Application of stable isotopes to examine N proportions within a simulated Aegiceras corniculatum wetland

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ABSTRACT
Salinity levels and drought status of coastal wetlands may be strongly affected by climate change, and changes in the nitrogen cycle of mangrove wetlands may also be affected. We established combinations of three salinity and water levels with applied stable isotope $^{15}$N to study the $^{15}$N distributions in the sediment and plants of a greenhouse-based simulated mangrove Aegiceras corniculatum wetland system. The stable isotope $^{13}$C and $^{15}$N distributions in different organs in response to high salinity stress. Compared to the $^{13}$C, the $^{15}$N values of plant organs increased with increasing water level in low salinity (10‰) and medium salinity (20‰) treatment groups but not in the high salinity (30‰) treatment group. This may attributed to A. corniculatum adjusting the $^{15}$N distribution in different organs in response to high salinity stress. Overall, the measured indicators exhibited different responses to salinity level and water level, suggesting that the changes in salinity and water levels have an impact on N cycling processes of wetland systems.

Introduction
Mangrove is a woody plant community found in the tropical and subtropical intertidal zones, and can be affected by the inland freshwater, sediments, nutrients and tidal variations [1]. This system has a high productivity and decomposition rate, and plays an important role in species survival and in the carbon balance in the coastal zone [1–7]. Over the past few decades, the global mangrove forests have undergone significant changes on distributive pattern and area, caused by a variety of factors including rising temperatures, changes in precipitation patterns, increased frequency and intensity of storms and sea level rise [8]. Some studies have shown that if the sea level rise is sufficiently slow and there are suitable area and environmental conditions, the mangrove forests are able to adapt gradually [1]. However, other studies have revealed that global climate change, especially air temperature, CO$_2$ precipitation, hurricanes and storms, sea-level rise, and human disturbance all strongly affect mangrove ecosystems [9–11]. Sea-level rise leads to salt water intrusion. Droughts caused by El Niño also can induce the rising salinity of the soil in mangrove wetland. The effect of changes in these environmental factors on nitrogen cycling processes in mangrove ecosystems has not yet to be studied.

Stable C isotopic composition, C:N and other indicators have been applied in food web studies of mangrove ecosystems, including biogeochemical cycles (such as belowground ecosystem C cycling), and other ecological processes, as well as the paleoenvironmental reconstruction, sea level rise, paleoclimate change, paleosalinity, and other study topics [12–21]. The use of $^{13}$C can reflect the long-term integrated physiological processes in plants, and has a certain degree of correlation with salinity; therefore the plant $^{13}$C can be seen as a characteristic indicator of physiological responses in the presence of changes in environmental factors [22]. Aegiceras corniculatum is a salt-secreting plant in the family Myrsinaceae, and is widely distributed in the intertidal zone of South China coast [22]. The current isotope studies [22, 23] on A. corniculatum have mainly focused on $^{13}$C, but little is known about the patterns of $^{15}$N distribution. However, the characteristics of the $^{15}$N variation in mangrove plant leaves coupling with

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the addition of the $\delta^{15}N$ labeled can be used to identify plant responses to environmental stress gradients.

Simulated tidal systems in greenhouses have been commonly used in the study of the responses of mangrove plant seedlings to changes in salinity, flooding, synthetic sewage, heavy metals and other factors [24–28]. *A. corniculatum* naturally distributed in the Pearl River estuary, South China was used for the current study. We established a simulated *A. corniculatum* wetland system in a greenhouse in Guangzhou, China, under different salinity levels and water levels (equivalent to different levels of drought). The stable isotope $^{13}C$ and $^{15}N$ value, C, and N content as well as the ratio of sediment and plants for each treatment group were analyzed. Additionally, $^{15}N$ labeled urea was applied to explore whether salinity or water level significantly affected the distribution of nitrogen in the simulated wetland system. The results will contribute to our understanding of the use of sediment N by plants under the impacts of different ecological factors. Furthermore, the storage and allocation of N element in the system will provide basic data for the future studies of the N cycle in mangrove wetland. The objectives of this study are focus on (1) Do the ecological factors of water level and salinity has some effects on the nitrogen cycle within the simulated mangrove wetland system? (2) Are there any differences between partitioning of stable isotopes of $^{15}N$ and $^{13}C$?

### Materials and methods

#### Materials

One-year-old *A. corniculatum* seedlings were purchased from Qi’ao Island, Zhuhai in early March 2015, where the viviparous seedlings were produced from hypocotyl. When the seedlings were planted, the average plant height was $17.1 \pm 2.0$ cm, stem base diameter was $4.4 \pm 0.6$ mm, and the number of leaves was $6 \pm 1$.

