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# Effects of N Addition Frequency and Quantity on *Hydrocotyle vulgaris* Growth and Greenhouse Gas Emissions from Wetland Microcosms

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Abstract: (1) Background: Increased attention has been paid to atmospheric nitrogen (N) deposition caused by human activities. N deposition quantity has seriously affected plant productivity and greenhouse gas emissions in wetlands, but the effects of N deposition frequency remain unclear. (2) Methods: We assembled microcosms, which contained vegetative individuals (ramets) of *Hydrocotyle vulgaris* and soil and subjected them to three frequencies (N addition 1, 2, and 14 times during the experimental period) crossed with three quantities (5, 15, and 30 g N m<sup>-2</sup> yr<sup>-1</sup>) for 90 days. (3) Results: The quantity of N addition significantly increased the root, stem biomass, and ramets number of *H. vulgaris*, but decreased the spike biomass. N addition quantity significantly promoted N<sub>2</sub>O emission and inhibited CH<sub>4</sub> emission but had no significant effect on CO<sub>2</sub> emission. The increasing frequency of N addition quantity. (4) Conclusions: In conclusion, N addition alters the reproductive strategy of *H. vulgaris* and enhances its invasiveness, promoting N<sub>2</sub>O emission but not the CO<sub>2</sub> equivalent of the *H. vulgaris*-soil system.

Keywords: greenhouse gas; growth; Hydrocotyle vulgaris; N addition frequency

## 1. Introduction

In recent years, more attention has been paid to the increasing atmospheric nitrogen (N) deposition caused by human activities [1]. N deposition has continued to increase over the past 50 years [2], influencing the structure and function of various ecosystems [3–5]. At present, N deposition flux in China is about 2.1 g N m<sup>-2</sup> yr<sup>-1</sup>, and that in Zhejiang Province is about 5 g N m<sup>-2</sup> yr<sup>-1</sup> [6]. It is projected that N deposition will increase in China by 2 to 3 times by 2100 [7]. In order to alleviate the contradiction between climate change and sustainable development of the wetland ecosystem, and understand the greenhouse gas emission mechanism, it is important to study the effect of atmospheric N deposition on plant growth and greenhouse gas emissions from the wetlands.

N is an essential element for plant growth, and N-limitation can affect physiological activities and development [8,9]. N deposition can increase plant biomass and decrease the root-to-shoot ratio within a certain supply range [10]. An increase in N availability may facilitate the successful invasion of exotic plants into new plant communities [11]. N deposition alters  $CO_2$  emission through an increase in plant biomass, a decrease in the C/N ratio of litter, and the mitigation of N restriction on microbial metabolism [12]. N deposition can affect CH<sub>4</sub> flux by altering the activity of methanogens



and methanotrophs [3,13], affecting the allocation of plant biomass, and thereby the impact on  $CH_4$  production, oxidation, and transport [10,14]. In addition, N deposition can increase N<sub>2</sub>O flux in wetlands because the growth and biomass accumulation of plants with N availability provide soil microorganisms with a more labile C source, which can be used as an energy source for denitrification [15,16].

Although the effects of N deposition on plant growth and greenhouse gas emissions have been extensively documented, results from the frequency of N deposition are controversial. N deposition frequency influences the resource acquisition of plants, which may provide an opportunity for plants to adapt to changes in nutritional conditions [17]. We predict that the effect of N deposition frequency on plants may be mediated by the quantity of N deposition. For example, under the conditions of low N deposition, the biomass of *Alternanthera sessilis* was higher in high-, compared to low-frequency N deposition frequency. For example, high frequency of N deposition can improve the biomass accumulation and root growth of forbs or grasses, but not of legumes [19]. Previous studies showed that there was an ephemeral peak of  $N_2O$  emissions after N addition [20]. Therefore, greenhouse gas emissions may be related to the frequency of N deposition. Results from modelling showed that a high frequency of N deposition can decrease CH<sub>4</sub> emission and increase CO<sub>2</sub> and N<sub>2</sub>O emissions [21]. More results from experiments are needed to observe some general patterns in the effects of N availability on the emission of greenhouse gases.

