# Assembling plant diversity mitigates greenhouse gas emissions and achieves high nitrogen removal when treating the low-C/N wastewater by constructed wetlands

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# Abstract

The low carbon-to-nitrogen (C/N) ratio in wastewater will inhibit pollutant removal, and more seriously, it will cause an increment of nitrous oxide ($N_2O$) emissions of constructed wetlands (CWs). Raising the C/N ratio of wastewater is an effective way to solve this problem, while it may cause secondary pollution and is costly. Assembling plant diversity promotes N removal, while the effects of plant diversity and increasing C/N ratio on global warming potential (GWP) combined by $N_2O$ and methane ($CH_4$) is lack of comparison. In this study, 108 CW microcosms were established to explore the effects of increasing the C/N ratio from 1 to 5 and assembling plant diversity on N removal and GHG emissions. Results showed that when the C/N ratio was 1, (1) increasing species richness reduced $N_2O$ and $CH_4$ emissions then reduced the GWP by 70%; (2) the presence of *Arundo donax* in microcosms reduced GWP by 72%; (3) an *A. donax* × *Tradescantia fluminensis* × *Reineckia carnea* mixture resulted in a high N removal and decreased the GWP per g N removal by 92% with a cost increment of 0.05 USD per $m^3$ wastewater treated; and (4) as the C/N ratio increasing to 5, the GWP per g N removal of monocultures was reduced by 96%, but the cost increased by at least 0.29 USD per $m^3$ wastewater treated. In summary, configuring plant diversity in CWs is an efficient, clean and cost-effective measure to treat wastewater with a low C/N ratio.


# 1 Introduction

In recent years, the demand for wastewater treatment around the world has increased sharply, with 80% of wastewater being discharged without any treatment (UNESCO 2021). The carbon-to-nitrogen (C/N) ratio of wastewater from different sources ranges from 1 to 132, and the C/N ratio will affect the performance of wastewater treatment facilities (Vymazal 2014; Sylla 2020). Theoretically, the N removal will be limited if the C/N ratio of wastewater was under 2.86 (Itokawa et al. 2001; Pelaz et al. 2018). In addition, wastewater treatment is accompanied by greenhouse gas (GHG) emissions (IPCC 2021). The total GHG emissions around the word by treating wastewater are 775 Mt $CO_2$-eq (IPCC 2014). Low C/N ratio in wastewater also promotes nitrous oxide ($N_2O$) emissions due to incomplete denitrification (Duan et al. 2021). Therefore, the measurement for treating wastewater with low C/N ratio urgently needs to be addressed.

Constructed wetlands (CWs) are the green measure to treat wastewater from dispersed sources (Vymazal et al. 2021). The $N_2O$ and methane ($CH_4$) emissions per kg N removed from CWs are only 3.4% and 0.1%, respectively, of those from wastewater treatment plants (Liu et al. 2012). However, CWs are commonly used to treat domestic wastewater with insufficient C sources (Sun et al. 2016; MEEPRC 2020). CWs are also used to treat piggery wastewater and landfill leachate, and the C/N ratio will be as low as 1 (Feng et al. 2020; Çakirgöz et al. 2021). The discharge of those wastewater with low C/N ratio is still increasing (Mander et al. 2014). Therefore, it is really urgent to optimize the CWs to meet the challenge posed by wastewater with a low C/N ratio.

Adding C sources is a common measure to enhance the wastewater treatment performance for wastewater with a low C/N ratio (Chen et al. 2020; Çakirgöz et al. 2021). Increasing the C/N ratio will enhance N removal (Nguyen et al. 2018) and reduce $N_2O$ emissions (Duan et al. 2021). The lowest levels of $N_2O$ emissions were reported at the C/N ratio of 5 (Wu et al. 2009) or 10 (Zhao et al. 2014; Guo et al. 2020). However, an excessive C/N ratio (such as 20) will also increase $N_2O$ emissions (Wu et al. 2009). In addition, excessive C may promote $CH_4$ emissions (Bhullar et al. 2013). Moreover, increasing the C/N ratio of wastewater means continuous investment and transportation, which increases the cost (Hussain et al. 2019). For example, adding methanol increases the infrastructure cost by 25 ~ 31% (CDM 2007). Therefore, it is urgent to find a more economical and effective method to reach high N removal and low global warming potential (GWP) when treating the low-C/N wastewater.