#### Establishment of greenhouse simulated wetland system

In a greenhouse in Guangzhou, China, 27 glass tank sets were utilized; each set was composed of one top glass box and one bottom glass box, and one electric pump per box. The power switch was controlled by a timer, and the daily pump operation was programmed to simulate the semidiurnal tide of Qi’ao Island, Zhuhai (high tide: 8:00–12:00 and 20:00–24:00; low tide: the remaining 16 h). The upper glass box (inner diameter: $0.75$ m (length) $\times 0.50$ m (width) $\times 0.55$ m (height)) was used for planting in 100 kg of sediment collected from Qi’ao, Zhuhai mangrove wetland (the sediment was pretreated by air drying and passed through 2 mm sieve; the thickness of sediment in the box was about 35 cm.). In each box, 15 *A. corniculatum* seedlings were planted. The bottom glass box was used to store tidal water at low tide. The simulated tidal water was prepared with salt and pure water [25], and pure water was used to compensate the amount of evaporated water each day at high tide [24, 27]. According to the salinity of Qi’ao, Zhuhai seawater (about 18.2‰) and soil salinity (about 13.4‰), three salinity gradients (10‰, 20‰ and 30‰) were set in the experiment [29]. In addition, three different water levels (equivalent to varying degrees of drought) were established as follows: the low water level was defined as the water level at 10 cm below the soil surface at high tide (not all soil being submerged); the medium water level was defined as the water level when the surface of the soil was just submerged at high tide; and the high water level was defined as 10 cm higher than the soil surface at high tide. The water levels were 20 cm below the soil surface at low tide for all treatment groups. In the greenhouse simulation experiment, there were a total of nine combinations of different salinity and water levels as shown in Table 1. For each treatment, there were three sets of glass tanks as replicates.

### Application of $^{15}N$ labeled nitrogen and sampling

The normal growth of *A. corniculatum* seedlings resumed after six months of cultivation [25]. At 9:00 am on November 10, 2015, 150 mg/L $^{15}N$-labeled (NH$_2$)$_2$CO (purchased from Shanghai Research Institute of Chemical Industry, enrichment: 5.15 atom%, batch No. 1921, weight: 150 g) was applied to the top glass box of each set at high tide. Subsequently, sampling was conducted after the simulation system had been operated for 120 days (at 15:00 on March 9, 2016 at low tide). From each glass box, three plants were randomly selected, and roots, stems, and mature leaves were processed. In the vicinity of each plant sampled (at 5 cm from the plant), one sediment sample was collected with a 2.3 cm diameter stainless steel sediment sampler (at 0–10 cm sediment depth with a wet weight of about 1 kg).

### Determination methods

#### Measurement of growth and biomass of plant

At the end of cultured experiment, stem height, stem base diameter, root length of plant were determined. After that, the plant was divided into root, stem and leaf parts. Fresh weight was calculated, and 10.0 g fresh

### Table 1. Treatment groups of the simulated system.

| Treatment group | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  |
|-----------------|----|----|----|----|----|----|----|----|----|
| Salinity/‰     | 10 | 10 | 10 | 20 | 20 | 20 | 30 | 30 | 30 |
| Water level     | Low| Medium| High| Low| Medium| High| Low| Medium| High|
samples of each organ were put into oven to obtain a constant weight at temperature of 80 °C.

The amount and distribution of nitrogen absorbed by the plant was calculated by the following formula [30]:

\[ {^{13}\text{N}} \text{ uptake amount (mg)} = \text{Biomass (g, DW)} \times N\% \times (\text{abundant of } {^{15}\text{N}} \text{ in samples} – \text{abundant of } {^{15}\text{N}} \text{ in nature}) \times 100 \times 1000 \]

Distribution rates (%) = \( {^{15}\text{N}} \text{ absorptive amount (mg)} \) by various organ/total \( {^{15}\text{N}} \text{ absorptive amount (mg)} \) by plant \times 100

**Sample preparation**

Plant and sediment samples were dried in the shade and then placed in a 60 °C incubator for 48 h [13]. They were then crushed and screened through a 0.154-mm sieve for the measurement of C/N content and isotope proportions. Because of their higher salt content, the sediment samples underwent further processing. After being dried in the 60 °C incubator, sediment samples (0.4 g) were soaked in 5% HCl for 1 day, then centrifuged and washed with 10-mL dH\(_2\)O, centrifuged again at 5000 rpm three times, and dried again in a 60 °C incubator for 48 h, followed by the measurement of C and N.

**Measurement of C/N content and isotope proportions**

Analysis of C/N content and the stable isotope composition was performed using an Elementar Analysensysteme (vario PYRO cube, Elementar, Germany) and an Isotope Mass Spectrometer (Isoprime 100, Isoprime, UK) in the State Key Laboratory of Isotope Geochemistry, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, China. First, the sample was put into a tin capsule and burnt into N\(_2\) and CO\(_2\). The generated gas was dried and separated for trichloroethylene (C/N mode), with the oxygenation time set at 90 s; the CuO and Cu, respectively, as oxidant and reductant; and the combustion and reduction temperatures, respectively, at 920 °C and 600 °C. Considering the accuracy of C/N content measurement, the relative standard deviation should be <1%. Next, the proportion of \( ^{13}\text{C} \) and \( ^{15}\text{N} \) was measured using mass spectrometry. Multipoint calibration was performed using the international standards IAEA-601, IAEA-NO\(_3\), and IAEA-N\(_2\). After repeated measurement of the standard, the accuracy error for \( ^{13}\text{C} \) and \( ^{15}\text{N} \) was <0.15 and <0.4‰, respectively.