Many studies have investigated the effects of N quantity on plant productivity and greenhouse gas emissions in wetlands. However, the effects of the frequency of N supply remain unclear. In this study, the *H. vulgaris* microcosms were subjected to three frequencies (2, 4, and 12 times with an N addition every 30, 15, and 5 days) with a respective three N quantities (adding 5, 15, and 30 g N m<sup>-2</sup> a<sup>-1</sup>) for 90 days. We aimed to test the following two hypotheses: (1) Increasing the quantity of N addition could promote the growth of *H. vulgaris*, which may be mediated by the frequency of N addition; (2) quantity and frequency of N addition could interactively affect greenhouse gas emissions.

## 2. Materials and Methods

### 2.1. Experimental Microcosm Set-up

*Hydrocotyle vulgaris* is a perennial clonal herb [22]. The species can recruit ramets through sprouts from nodes by asexual reproduction, and seeds can also be produced for sexual reproduction [23]. *H. vulgaris* was introduced to China in the 1990s as a horticultural ornamental plant [24]. *H. vulgaris* can expand quickly in some wetlands in China, and it is considered a highly invasive species due to its ability to block rivers and canals and replace native species [24]. In this experiment, plants of *H. vulgaris* were collected from the Xixi wetland in Hangzhou, Zhejiang Province, China, and they were propagated vegetatively in a greenhouse at the Forest Science Co. Ltd. of Beijing Forestry University in Beijing. We selected 226 similar-sized single node ramets with a leaf and a few roots. For the initial measurement, 10 ramets were used, and 216 ramets were used for the assembled 54 microcosms in the experiment. The average initial dry mass was  $0.13 \pm 0.01$  g (mean  $\pm$  s.e, n = 10). The field soil contained  $5.8 \pm 0.005$  mg C g<sup>-1</sup>,  $0.57 \pm 0.006$  mg total N g<sup>-1</sup>, and  $0.58 \pm 0.04$  mg total P g<sup>-1</sup>.

## 2.2. Experimental Design

The experiment was a factorial design with three quantities (low, medium, and high N quantity) crossed with three frequencies (low, medium, and high N frequency). Based on N deposition in the Eastern Zhejiang Province, China, and N addition quantities used in previous studies [25], we set 5, 15, and 30 g N m<sup>-2</sup> yr<sup>-1</sup>, which referred to low, medium, and high N, respectively [6]. After transplantation for 8 days, we applied three frequencies of N addition, i.e., N addition every 5 days (high frequency, 14 times), every 30 days (medium frequency, twice), and once (low frequency) during the whole experiment to simulate the wet deposition frequency of N in summer in the Zhejiang

Province. There were nine treatments, and each treatment had six replicates (pots), 54 pots in total. For N addition treatment, ammonium nitrate ( $NH_4NO_3$ ) mixed with 200 mL of deionized water was sprayed and then rinsed with 200 mL of deionized water.

The experiment was carried out in the greenhouse of Forest Science Co. Ltd. of Beijing Forestry University from 6 June to 3 September 2016. The soil moisture content remained around 60%, and the air temperature was 27–36 °C during the experimental period.

## 2.3. Measurement of Growth Index

The number of ramets was counted and the leaf area of *H. vulgaris* was measured when plants were harvested (obtained by WinFOLIA Pro 2004a, Regent Instruments, Inc., QC, Canada). Then, we separated the roots, stems, stalk, leaves, and spikes. The plant samples were oven dried at 70 °C for 72 h and weighed, and biomass was expressed as g pot<sup>-1</sup>.