As an important part of the ecological structure of CWs, plants play a key role in pollutant removal and GHG emissions (Brisson et al. 2020; Vymazal 2020). Optimizing the plant community may enhance N removal and reduce GHG emissions in CWs (Maucieri et al. 2017; Brisson et al. 2020). First, increasing plant richness promotes the complementary utilization of resources by plant communities and enhances N removal (Cardinale 2011; Brisson et al. 2020). Moreover, increasing plant species richness promotes plant biomass production to mitigate $N_2O$ and $CH_4$ emissions (Du et al. 2020; Luo et al. 2020). However, increasing plant richness promotes $N_2O$ emissions due to the increase in plant C secretion (Han et al. 2016). Notably, plant identities are also prominent (Abalos et al. 2014; Jesus et al. 2018). For example, CWs consisting of *Arundo donax* have higher N absorption than CWs consisting of other species (Kouki et al. 2012; Sylla 2020), and CWs with *A. donax* or *Canna indica* have lower $N_2O$ emissions than those without them (Luo et al. 2020). Nevertheless, studies on the effects of plant diversity on CW ecosystem functioning have mostly been carried out in inorganic (C-free) wastewater treatment systems (Luo et al. 2016; Han et al. 2021). One study showed that the effects of mixing 4 plant species on N removal as well as $N_2O$ emissions were equivalent to that of increasing the C/N ratio to 1 when treating C-free wastewater (Han et al. 2016). Whether assembling plant diversity is a suitable alternative to increasing the C/N ratio to a higher level (5) to effectively solve the problem raised by treating wastewater at a low C/N ratio still needs to be explored.

In this study, 108 simulated vertical flow CW microcosms were set up to compare the effects of increasing C/N ratio from 1 to 5 and assembling plant diversity (species richness and species identity) on ecosystem functioning (N removal and GHG emissions) in CWs. The aims were to study (1) the changes in the N mass removal rates and GHG emissions of microcosms with an increment in C/N ratio from 1 to 5; (2) effects of plant diversity on the N mass removal rates and GHG emissions of microcosms at C/N ratios of 1 and 5; and (3) the costs and benefits of species combinations with high N removal and low GWP when treating wastewater at a C/N ratio of 1.

## 2 Methods

# 2.1 Experimental design

In late March 2019, the total of 108 microcosms (46 cm × 35 cm × 22 cm) were set up in Sandun town (30° 21' N, 120° 02' E), Hangzhou city. Washed sand (diameter = 0.5 ~ 3 mm) was used as the substrate of each microcosm (Fig. S1).

The species richness in plant communities was 1, 3, or 4 (Fig. 1; Fig. S2). Two frequently used species in CWs, *A. donax* L. and *Oenanthe javanica* (Blume) DC., and two ornamental species, *Reineckea carnea* (Andr.) Kunth., and *Tradescantia fluminensis* Vell., were chosen (Fig. S2). The four species are morphologically different, and have different resources utilization mode (Table S1). The nine plant combinations (4 monocultures of each species, 4 three-species mixtures and 1 four-species mixture) and six replicates for each combination were set up based on a randomized block design. The planting density was 12 plants per microcosm. For the mixtures, the individual number of each species was the same, and different plant species were planted adjacent to each other to ensure the evenness of each species.

The synthetic wastewater was according to Hoagland and Arnon (1950), and sucrose was added to the water as the C source (Table 1). This study set the C/N ratio (calculated as COD/N) at 1 and 5 (Table 1). The research group has shown that plant diversity can improve N removal when treating low-C/N ratio wastewater, but there is a lack of research on GWP (Han et al. 2016; Du et al. 2020). Therefore, a C/N ratio of 1 was set in this study to further explore the various plant combinations in one treatment. The research group has also shown that in CWs, the promotion of increasing plant richness on N removal was equivalent to that of increasing the C/N ratio from 0 to 1 (Han et al. 2016). Previous studies suggested the C/N ratio of 5 was conducive to N removal and $N_2O$ reduction at the same time (Wu et al. 2009). Therefore, in this study, a C/N ratio of 5 was set in another treatment for comparing the effect of plant diversity and increasing C/N ratio on N removal and GWP. The pH of wastewater was approximately 6. Each microcosm was fed with wastewater (7 L) once every 10 days. The experiment lasted until the beginning of July.

Table 1
Combination and concentration of nutrients in the simulated wastewater.

| Macroelement | Concentration ($g\ L^{-1}$) | | Microelement | Concentration ($mg\ L^{-1}$) | |
|---|---|---|---|---|---|
| | C/N = 1 | C/N = 5 | | C/N = 1 | C/N = 5 |
| $Ca(NO_3)_2·4H_2O$ | 0.71 | 0.14 | $H_3BO_3$ | 2.86 | 0.57 |
| $CaCl_2·2H_2O$ | 0.30 | 0.06 | $MnCl_2·4H_2O$ | 1.81 | 0.36 |
| $(NH_4)_2SO_4$ | 1.19 | 0.24 | $ZnSO_4·7H_2O$ | 0.22 | 0.04 |
| $KH_2PO_4$ | 0.14 | 0.03 | $CuSO_4·5H_2O$ | 0.08 | 0.02 |
| $MgSO_4·7H_2O$ | 0.49 | 0.10 | $H_2MoO_4·4H_2O$ | 0.09 | 0.02 |
| $C_{12}H_{22}O_{11}$ | 0.30 | 0.30 | $FeSO_4·7H_2O$ | 5.56 | 1.11 |
| | | | $Na_2EDTA$ | 7.44 | 1.49 |

