g) were transferred to 309 mL sterile glass bottles with 150 mL sterile anoxic pure water. We then supplied nitrate as a denitrification substrate from sterile anoxic stock solutions of $NaNO_3$, to give a final concentration of 10 mM nitrate. The treatments without nitrate addition were used as blank controls. Each treatment was performed in independent triplicates, corresponding to the three adjacent *P. fraseri* trees. All bottles were sealed with sterile big butyl rubber stoppers and caps, then evacuated and flushed with helium (He) gas (99.999% purity) for five cycles, and finally vented to atmospheric pressure. Thereafter, all anoxic bottles were incubated at 28 °C in the dark for 7 h.

At the beginning (0 h) and end (7 h) of the incubation experiment, 2 mL of solution samples were collected and centrifuged (14000*g* for 3 min) to remove pellets. The supernatants were used for the subsequent measurement of NO_3^-, NO_2^-, and NH_4^+ contents. At the end of incubation, 6 mL of headspace gas was sampled and injected into 120 mL crimped serum bottles with pre-filled pure He. Before gas sample injection, 6 mL of He gas has been withdrawn from the serum bottles. These serum bottles were used for gas measurements as described below.

2.4. Analytical Methods. The total amounts of C, N, and S of leaves were determined using an element analyzer (Vario MAX CNS, Germany) (Table S1 of the Supporting Information). The concentrations of NO_3^-, NO_2^-, and NH_4^+ were quantified using a flow injection analysis (FIA) system (AutoAnalyzer 3, Bran Luebbe/SEAL Analytical, Norderstedt, Germany). The contents of N_2O and CO_2 were quantified by an Agilent 7890A gas chromatograph (Palo Alto, CA, U.S.A.). Dissolved N_2O in the aqueous phase was calculated from headspace N_2O using the Ostwald coefficient, with a value of 0.5502 (liters of gas per liter of water at 1 atm partial pressure) for N_2O at 28 °C.[33,34] The sum of headspace and aqueous N_2O represents total N_2O produced in the vessel. Likewise, the total CO_2 content was calculated using an Ostwald coefficient for CO_2 of 0.7722 at 28 °C.[34] The rates of nitrate consumption, denitrification-derived N_2O emission, and anoxic respiration were calculated on the basis of changes in nitrate, N_2O, and CO_2 over the entire incubation, respectively.

2.5. Statistical Analysis. The statistical analysis was conducted with R (version 4.1.2). The Shapiro−Wilk normality test and Levene test were performed to evaluate the distribution and variance of data sets, respectively. Unpaired two-tailed Student's *t* test (normal distribution and equal variance), Welch's *t* test (normal distribution and unequal variance), or Wilcoxon rank-sum test (abnormal distribution) was used to determine the statistical difference of all data between rainy and sunny treatments at $p < 0.05$. Spearman rank correlation analysis was used to evaluate the relationship among independent variables at $p < 0.05$. All experimental results are given on an oven-dry basis.

3. RESULTS AND DISCUSSION

3.1. Abundance of Nutrient-Cycling and Denitrifying Bacteria. The functional genes involved in bacterial CNPS cycling were quantified on the basis of HT-qPCR of the targeted genes and were normalized to bacterial 16S rRNA genes. A total of 41 targeted functional genes were detectable on the *P. fraseri* phyllosphere (Table S3 of the Supporting Information). Rainy days appeared to slightly enhance relative abundances of most CNPS cycling genes (38 in total) on leaf surfaces compared to sunny days but without significant differences (Table S3 of the Supporting Information). Other studies have reported that short-term disturbances, such as rain events,[35−37] just exerted a weaker and negligible effect on foliar bacterial communities when compared to long seasonal patterns.[38,39] Our results are generally in line with these reports. Nonetheless, we observed that the 38 CNPS cycling genes exhibited the same trend; i.e., rainy days caused a higher relative abundance on average than that of sunny days, although showing no significant changes (Table S3 of the Supporting Information). The trend that rainfalls within a season tend to enhance the relative abundances of phyllosphere CNPS cycling genes may be explained by two plausible mechanisms. First, the rainfall event is along with microbial mass dispersal carrying airborne microbial genes and cells onto the leaf surfaces for colonization.[40−42] Second, rainfalls could increase the mobility and accessibility of nutrients for the growth of phyllosphere microbiota and could also introduce external nutrients, such as precipitated N.[12,43]