#### 2.4. Measurement of Greenhouse Gas Emissions

We measured the greenhouse gas emissions from the microcosms (pots with soil and ramets) four times during the experiment. Gas sampling was carried out once every 15 days after the first N addition. The measurement was performed using a static opaque chamber and gas chromatography [26]. The chamber was formed by an opaque, one-end sealed PVC tube with a diameter of 16 cm and a height of 50 cm. The chamber was sealed for 90 min to capture greenhouse gases before the measurements. Gas samples (40 mL) were collected at 30 min intervals over 90 min using plastic syringes. All samples were collected between 9:00 and 15:00 and then analyzed using gas chromatography (Agilent 7890A, Agilent Co., Santa Clara, CA, USA) within 24 h. CO<sub>2</sub> and CH<sub>4</sub> were analyzed by a flame ionization detector (FID) to analyze the concentration, and N<sub>2</sub>O was analyzed by an electron capture detector (ECD), the carrier gas was N<sub>2</sub>, the operating temperature of FID was 300 °C, and the ECD was 350 °C.

The fluxes of the three greenhouse gases were calculated from the slope of the linear regression between the gas concentration and the sampling time (0, 30, 60, and 90 min after chamber closure). The formula for the fluxes is [27]:

$$F = \frac{MPT_0}{V_0 P_0 T} H \frac{dc}{dt} \tag{1}$$

where *F* is the emission flux (mg m<sup>-2</sup> h<sup>-1</sup>); *M* is the molar mass of the gas.  $P_0$  and  $T_0$  are the air pressure and temperature under standard conditions, 110.325 k Pa and 273.15 K, respectively.  $V_0$  is the molar volume of the target compound under standard conditions and is 22.41 L mol<sup>-1</sup>. *H* is the height of the gas-sampling box (m). *P* and *T* are the air pressure (kPa) and temperature (K) at the sampling point, and *dc* is the concentration change of the target gas in the collection box within *dt*.

## Estimation of CO<sub>2</sub> Equivalent

According to the IPCC (Intergovernmental Panel on Climate Change) report, the warming effect of  $CH_4$  is 25 times greater than that of  $CO_2$  and the warming effect of  $N_2O$  is 298 times that of  $CO_2$  [28], based on the 100-year scale. We used the  $CO_2$  equivalent to show the combined effect of the three greenhouse gases.  $CO_2$  equivalent is calculated as:

$$CO_2 equivalent = EM(CH_4) \times 25 + EM(N_2O) \times 298 + EM(CO_2)$$
(2)

where EM (CH<sub>4</sub>) is the cumulative emissions of CH<sub>4</sub> during the experiment, EM (N<sub>2</sub>O) is the cumulative emissions of N<sub>2</sub>O during the experiment, and EM (CO<sub>2</sub>) is the cumulative emissions of CO<sub>2</sub> during the experiment.

EM is calculated as:

$$EM = \sum_{i=1}^{n} \frac{(F_i + F_{i-1})}{2} \times (t_i - t_{i-1}) \times S \times 24$$
(3)

where EM is the cumulative emissions, *F* is the gas flux, *t* is the number of days from the beginning of the experiment, *i* is the number of measurements, and *S* is the area of each pot.

#### 2.5. Data Analysis

A two-way ANOVA, followed by Tukey tests, was used to examine the effects of the quantity (low, medium, and high quantity) and frequency (low, medium, and high frequency) of N addition and their interaction in the growth of *H. vulgaris* (biomass, root/shoot, No. of ramets, leaf area, specific leaf area) and the greenhouse gas emissions (CO<sub>2</sub>, CH<sub>4</sub>, N<sub>2</sub>O, and CO<sub>2</sub> equivalent) from the *H. vulgaris* soil system. Correlation analysis was used to examine the relationship between the biomass of *H. vulgaris* and the greenhouse gas emissions. Prior to the analyses, the data were tested for normality and underwent Levene's test. All data satisfied normal distribution and homogeneity of variance. All data analysis was conducted in the statistical software SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Effects were considered to be significant if p < 0.05.