**Statistical analysis**

The average and standard deviation of parallel data of each treatment group were calculated using Excel 2007 (Microsoft Corporation, USA), and corresponding charts were plotted. SPSS (Version 17.0, IBM Corp., USA) was used to conduct statistical analysis, single tailed analysis of variance (ANOVA) was performed on the C and N contents, C:N, as well as \( \delta^{13}\text{C} \) and \( \delta^{15}\text{N} \) value of sediment samples from the same source or the same plant organ, as well as stem height and stem base diameter, biomass of plant, and significance was tested using least significant difference. To examine the differences in the values of samples from different sources (sediment, roots, stems, leaves), a paired sample t test was performed on the mean values. For the analysis of effect of salinity and water levels, the main effect model in the univariate general linear model was used for variance test without adjusting for interactions.

**Results and analyses**

**C content of sediment and in A. corniculatum**

The measured C content of the sediment and the roots, stems, leaves of *A. corniculatum* are shown in Figure 1. The C content of the sediment was 2.19 ± 0.15% in average. No significant differences in the sediment C content were found between each salinity treatment group (p = 0.505) and water level treatment group (p = 0.606). The sediment C content was significantly lower than those of plant roots, stems and leaves (23.46 ± 1.55% – 49.85 ± 3.35% (p < 0.001)). The ANOVA results suggested that the effects of salinity (p = 0.576) and water level (p = 0.651) on sediment C content were not significant.

The average C content of the plant organs in each group were in the following order: roots (30.29 ± 3.66%) < stems (39.89 ± 0.28%) < leaves (46.89 ± 4.43%), and extremely significant differences were present between these three organs (p < 0.001).

The root C content in the 30‰ salinity treatment group (27.03 ± 3.18%) were slightly lower than those of the 10‰ salinity treatment group (30.99 ± 3.56%) and 20‰ salinity treatment group (32.85 ± 2.09%), but there was no significant difference (p = 0.130). The root C content in the high water level treatment (28.56 ± 6.05%) were slightly lower than those of the low water treatment group (31.24 ± 2.89%) and medium water level treatment group (31.08 ± 1.38%), but no significant difference was observed (p = 0.667). The ANOVA results demonstrated that the effects of salinity (p = 0.189) and water level (p = 0.563) on the root C content were not significant.

The stem C content of each salinity treatment group (p = 0.423) and water level treatment group (p = 0.273) were almost equal. The ANOVA results showed that the effects of salinity (p = 0.379) and water level (p = 0.282) on the stem C content were not significant.

The leaf C content of the 30‰ salinity treatment group (43.36 ± 6.87%) were slightly lower than those of the 10‰ salinity treatment group (48.15 ± 1.53%) and the 20‰ salinity treatment group (49.16 ± 0.37%), but no significant difference was observed (p = 0.254). The leaf C content of the high water level treatment group (43.99 ± 7.42%) were slightly lower than those of low water level treatment group (47.58 ± 1.30%) and medium water level treatment group (49.09 ± 1.08%), but no significant difference was observed (p = 0.401).
The sediment N content were significantly lower than those of plant roots, stems and leaves (0.25 ± 0.02% to 1.05 ± 0.07%; \( p < 0.001 \)). The ANOVA results showed that the effects of salinity (\( p = 0.766 \)) and water level (\( p = 0.250 \)) on sediment N content were not significant.

The ANOVA results showed that the effects of salinity (\( p = 0.253 \)) and water level (\( p = 0.343 \)) on leaf C content were not significant.

**N content of sediment and in A. corniculatum**

The measured N content of the sediment and the roots, stems, leaves of *A. corniculatum* are shown in Figure 2. The N content of the sediment was 0.14 ± 0.01% on average; no significant differences in sediment N content were found between each salinity treatment group (\( p = 0.813 \)) and water level treatment group (\( p = 0.152 \)). The sediment N content were significantly lower than those of plant roots, stems and leaves (0.25 ± 0.02% to 1.05 ± 0.07%; \( p < 0.001 \)). The ANOVA results showed that the effects of salinity (\( p = 0.766 \)) and water level (\( p = 0.250 \)) on sediment N content were not significant.