Note that, of all detectable CNPS cycling functional genes, *nirK* and *nirS* are among the only three genes (another gene is *frdA* responsible for C fixation) that showed moderate evidence for difference ($p < 0.05$) between rainy day and sunny day samples (Table S3 of the Supporting Information and Figure 1b). The denitrification marker genes *nirK* and *nirS* encode nitrite reductase that catalyzes the hallmark step of the denitrification process by converting nitrite to nitrogenous gases. We found moderate evidence ($p = 0.029$ for *nirK* and $p = 0.041$ for *nirS*) that rains can enhance the relative abundance of these genes on the phyllosphere. As shown in Figure 1b, leaves sampled on rainy days had an average abundance of 0.803% for the *nirK* gene and 1.45% for the *nirS* gene, 215.04 and 72.18% higher than those of leaves collected on sunny days, respectively. For other functional genes associated with complete denitrification, rains tended to slightly enhance the abundance of the *narG* gene that encodes nitrate reductase and the *nosZ* gene that encodes N_2O reductase but without significant differences (Figure 1b). The *napA* gene that can also encode nitrate reductase was not detectable in all samples (Table S3 of the Supporting Information).

The existence of denitrification-related genes in the phyllosphere epiphytic environment of terrestrial plants has been reported for farmland and forest vegetation[31] as well as high-mountain neotropical ecosystems,[44] albeit without explicitly validating the metabolic activities in those studies. Similar findings have been observed for floating plants. For example, denitrifying functional genes were found in epiphytes of floating macrophytes from a freshwater lake, as revealed by traditional qPCR analysis.[45] A metagenomic shotgun sequenc-

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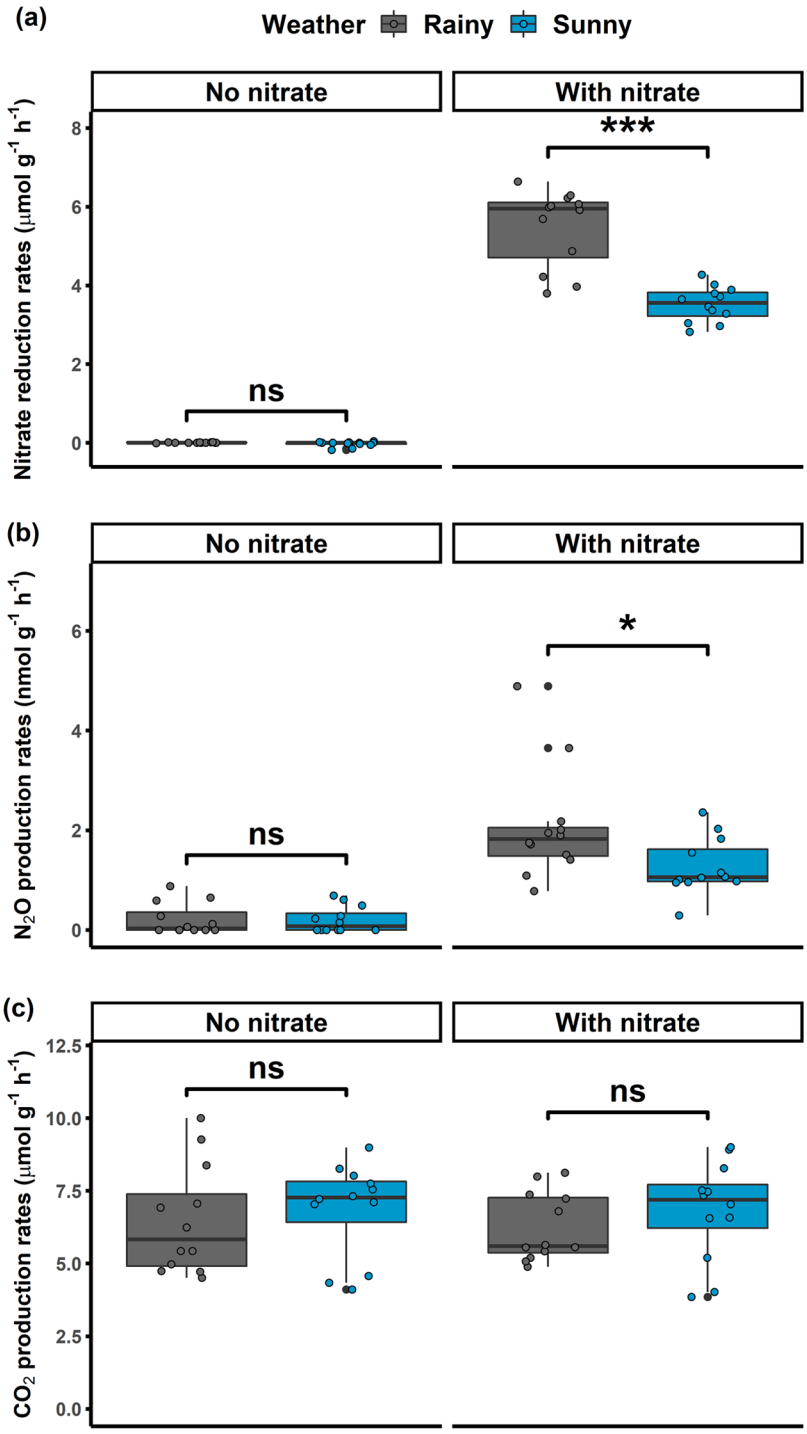