#### 3. Results

#### 3.1. Effects of the Quantity and Frequency of N Addition on the Growth of Hydrocotyle Vulgaris

The quantity and frequency of N addition had a significant interactive effect on the total biomass and stem biomass (Table 1). Under high frequency of N addition, the total biomass and stem biomass were significantly lower in the low, compared to the medium N quantity treatment. We did not find a significant difference between the three quantity treatments in either the low or medium frequency treatment (Figure 1A,F). The frequency of N addition significantly affected the root-to-shoot ratio and did not impact other growth indexes of *H. vulgaris* (Table 1). High and medium N quantity increased the root-to-shoot ratio (Figure 2A), decreased the spike biomass of *H. vulgaris* (Table 1, Figure 1E), and increased the root biomass under high-frequency treatment (Figure 1B). High quantity of N addition significantly increased the number of ramets and leaf area (Table 1, Figure 2E,C), and increased SLA under the low-frequency treatment (Figure 2D).

Variable	Frequency (F)		Quantity (Q)		$F \times Q$	
	F <sub>2,45</sub>	p	$F_{2,45}$	р	F <sub>4,45</sub>	p
Total mass	0.43	0.653	0.25	0.780	2.88	0.033
Root mass	2.60	0.085	3.73	0.032	2.32	0.071
Leaf mass	1.52	0.230	1.08	0.348	1.56	0.202
Stalk mass	1.24	0.298	1.65	0.203	0.84	0.507
Stem mass	1.27	0.29	5.45	0.008	2.73	0.041
Spike mass	0.20	0.819	21.78	<0.001	2.20	0.084
Root/shoot	4.02	0.025	5.30	0.009	0.71	0.59
No. of ramets	0.88	0.422	20.84	<0.001	0.18	0.948
Leaf area	0.85	0.433	9.51	<0.001	0.93	0.455
SLA	0.62	0.542	5.06	0.010	1.14	0.348

Table 1. Effects of frequency and quantity of N addition on the growth of Hydrocotyle vulgaris.

Note: *F*, degree of freedom and *p* values are shown. Values with p < 0.05 are in bold.



**Figure 1.** Effects of the quantity and frequency of N addition on the biomass of *H. vulgaris*. Bars and error bars show means  $\pm$  SE (n = 6). Different letters indicate significant differences between quantities of N addition treatment ( $p \le 0.05$ ).



**Figure 2.** Effects of the quantity and frequency of N addition on the growth of *H. vulgaris*. Bars and error bars show means  $\pm$  SE (n = 6). Different letters indicate significant differences between quantities of N addition treatment ( $p \le 0.05$ ).

#### 3.2. Effects of N addition Quantity and Frequency on Greenhouse Gas Emissions

The significant interactive effect between frequency and quantity of N addition affected  $N_2O$  emission from the *H. vulgaris* microcosms (Table 2).  $N_2O$  emission was significantly higher in high- than in low- and medium-quantity N treatments, respectively (Figure 3A). CH<sub>4</sub> emission was significantly higher in low- than in high-quantity N treatments with a low frequency, and it was also higher in low- than in medium- and high-quantity N treatments with a high frequency (Figure 3B). We did not find any significant effect of N frequency or N quantity on CO<sub>2</sub> emissions from the *H. vulgaris* microcosms (Figure 3C). There was no significant effect of N addition frequency and quantity on the CO<sub>2</sub> equivalent (Figure 3D).

**Table 2.** Effects of frequency and quantity of N addition on greenhouse gas emissions from the plant–soil system.

Variable	Frequency (F)		Quantity (Q)		$F \times Q$	
	F <sub>3,56</sub>	р	F <sub>3,56</sub>	р	F <sub>3,56</sub>	р
N <sub>2</sub> O emission	8.45	0.001	55.75	<0.001	11.14	<0.001
CH <sub>4</sub> emission	2.20	0.122	6.75	0.003	0.60	0.665
$CO_2$ emission	2.31	0.111	0.14	0.871	1.82	0.141
$CO_2$ equivalent	2.56	0.089	0.66	0.524	1.25	0.304



Note: *F*, degree of freedom and *p* values are shown. Values with p < 0.05 are in bold.