The average N content of plant organs in each group increased in the following order: roots (0.36 ± 0.11%) < stems (0.39 ± 0.10%) < leaves (0.89 ± 0.11%), and the leaf N content were significantly higher than those of roots and stems, between which no significant differences were found (\( p = 0.316 \)).
The leaf N content of the salinity 30‰ treatment group (0.90 ± 0.06%) were not significantly different from those of the salinity 10‰ treatment group (0.86 ± 0.18%) and the 20‰ salinity treatment group (0.89 ± 0.09%; p = 0.903). The leaf N content of the high water level treatment group (0.96 ± 0.10%) were significantly higher than those of the low water level treatment group (0.79 ± 0.10%) (p = 0.045). However, they were not significantly different from those of the medium water level treatment group (0.90 ± 0.06%; p = 0.392). At the same salinity level, the leaf N content of other treatment groups increased with increasing water level, except for those of the high salinity level + high water level treatment group. The ANOVA results demonstrated that the effects of salinity (p = 0.865) and water level (p = 0.212) on leaf N content were not significant.

The root N content of the 30‰ salinity treatment group (0.41 ± 0.14%) were slightly higher than those of the 10‰ salinity treatment group (0.34 ± 0.10%) or 20‰ salinity treatment group (0.34 ± 0.11%), but no significant difference was observed (p = 0.727). The root N content in the same salinity treatment group showed an increasing trend with increasing water level. The N content in the high water level treatment group (0.49 ± 0.05%) were significantly higher than those of the low water level treatment group (0.26 ± 0.01%, p = 0.001) and medium water level treatment group (0.34 ± 0.06%, p = 0.008). However, no significant difference was found between N contents of the medium and low water level treatment groups (p = 0.091). The ANOVA results suggested that the effect of salinity on root N content was not significant (p = 0.081), but the increase in the water level significantly elevated the root N content. This effect was significant (p = 0.002).

The stem N content of the salinity 30‰ treatment group (0.50 ± 0.13%) were significantly higher than those of the 10‰ salinity treatment group (0.33 ± 0.01%; p = 0.038). However, they showed no significant difference from those of the 20‰ salinity treatment group (0.35 ± 0.04%; p = 0.054). The stem N content of the high water level treatment group (0.45 ± 0.16%) were slightly higher than those of the low water level treatment group (0.35 ± 0.05%) and medium water level treatment group (0.38 ± 0.06%), but no significant differences were present (p = 0.494). The stem N content in the same salinity treatment group showed an increasing trend with increasing water level, and the range of the variation in stem N content was smaller than that of the root N content. The ANOVA results showed that the effects of salinity (p = 0.065) and water level (p = 0.241) on the stem N content were not significant.

The leaf N content of the salinity 30‰ treatment group (0.90 ± 0.06%) were not significantly different from those of the salinity 10‰ treatment group (0.86 ± 0.18%) and the 20‰ salinity treatment group (0.89 ± 0.09%; p = 0.903). The leaf N content of the high water level treatment group (0.96 ± 0.10%) were significantly higher than those of the low water level treatment group (0.79 ± 0.10%) (p = 0.045). However, they were not significantly different from those of the medium water level treatment group (0.90 ± 0.06%; p = 0.392). At the same salinity level, the leaf N content of other treatment groups increased with increasing water level, except for those of the high salinity level + high water level treatment group. The ANOVA results demonstrated that the effects of salinity (p = 0.865) and water level (p = 0.212) on leaf N content were not significant.

**C:N ratio of sediment and in A. corniculatum**

The results of C:N ratio in the sediment and roots, stems, leaves of A. corniculatum are shown in Figure 3. The sediment organic matter C:N ratio was 15.3 ± 0.6 in average. The sediment N content showed no significant differences between salinity treatment groups (p = 0.753) and water level treatment groups (p = 0.123). However, the sediment N content were significantly lower than the N content in plants (41.2 ± 2.4 – 129.2 ± 9.1; p = 0.000). The ANOVA results indicated that the effects of salinity (p = 0.670) and water level (p = 0.201) on sediment C:N ratio were not significant.

The average C:N ratios of different in plant organs decreased in the following order: stems (106.1 ± 20.5) > roots (90.5 ± 29.6) > leaves (53.5 ± 8.2). Root C:N was significantly lower than stem C:N (p = 0.045), but significantly higher than leaf C:N.
The leaf C:N of the 30‰ salinity treatment group (47.9 ± 6.1) was slightly lower than those of 10‰ salinity treatment group (57.3 ± 11.4) and the 20‰ salinity treatment group (55.4 ± 5.4), but no significant difference was present (p = 0.377). The leaf C:N of the high water level treatment group (45.5 ± 4.3) was significantly lower than that of the low water level treatment group (60.6 ± 7.5; p = 0.017), but was not significantly different from that of medium water level treatment group (54.5 ± 4.8; p = 0.098). The ANOVA demonstrated that the effect of salinity on leaf C:N effect was not significant (p = 0.051), but an increase in the water level significantly reduced the leaf C:N (p = 0.012).