Figure 2. (a) nitrate consumption rate, (b) denitrification-derived N_2O emission rate, and (c) anoxic respiration rate in microcosms of leaves (sampled on rainy or sunny days) through the entire incubation (n = 12). An asterisk denotes a significant statistical difference between treatments (*, $p < 0.05$; ***, $p < 0.001$), and ns indicates that the statistical difference is not significant.

ing study implied genes for phyllosphere endophyte denitrification of a floating fern.[21] Note that the classic clade I *nosZ* gene was measurable in all samples, whereas the atypical clade II *nosZ* gene was not detected. A substantial fraction of microorganisms bearing clade II *nosZ* is composed of non-denitrifying N_2O reducers, which could be true sinks of the greenhouse gas N_2O but without producing it. In the past decade, emerging evidence have demonstrated the prevalence of clade II *nosZ* genes in bacterial and archaeal genomes in many environments, such as soils,[33,46] sediments,[47,48] and a bioreactor,[49] yet very little is known about their presence in the plant phyllosphere. In this study, however, we did not observe the residence of phyllosphere epiphytes that harbored clade II *nosZ* genes. Together, our results indicated that the *nirK*- and *nirS*-bearing denitrifiers are more sensitive to hydrological perturbations than *narG*-type nitrate reducers and clade I *nosZ*-type N_2O reducers.

3.2. Denitrification Activity. To measure the denitrification activity, we introduced the method routinely used in soil biogeochemistry into our study on phyllosphere microbes. In

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brief, we incubated the leaves under ideal denitrifying conditions, i.e., anoxic and nitrate replete. To the best of our knowledge, we are the first to apply this method for studying phyllosphere microbes. The phyllosphere of terrestrial plants is detached from soil, making the leaf surface an oligotrophic microhabitat.[50] Hence, the organic carbon source was not added, and only nitrate was supplemented as the electron acceptor in our microcosm. It has to be noted that the acetylene inhibition method,[51] which is often used for measuring soil potential denitrification activity (represented as total N_2O and N_2 emissions) owing to the inhibitory properties of acetylene on the N_2O reduction step, was not adopted here. The reason is that acetylene could serve as a carbon source for soil microbes[52] and, therefore, may supply an unwanted external carbon source for microbes inhabiting the nutrient-deficient phyllosphere. Therefore, we rather assessed the denitrification activity in the combination of the observed nitrate reduction rates and denitrification-derived N_2O emission rates in acetylene-free nitrate-amended treatments.

We found very strong evidence ($p < 0.0001$) that rainy days stimulated the nitrate reductase activity of phyllosphere microbes compared to sunny days. As shown in Figure 2a, leaves sampled on rainy days had an average nitrate consumption rate of 5.48 μmol of NO_3^- g^{-1} h^{-1}, 55.36% higher than that of leaves collected on sunny days. The occurrence of dissimilatory nitrate reduction to ammonium (DNRA, $NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+$) could be excluded, because the end product NH_4^+ was not detected at the beginning and end of the 7 h incubation, including treatments both with and without nitrate. Hence, the observed nitrate reduction could be attributed to denitrification. This is further supported by the well-known fact that DNRA is unfavorable under electron donor (e.g., organic carbon) limiting and nitrate excess conditions.[53,54]