**Figure 3.** Effects of frequency and quantity of N addition on greenhouse gas emissions from the plant–soil system. Bars and error bars show means  $\pm$  SE (n = 6). Different letters indicate significant differences between quantities of N addition treatment ( $p \le 0.05$ ).

### 3.3. The Relationship between Greenhouse Gas Emissions and the Biomass of H. Vulgaris

Cumulative emission of CO<sub>2</sub> was significantly positively correlated with leaf biomass (p = 0.004, Figure 4A). In addition, the cumulative emission of CH<sub>4</sub> was linearly positively correlated with spike biomass (p < 0.001, Figure 4C). In contrast, the cumulative emission of N<sub>2</sub>O was linearly negatively correlated with spike biomass (p = 0.001, Figure 4B).



Figure 4. Relationships between greenhouse gas emissions and the growth of *H. Vulgaris*.

## 4. Discussion

#### 4.1. Effects of N Addition Quantity and Frequency on the Growth of H. Vulgaris

N addition directly promoted the growth of *H. vulgaris* in our study. High N addition significantly increased the root and stem biomass, the root-to-shoot ratio (R/S), the number of ramets, leaf area, and the specific leaf area of *H. vulgaris*. The results indicated that the growth of *H. vulgaris* benefited from sufficient nutrient conditions, which was consistent with previous findings [29]. Our results also confirmed that appropriate N addition can promote plant growth, thus increasing plant productivity [10].

Notably, the increase in belowground biomass and R/S in our study provided evidence to support the optimal partitioning theory rather than isometric theory [30,31]. In order to adapt to the changing habitat conditions, *H. vulgaris* allocated more biomass to the root and stem systems in the high and medium N addition treatments. The N-mediated increase in R/S may be due to the experimental period and the stage of plant development [32,33]. A previous study showed that the R/S of herbs increased with N addition for two years, and then decreased after four years [34]. Therefore, we proposed that the short-term N addition, which increased the R/S, could change the adaptive

strategy of *H. vulgaris* in response to the increasing N availability. This seems plausible, as the roots and stems are the asexual reproductive organs of *H. vulgaris*, therefore, reproduction could become particularly important when the plant growth was assured.
High N addition increased the number of ramets of *H. vulgaris* and reduced the plant's ability to spike, which could be a reproductive strategy of *H. vulgaris* to increase performance. The selection of the reproductive strategy of clonal plants depends on their own genetic condition and the abundance of resources [35]. There is an obvious trade-off in resource investment between sexual and asexual reproduction. Increasing the allocation of resources to asexual reproduction may be a reproductive strategy for clone plants in sufficient nutrient conditions [35,36]. High N addition can promote clone

statement, which indicates the adaptive strategy of *H. vulgaris* to the increase in availability of soil N. The quantity and frequency of N addition had a significant interactive effect on the total biomass and stem biomass of *H. vulgaris*. The results were consistent with a previous study [18]. Under low-quantity N addition, the total and stem biomass were significantly lower in the highcompared to the medium- and low-frequency N treatments. However, the frequency of N addition did not affect the growth of *H. vulgaris* in the medium- and high-quantity N treatment groups. A possible explanation is that the medium quantity of N addition (15 g N m<sup>-2</sup> yr<sup>-1</sup>) was able to meet the demand of the plants, and the frequency of N addition may not affect the growth of plants that have sufficient resources [18].

reproduction and increase the risk of *H. vulgaris* invasion. This may further corroborate the foregoing