δ¹³C value of sediment and A. corniculatum organs

The measured δ¹³C value in the sediment and the roots, stems, leaves of A. corniculatum are shown in Figure 4. The sediment organic matter δ¹³C value was −25.12 ± 0.30‰ in average. This is significantly higher than those in the plant (−32.27 ± 0.33‰ to −29.28 ± 0.15‰; p < 0.001). No significant difference was found in the δ¹³C value between salinity treatment groups (p = 0.814) and water level treatment groups (p = 0.456). The ANOVA results indicate that the effects of salinity (p = 0.014) and water level (p = 0.001) on root C:N ratio were significant and strongly significant, respectively.

The stem C:N ratios of the 30‰ salinity treatment (83.5 ± 19.0) were significantly lower than those of the 10‰ salinity treatment group (119.3 ± 2.3; p = 0.016) and the 20‰ salinity treatment group (115.4 ± 12.5; p = 0.025), but no significant difference was found between the 10‰ salinity and 20‰ salinity treatment groups (p = 0.727). The stem C:N ratios of the high water level treatment (94.6 ± 28.8) were slightly lower than those of the low water level treatment group (116.1 ± 16.4) and the medium water level treatment group (107.6 ± 15.0), but no significant difference was observed (p = 0.493). The ANOVA results suggested that salinity significantly reduced the stem C:N (p = 0.016), but the effect of the water level on stem C:N was not significant (p = 0.105).
The measured δ¹⁵N value in the sediment and the roots, stems, leaves of A. corniculatum are shown in Figure 5. The sediment  δ¹⁵N value was 23.5 ± 21.5‰ in average, significantly lower than those in the plant (32.3 ± 0.3‰ to 29.3 ± 0.2‰; p < 0.001). The sediment δ¹⁵N value of the groups treated with the same salinity level increased with increasing water level. The sediment δ¹⁵N value of 30‰ salinity treatment (19.2 ± 18.7‰) was not significantly different from the 10‰ salinity treatment group (25.5 ± 25.2‰; p = 0.936). However, the sediment δ¹⁵N value of the high water level treatment group (51.3 ± 9.5‰) was significantly greater than that of the low water level treatment group (7.7 ± 1.6‰; p < 0.001) and the medium water treatment group (11.4 ± 1.3‰; p < 0.001). No significant difference (p = 0.455) was observed between the low water level treatment group and the medium water level treatment group. The ANOVA results showed that the effect of salinity on the sediment δ¹⁵N value was not significant (p = 0.327), while the effect of water level on the sediment δ¹⁵N value was strongly significant (p = 0.001). That is, the increase of water level significantly increased the sediment δ¹⁵N value.

The average δ¹³C value of the roots, stems and leaves of plants treated with different salinity levels and water levels decreased in the following order: root (−29.50 ± 0.24‰) > stem (−30.56 ± 0.40‰; p = 0.004) and significantly higher than the 20‰ salinity treatments (−30.10 ± 0.16‰; p = 0.042). In addition, there was no significant difference between the water level treatment groups (p = 0.908). The ANOVA results showed that the increase in the salinity significantly enhanced the root δ¹³C value (p = 0.038), but the effect of water level on the root δ¹³C value was not significant (p = 0.732).

The stem δ¹³C value of the 30‰ salinity treatment group (−30.01 ± 0.61‰) was significantly higher than that of the 10‰ salinity treatment group (−31.86 ± 0.39‰) (p = 0.004) and significantly higher than that of 20‰ salinity treatment group (−30.80 ± 0.14‰) (p = 0.042), but no significant difference (p = 0.973) was observed between the water level treatment groups. The ANOVA results showed that an increase in the salinity significantly increased the stem δ¹³C value (p = 0.029), while the effect of water level on the stem δ¹³C value was not significant (p = 0.899).

The leaf δ¹³C value of the 30‰ salinity treatment group (−30.06 ± 0.60‰) was significantly higher than that of the 10‰ salinity treatment group (−31.65 ± 0.33‰; p = 0.021). However, it was not significantly different from that of the 20‰ salinity treatment group (−30.65 ± 0.29‰; p = 0.113), and there was no significant difference (p = 0.705) between the water level treatment groups. The ANOVA results indicate that an increase in the salinity significantly increased the leaf δ¹³C value (p = 0.039), while the effect of water level on leaf δ¹³C value was not significant (p = 0.737).
root, stem, and leaf $\delta^{15}$N value of the 10‰ salinity and 20‰ salinity treatment groups increased with increasing water level. The $\delta^{15}$N value for the 30‰ treatment group decreased in the following order: medium water level > low water level > high water level, which may be because the plants may adjust the absorption and distribution of $\delta^{15}$N due to the presence of high salinity stress. If the 30‰ salinity treatment group was excluded, the comparison of only the 10‰ salinity treatment group and 20‰ treatment groups showed that the effect of salinity on the $\delta^{15}$N value in plant roots ($p=0.695$), stems ($p=0.910$) and leaves ($p=0.855$) was not significant. However, an increase in water level significantly increased the $\delta^{15}$N value in roots ($p=0.024$), stems ($p=0.047$), and leaves ($p=0.034$).