## 2.2 Sample collection and measurements

GHGs mainly include carbon dioxide ($CO_2$), $N_2O$, and $CH_4$ (IPCC 2021). In this study, GHGs were represented by $N_2O$ and $CH_4$ without considering $CO_2$, as $CO_2$ from herbaceous plants is a short-cycle gas and be not a contributor to the greenhouse effect (IPCC 2021). Gas samples were taken by closed static chamber method (Cheng et al. 2007) at the end of the experiment for horizontal comparison between the two treatments. Transparent PVC chambers (48 cm × 35 cm × 110 cm) enclosed all the plants and were made airtight by inserting the bottoms into water. During the pre-experiment, one microcosm was randomly selected for sampling and determination for each block, and sampling was performed at 0, 3, 5, 10, 20, 30, 40, 50 and 60 minutes after the chambers were made airtight. Results showed that the concentrations of $N_2O$ and $CH_4$ increased linearly in the first 20 minutes. Therefore, during the formal test, the sampling time is determined as 20 minutes after the chambers were made airtight. A vacuum gas collection tube was used to collect gas samples from each microcosm. At the same time, the temperature and humidity in the chamber were measured by a hygrothermograph. Gas concentrations were determined by gas chromatography (Agilent 7820, Agilent Technologies Inc., USA).

After gas sampling, a water sample (100 mL) was taken from each microcosm. The concentrations of ammonium ($NH_4^+$-N), nitrite ($NO_2^-$-N), and nitrate ($NO_3^-$-N) were determined by automated colorimetry (CleverChem-Anna, DeChem-Tech.GmbH, Germany). The total N (TN) concentration was analyzed by a nondispersive infrared detector (Multi N/C 3100, Analytik Jena AG, Germany). A multiparameter water quality meter (Bante 900P, Bante Instruments, China) was used to determine oxidation-reduction potential (ORP) and dissolved oxygen (DO).

After water sample collection, all the plants were harvested. The plants were oven-dried (65°C) to a constant weight to measure the aboveground and belowground biomasses. The N concentrations in plant tissues were detected by an elemental analyzer (Flash HT2000, Thermo Finnigan, Germany). To determine the N pool in the substrate, a fresh substrate sample from each microcosm was weighed at 150 g, and the N concentration of each sample was measured after extract by KCl solution (2 mol $L^{-1}$). Bulk density was determined with the ring-cutting method.

# 2.3 Calculations

The calculation of GHG emissions followed the method described by Cheng et al. (2007),

$$E_i = (\rho \times d_c \times V \times T_0) / (d_t \times A \times T)$$

1

where $E_i$ is the emissions rate of $N_2O$ or $CH_4$ (mg $m^{-2}$ $day^{-1}$),$\rho$ is the density of every gas (mg $m^{-3}$) under normal conditions, $d_c$ is the change in gas volume fraction ($m^3$ $m^{-3}$), $V$ is the volume ($m^3$) of the air in chamber, $T_0$ is the reference temperature (273 K), $d_t$ is the sampling duration (min),$A$ is the bottom area ($m^2$) of the chamber, and T is the experimental temperature (K).

The total GWP (g $CO_2$-eq $m^{-2}$ $day^{-1}$) was calculated as the sum of $CH_4$ and $N_2O$ emissions multiplied by 27 and 273, respectively (IPCC 2021).

The TN mass removal rate (NR, mg $m^{-2}$ $day^{-1}$) was calculated as,

$$\mathrm{NR} = (\mathrm{c_i} \times \mathrm{V_i} - \mathrm{c_e} \times \mathrm{V_e}) / (\mathrm{S} \times \mathrm{D})$$

2

where $c_i$ is the influent TN concentration (mg $L^{-1}$), $V_i$ is the total influent volume during the whole operation, $c_e$ is the effluent TN concentration of the end of the last hydraulic cycle (mg $L^{-1}$), $V_e$ is the total effluent water volume (L),$S$ is the microcosm's bottom area ($m^2$), and$D$ is the total experimental time (days).