In the nitrate-free controls, N_2O was detectable and accumulated with lower rates ($p < 0.0001$ for rainy days and $p < 0.0001$ for sunny days) than corresponding nitrate-amended treatments (Figure 2b). These results, on one hand, revealed the *in situ* metabolic activity of N_2O-producing anaerobes on the phyllosphere of sampled leaves and, on the other hand, showed the stimulation effects of the denitrifying substrate nitrate on N_2O emissions derived from denitrification. Further, there was moderate evidence ($p = 0.045$) that rainfalls enhanced denitrification-derived N_2O emissions of phyllosphere-associated microbiota. The average N_2O emission rate of leaves sampled on rainy days (2.07 nmol of N_2O g^{-1} h^{-1}) was 63.1% larger than that of leaves collected on sunny days (Figure 2b) and was positively correlated ($p < 0.05$) with the relative abundances of denitrification marker genes (*nirK* and *nirS*) in the rainy day samples than sunny day samples (Figure 1b). The observed N_2O emissions, together with the genetic evidence of denitrifiers as discussed above, demonstrated the functional potential of denitrification microbes on the phyllosphere. In addition, the higher denitrification-driven N_2O emission rates of rainy day leaf epiphytes may be attributed to the more abundant *nirK*- and *nirS*-harboring denitrifiers than those of sunny day leaves. In contrast, there was no clear correlation ($p = 0.272$) between nitrate reduction rates and the *narG* gene abundance, which likely needs to be explained at the transcript or protein level that was not examined in this study.

Both rainfalls ($p = 0.597$ for nitrate-free treatments and $p = 0.345$ for nitrate-amended treatments) and nitrate addition ($p = 0.717$ for rainy days and $p = 0.817$ for sunny days) showed no evidence for any effect on the anoxic respiration rates (CO_2 emission rates) across all microcosms (Figure 2c). Although rainfalls and nitrate addition promoted denitrification activity, their effects on the overall anoxic respiration rates seemed to be negligible because denitrifiers only accounted for a small fraction of total bacteria.

3.3. Environmental Implications. Our results showed the genetic (gene abundances) and functional (enzyme activities) potential for epiphytic denitrification of the tree *P. fraseri* in a suburban park and revealed the stimulation effects resulting from rainfalls, which serve as the predominant hydrological perturbation for terrestrial trees and shrubs. We argue that the plant phyllosphere, as a previously overlooked microbial habitat for denitrifying bacteria, may contribute to the mitigation of urban reactive N by denitrifying nitrate or nitrite to nitrogenous gases and releasing them into the atmosphere. Thus, planting more trees in urban areas is expected to help in minimizing the transport of reactive N to soils and groundwater. On the one hand, the tree canopy can retain some rainfalls and, thus, reducing nutrient leaching.[55] On the other hand, phyllosphere complete denitrifiers could respire nitrate or nitrite to the N_2 end product, particularly at night when receiving no sunlight and the stomata are closed. Furthermore, we conclude that, by enhancing the denitrifier abundance and denitrification function of the tree phyllosphere, rainfalls could promote the biological removal of N on urban tree leaf surfaces that accumulated before and after rain events via dry deposition as well as during rainfalls via wet deposition. On the basis of this, phylloremediation technologies[26,27] could be developed for biological N removal from urban ecosystems. As a straightforward example, for cities in relatively dry regions, increasing the frequency of irrigation on urban evergreen plants may improve the removal of reactive N through complete denitrification. Nonetheless, such phylloremediation technologies should make efforts not only to maximize the rates and extent of phyllosphere denitrification but also to minimize the byproduct flux of N_2O, thereby curbing the greenhouse gas emissions.

4. CONCLUSION

In summary, this study showed the residence of denitrifying bacteria on the tree phyllosphere and indicated that rainfalls could increase the genetic and functional potential for leaf epiphytic denitrification. Our findings implied that phyllosphere bacteria could denitrify and detoxify NO_x to the gaseous forms and, therefore, may play an unheeded role in alleviating urban N pollution. Future phylloremediation technologies may take advantage of hydrological perturbations, facilitating tree phyllosphere denitrification for N removal. Nonetheless, how the phyllosphere denitrifiers and the denitrification process, including its N_2O emission patterns, respond to rainfalls may differ for various urban greening trees or even different canopy positions of the same tree, likely owing to the dependence upon species-specific tree traits. This merits more research digging into the ecophysiology of urban phyllosphere denitrifying microbes with diverse tree species at larger spatial and temporal scales, particularly in the context of global change.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsearthspacechem.2c00061.

Elemental composition of sample leaves and statistical p values between treatments (Table S1), general information on the bacterial 16S rRNA gene and 72 CNPS cycling genes in the gene chip (Table S2), and relative abundance of each detected gene and statistical p values between treatments (Table S3) (PDF)

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Notes

The authors declare no competing financial interest.

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