The root $\delta^{15}$N value of the 30‰ salinity treatment group (675.1 ± 164.2‰) was lower than that of the 10‰ treatment group (1028.9 ± 486.4‰) and 20‰ salinity treatment group (852.8 ± 535.8‰), but no significant difference was observed ($p=0.624$). The root $\delta^{15}$N value of the high water level treatment group (1187.1 ± 537.1‰) was not significantly different from that of the low water level treatment group (547.5 ± 48.5‰) and the medium water level treatment group (822.3 ± 211.8‰; $p=0.141$). The ANOVA results indicated that the effects of salinity ($p=0.519$) and water level ($p=0.193$) on root $\delta^{15}$N value were not significant.

The stem $\delta^{15}$N value of the 30‰ salinity treatment group (815.1 ± 127.3‰) was slightly lower than that of the 10‰ salinity treatment group (1219.8 ± 733.4‰) and the 20‰ salinity treatment group (1150.2 ± 699.8‰; $p=0.681$). The stem $\delta^{15}$N value of the high water level treatment group (1505.8 ± 677.0‰) was slightly higher than that of the low water level treatment group (638.3 ± 195.3‰), but slightly lower than that of the medium water level treatment group (1041.1 ± 327.1‰).

However, there was no significant difference between the three groups ($p=0.138$). The ANOVA results showed that the effects of salinity ($p=0.519$) and water level ($p=0.193$) on the stem $\delta^{15}$N value were not significant.

The leaf $\delta^{15}$N value of the 30‰ salinity treatment group (1240.4 ± 261.8‰) was lower than that of the 10‰ treatment group (1623.2 ± 521.3‰) and the 20‰ salinity treatment group (1605.2 ± 755.4‰), but there was no significant difference ($p=0.129$) between the three groups. The leaf $\delta^{15}$N value of the high water level treatment group (1781.4 ± 685.6‰) was slightly lower than that of the low water level treatment group (1012.5 ± 158.3‰) and the medium water level treatment group (1674.9 ± 187.6‰), but the differences between the three groups were not significant ($p=0.651$). The ANOVA results suggested that the effects of salinity ($p=0.542$) and water level ($p=0.184$) on leaf $\delta^{15}$N value were not significant.

### Plant growth and nitrogen absorption

The increment of stem height and stem diameter, biomass of different plant organs determined is shown in Table 2. Calculated absorption amount of $\delta^{15}$N by different plant organs is shown in Table 3. Table 2 showed that the plant seedlings had the lowest values of all growth indexes in treatment group with high salinity coupling with high water level, which meant the combine effect of these two factors had extremely significant effects on the plant growth.

Table 3 showed that total absorption amount of nitrogen were relative high in the 10‰ salinity treatment group, but relative lower in the 30‰ salinity treatment group. The lowest value of absorption amount of nitrogen was in the treatment of 30‰ salinity and high water level treatment.
The proportion of $^{15}$N absorbed by plant in different plant organs are shown in Figure 6. Figure 6 showed that most $^{15}$N absorbed were in leaves except for the treatment group of 30‰ salinity and high water level which was the highest in stem. With the enhancing of the water level treatment, the proportion increased in root.

**Discussions**

Salt accumulation and reduced water use efficiency in plants can lead to a decline in the net primary productivity, growth and seedling survival rate of mangrove forests, and may also change the competitive relationships among mangrove species [9]. With the changes in environmental factors, the C content, N content, C:N ratio, δ$^{13}$C and δ$^{15}$N value of each component in the habitat are likely to change.

**C/N content and C:N ratio**

Plant C:N ratio is very effective in the determination of continuous environmental changes in mangrove ecosystems [13]. Because vascular plants have a high cellulose content, their C:N ratios are greater than 12 [13].

The results of the current study showed that an increase in either salinity or water level significantly reduced the root C:N ratio, high salinity significantly reduced the stem C:N ratio, and high water level significantly reduced the leaf C:N, suggesting various responses of different organs to salinity and water level. The average leaf C content, N content, and C:N of *A. corniculatum* in each treatment group were 46.89%, 0.89%, and 53.5, respectively, which were slightly higher, lower, and much higher than the C content (39.7%), N content (3.0%) and C:N ratio (10.2–22.7) of the 1–6 month-old leaves of *Acrostichum aureum* from La Mancha in Mexico [31]. Since *Ac. aureum* is a fern and the sample was young leaves, the measured results showed major differences from those of the current study. In this study, the root, stem, leaf C:N ratios (53.5–106.1) of *A. corniculatum* were close to those of *Lumnitzera racemosa* from Belize (46.5–116.1) [32], but slightly higher than those of *L. racemosa* from Florida, USA (42.2–76.0) [33].