#### 4.2. Effects of the Quantity and Frequency of N Addition on Greenhouse Gas Emissions from Microcosms

 $N_2O$  emission is an important pathway of the soil nitrification–denitrification process [37,38]. N addition significantly increased  $N_2O$  emission in our study, which was consistent with the results from previous studies [16,39,40]. N addition can provide a sufficient N source for nitrification–denitrification and promote the production of  $N_2O$  [41,42]. N addition promoted the root growth of *H. vulgaris*, which could provide more C source for soil microorganisms during denitrification and thereby promote  $N_2O$  emission [15,16]. Previous studies have shown that the peak of  $N_2O$  emission after N addition lasted for a few days and then declined [20]. The accurate estimation of greenhouse gas emission may have been biased, as we measured the  $N_2O$  emission at a low frequency during the experiment, which may have missed the peak emission of the  $N_2O$ . In future experiments, continuous measurements during the experiment will be needed. It should be noted that this response of  $N_2O$  emission pattern to the frequency of N addition did not affect the CO<sub>2</sub>eq estimates, because  $N_2O$  emission was low, although the warming effect of  $N_2O$  is 298 times higher than that of CO<sub>2</sub> [3].

CH<sub>4</sub> emission is related to the methanogens and methanotrophs in the soil [4,43]. N addition can increase the availability of soil N, thereby altering the microbial processes of CH<sub>4</sub> production and oxidation [44,45]. CH<sub>4</sub> emission significantly decreased with the increase in N addition. Studies showed that NH<sub>4</sub><sup>+</sup> may inhibit the activity of methanotrophs and NO<sub>3</sub><sup>-</sup> may promote it [45,46]. In this experiment, we used ammonium nitrate (NH<sub>4</sub>NO<sub>3</sub>) as an N source, which may promote or inhibit CH<sub>4</sub> emission. In the high- and medium-quantity N addition treatments, more NH<sub>4</sub><sup>+</sup> was absorbed by *H. vulgaris* and more NO<sub>3</sub><sup>-</sup> remained in the soil, which may have inhibited CH<sub>4</sub> emission. There was a significant positive correlation between CH<sub>4</sub> emission and the spike biomass of *H. vulgaris*. This may be related to the well-developed aerenchyma in the spike, where CH<sub>4</sub> produced from the soil can be

released directly into the atmosphere, reducing the amount of oxidized methane [47]. CH<sub>4</sub>-positive emission was observed under the condition of 60% soil moisture content, which was consistent with previous studies [26,48].

 $CO_2$  emission is related to the respiration of plants and soil. N addition did not significantly affect  $CO_2$  emission in our study. This may be attributed to the low soil C/N ratio (about 10) in our experiment. N addition may further decrease the soil C/N ratio, accentuating the limitation of C to soil microbial activity [12]. The meta-analysis also showed that N addition significantly reduced soil microbial respiration and increased plant root respiration, but had no significant influence on total belowground respiration [5]. We found that  $CO_2$  emission from the *H. vulgaris* microcosms was significantly positively correlated with the leaf biomass of *H. vulgaris*, while N addition did not significantly affect leaf biomass. This result was consistent with previous studies [26,49]. This may explain the gas exchange function of plant leaves, where the  $CO_2$  produced from plant respiration could be released into the atmosphere through the stomata [50]. The greenhouse gas emissions were affected not only by leaf and spike biomass, but also by other factors such as stomata number, stomatal conductance, and soil microorganisms, which needs further study.

## 5. Conclusions

In conclusion, increasing the quantity of N addition was beneficial to root and stem biomass growth in the high-frequency N addition group, which increased the number of ramets and decreased the spike biomass of *H. vulgaris*, implying that high N addition can shift the reproductive strategy of *H. vulgaris* from sexual reproduction to clone reproduction. N addition quantity significantly promoted N<sub>2</sub>O emission and inhibited CH<sub>4</sub> emission of the *H. vulgaris* soil system and did not significantly affect CO<sub>2</sub> emission and CO<sub>2</sub> equivalent. Further studies are needed to reveal the reasons behind these findings.

**Author Contributions:** J.-Q.G., Q.-W.L. and M.-H.S. designed the experiments and wrote the manuscript; Q.-W.L., X.-Y.Z., J.-F.L., and Y.Y. performed the experiments and revised the manuscript; Q.-W.L. and J.-Q.G. analyzed the data. Q.-W.L., X.-Y.Z., J.-Q.G., M.-H.S., J.-F.L., and Y.Y. approved the final manuscript.

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