**δ$^{13}$C value**

The δ$^{13}$C value of soil organic carbon (SOC) from high tidal flats where *A. corniculatum* is dominant is relatively stable at a depth of 0–20 cm, reflecting the high productivity and rapid decomposition of organic matter in mangrove forests. The SOC δ$^{13}$C is the most abundant at the 20–70 cm depth [15]. The average particulate organic matter δ$^{13}$C values of the upper, middle and outer Vietnam Red River estuaries are −27.88‰, −28.12‰ and −24.83‰, respectively [13]. Among these, the value for the middle estuary is very close to that of the mangrove plant leaves, indicating that the source is from C-3 terrestrial plants (including mangrove leaf litter) [13].

The δ$^{13}$C value of mangrove plants in most regions are lower than those of common terrestrial plants (−27‰), which may be related to their coastal tidal habitat. The δ$^{13}$C value in plants is mainly affected by three factors: (1) inorganic C source for photosynthesis; (2) C isotope fractionation for the CO$_2$ diffusion through the stomata; (3) C fixation by carboxylase in photosynthesis. The leaf δ$^{13}$C value is mainly determined by photosynthesis and stomatal behavior [22]. Wei et al. [23] found that *A. corniculatum* leaves had a low δ$^{13}$C value in a greenhouse with suitable salinity (15‰), and the seedling root and leaf δ$^{13}$C values were slightly (not significantly) affected by the addition of Cd. Wooler et al. [32] found that the δ$^{13}$C value in samples from Florida (average
The average δ¹³C value of mangrove plants is generally reported to be lower than that of their sediments, which is the effect of plant physiological activities on the δ¹³C fractionation [31]. The plant δ¹³C fractionation occurs in presence of flooding and salinity stress, and the plant tissue δ¹³C is associated with the water use under the impacts of environmental factors [22]. The shrubby mangrove plants (height < 1.5 m) have higher water use efficiency than the tree species with a height of 5–10 m, and have a higher δ¹³C value [33].

Ghashghaie [34] showed that there are significant changes in δ¹³C values of different plant organs, such as roots, leaves, stems, flowers, fruits, seeds. In the current study, the average δ¹³C value of plant roots, stems and leaves of different salinity and water level treatment groups decreased in the following order: roots (−30.05‰) > leaves (−30.79‰) > stems (−30.89‰). This is consistent with previous results, in which the root exhibited the highest δ¹³C value; for example, Werth and Kuzyakov [35] reported that the enrichment of δ¹³C in C-3 plant roots was 1.2‰ higher than that in the entire branch, or Bowling et al. [36] suggested that the same measure was 2.3‰ higher than that of the blade. Old leaves contain more δ¹³C-consuming lipids and lignin and transport C to the young leaves, and young leaves contain more δ¹³C-enriched cellulose [37]. The C and N assimilations in the young and old leaves of Ac. danaeifolium and Ac. aureum lead to two opposite gradients of δ¹³C and δ¹⁵N. That is, compared to old leaves, δ¹³C is enriched and δ¹⁵N is depleted in young leaves [31]. When the leaves develop and reach maturity, the δ¹³C value of the old leaves in Ac. aureum is lower than that of young leaves because of ¹³C depletion and ¹⁵N enrichment. This can be explained by the different mechanisms of C and N assimilations, the transportation of these photosynthetic products from old leaves to the young leaves, and the fact that the plant leaf δ¹³C value increases with the accumulation of Na [31]. The plant tissue δ¹³C is associated with its inherent salt tolerance, and therefore, there are differences in δ¹³C among different species or different organs of the same plant [22].

In the current study, the fact that the increase in the salinity significantly increased the root, stem and leaf δ¹³C value in A. corniculatum demonstrates that salinity is closely related to the plant tissue δ¹³C content; however, the effect of water level on the root, stem and leaf δ¹³C value was not significant. The antioxidant activity of A. corniculatum may play an important role in the salinity and drought stress [38, 39]. Exogenous application of glycine betaine has a positive effect for the A. corniculatum under drought stress, which is favorable for mangrove plants to mitigate adverse effects [39]. Cardona-Olarte et al. [26] showed that under salinity and hydroperiod treatments, L. racemosa was more sensitive to the treatments than Rhizophora mangle, and the effect of hydroperiod was more significant than that of salinity. Mangrove plants absorb salts through their roots, but A. corniculatum and other plants retain only water molecules and the necessary inorganic salts, and expel excess salt through salt glands in the leaves. The process is regulated by hypodermal cells, which can store either salts or water, and the NaCl concentration of the xylem is relatively high; about 1/10 that of sea water [1].

δ¹⁵N value

The δ¹⁵N value of mangrove forests reflects the overall nutritional status of ecosystems [40]. In the current study, the increased water level significantly increased the sediment δ¹⁵N level, which may be because the δ¹⁵N labeled nitrogen dissolved in water migrated to a higher sediment position with the tidal water and the sediment sampling location was relatively high. The measured sediment organic matter δ¹⁵N value was 6.1 ± 0.4‰–58.8 ± 1.3‰, with an average of 23.5 ± 21.5‰. Because of the artificially added δ¹⁵N markers, the δ¹⁵N values in this study were significantly higher than those of the sediment from the northeast wetlands of Gulf of Mexico (1.8–10.4‰) [41], and the average values of the sediments from the mangrove forests at Ko Muk (2.8 ± 1.2‰) and Ko Tariibong estuaries (0.7–4.7‰) [42].

The plant N content and δ¹⁵N value in each treatment group exhibited a consistent trend and increased in the following order: roots < stems < leaves. Extremely large significant differences were observed between the three organs. With the exception of the leaf N content, the root and stem N contents displayed a clear increasing trend with increasing water level. The δ¹⁵N value of roots, stems, and leaves in the low salinity (10‰) and medium salinity (20‰) treatment groups also exhibited an increasing trend with increasing water level, which is generally consistent with the trend of N content. However, significant changes were found in the high salinity (30‰) treatment group, presumably because A. corniculatum adjusted the allocation of δ¹⁵N in different organs after being subjected to high-salinity stress. Similarly, there is no significant difference between Florida L. racemosa and Belize L. racemosa in terms of the average δ¹⁵N value (Florida: 0.6‰, Belize: 0.3‰) [43]. Under the impact of seabird droppings (with a δ¹⁵N value of about 14.0‰), the δ¹⁵N value of L. racemosa could reach up to 11.4–12.3‰, revealing that the plant composition changes in response to the changes in N source [43].

The characteristics of the δ¹⁵N variation in mangrove plant leaves can be used to identify plant responses to environmental stress gradients (such as water and nutrient limitation) and regulatory mechanisms (such as salinity and S toxicity), and are subject to the effects of inorganic N, soluble phosphate and pore water sulfide.
Table 4. Correlation analysis between nitrogen and growth of A. corniculatum by effects of salinity and drought stress.

| Plant organ | Item                              | Stem height (cm) | Stem base diameter (mm) | Total biomass (g) |
|-------------|-----------------------------------|------------------|-------------------------|------------------|
| Root        | Total nitrogen                    | −0.314           | −0.213                  | −0.033           |
| Root        | Assimilation amount of $^{15}$N   | 0.688*           | 0.840**                 | 0.853**          |
| Stem        | Total nitrogen                    | −0.803**         | −0.812**                | −0.643           |
| Stem        | Assimilation amount of $^{15}$N   | 0.811*           | 0.880**                 | 0.903**          |
| Leaf        | Total nitrogen                    | −0.033           | 0.295                   | 0.514            |
| Leaf        | Assimilation amount of $^{15}$N   | 0.750*           | 0.800**                 | 0.835**          |

*Indicates significant correlation at 0.05 levels; **Indicates extremely significant correlation at 0.01 levels.

Table 5. Analyses of variance on plant growth of A. corniculatum.

| Analyses of variance (F value and significant) | C content (%) | N content (%) | C:N ratio | $\delta^{13}$C (%) | $\delta^{15}$N (%) | Stem height (cm) | Stem base diameter (mm) | Total biomass (g) |
|-----------------------------------------------|---------------|---------------|-----------|---------------------|---------------------|-------------------|------------------------|------------------|
| Salinity (%)                                  | 1.97          | 1.45          | 2.40      | 3.69*               | 4.08*               | 207.40**          | 62.83**                | 39.08**          |
| Water level                                   | 2.22          | 3.97*         | 9.14**    | 2.36                | 20.59***            | 1.16              | 1.12                   | 1.69             |
| Salinity × water level                        | 2.01          | 3.53*         | 7.38**    | 3.47*               | 11.80**             | 24.37**           | 16.52**                | 10.56**          |

*Indicates significant correlation at 0.05 levels; **Indicates extremely significant correlation at 0.01 levels.

Conclusions

1. The effects of salinity and water level on the behavior of the N cycle within the simulated mangrove A. corniculatum wetland system were complex; the responses of different plant organs to salinity and water level were not completely consistent. Results showed that salinity had no significant effect on the C and N content, and C:N ratio, but had significant or extremely significant effects on the $\delta^{13}$C and $\delta^{15}$N values, plant stem height, stem base diameter, and total biomass.

2. In the current study, the increase in salinity significantly increased root, stem and leaf $\delta^{15}$N value of Ac. aureum, because the plant tissue $\delta^{13}$C values was related to its inherent salt tolerance [22], but there were no significant relationship with the water level treatments. The $\delta^{15}$N values were strongly affected by different salinity and water level treatments, indicating that the behavior of the N cycle was somewhat difference with the C cycle, and affected by both salinity and water level.

3. Most of $^{15}$N absorbed by plant tissues were in leaves except for the highest salinity and high water level treatment, indicating that increasing water level, the proportion increased in the root. This result may be because A. corniculatum adjusted the $\delta^{15}$N allocations among all organs after being subjected to high-salinity stress.
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Disclosure statement

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