The N budget of the microcosm was calculated by the mass balance method (Geng et al. 2019),

$$M_i = M_e + M_p + M_s + M_d + M_a$$

3

where $M_i$ is the amount of N supplied (mg), $M_e$ is the amount of N remaining in the effluent (mg), $M_p$ is the amount of plants N uptake (mg), $M_s$ is the amount of N adsorbed by the substrate (mg), $M_d$ is the amount of N used for denitrification, and $M_a$ is the amount of N lost by ammonia volatilization. Among these variables, $M_a$ was less than 1% of the total N removal in a previous study with the same influent N concentration, so it was ignored in this study (Luo et al. 2020).

The plant uptake ($N_p$, mg N m$^{-2}$ day$^{-1}$), denitrification ($N_d$, mg N m$^{-2}$ day$^{-1}$), and substrate adsorption ($N_s$, mg N m$^{-2}$ day$^{-1}$) rates were calculated as,

$$N_p = M_p / (S \times D)$$

4

$$N_d = M_d / (S \times D)$$

5

$$N_s = M_s / (S \times D)$$

6

where $S$ is the microcosm's bottom area (m$^2$), and $D$ is the total experimental time (days).

The transgressive under-emission effect was calculated according to Luo et al. (2016),

$$D_{mix-GHG} = (Mix(Ei) - Min(Ei)) / Min(Ei)$$

7

where $Mix(Ei)$ is the GHG emissions or GWP in the mixtures and $Min(Ei)$ is the lowest GHG emissions or GWP in the relevant monocultures. Similar to the transgressive under-depletion effect (Palmborg et al. 2005), $D_{mix-GHG} < 0$ suggests that the GHG emissions in mixtures were lower than those in the monoculture with the lowest emissions, and this state is called transgressive under-emission (Luo et al. 2016).

## 2.4 Statistical analysis

The data were analyzed to investigate the impacts of plant species richness, identity, and composition on CW ecosystem functioning. At first, the data were subjected to the Kolmogorov-Smirnov test for normality and Levene's test for the variance equality. The GHG emissions data were natural logarithm transformed to meet the analysis of variance statistical assumptions. To meet the statistical requirements, the $N_2O$ emissions data at a C/N ratio of 5 were added by one when taking the logarithm. The effects of species richness and composition on the N mass removal rate and N removal pathways were analyzed by the Kruskal-Wallis test. Independent sample t-tests were used to define the significant differences in GHG emissions or N removal under two C/N ratios as well as the differences in combinations with or without a particular species. The difference between $D_{mix-GHG}$ and 0 was detected by a one-sample t-test. The analyses were performed in SPSS 20 (SPSS Inc., Chicago, IL, USA) and R 4.1.2. at a statistical significance level of $\alpha = 0.05$.

# 3 Results

## 3.1 Effects of plant diversity on $N_2O$ and $CH_4$ emissions under two C/N ratios

When the C/N ratio was 1, the $N_2O$ emissions of the microcosms decreased with increasing species richness ($P < 0.05$; Fig. 2a), and the lowest $N_2O$ emissions occurred in the microcosms with the *T. fluminensis × R. carnea × A. donax* mixture, while the highest $N_2O$ emissions were observed in the monoculture of *T. fluminensis* ($P < 0.05$; Fig. 2b). To allocate *A. donax* and *R. carnea* in the community reduced $N_2O$ emissions (Table 2). When the C/N ratio was increased to 5, the $N_2O$ emissions of all microcosms were reduced ($P < 0.05$; Fig. 2b), but there were no differences among the combinations ($P > 0.05$; Fig. 2b).

**Table 2** Emissions of $N_2O$ and $CH_4$ (mg $m^{-2}$ $day^{-1}$), the GWP (g $CO_2$ eq $m^{-2}$ $day^{-1}$), N removal rate (mg N $m^{-2}$ $day^{-1}$), plant uptake, denitrification, and substrate adsorption rate (mg N $m^{-2}$ $day^{-1}$) in the microcosms with or without the four plant species.

| Response variable | C/N ratio | Presence of a species | | | |
|---|---|---|---|---|---|
| | | *Oenanthe javanica* | *Tradescantia fluminensis* | *Reineckia carnea* | *Arundo donax* |
| $N_2O$ emission | 1 | ↔ | ↔ | ↘ | ↘ |
| | 5 | ↔ | ↔ | ↔ | ↗ |
| $CH_4$ emission | 1 | ↘ | ↔ | ↗ | ↘ |
| | 5 | ↔ | ↔ | ↔ | ↔ |
| GWP | 1 | ↔ | ↔ | ↘ | ↘ |
| | 5 | ↔ | ↔ | ↔ | ↔ |
| N removal | 1 | ↘ | ↔ | ↔ | ↗ |
| | 5 | ↔ | ↔ | ↔ | ↗ |
| Plant uptake | 1 | ↗ | ↔ | ↔ | ↗ |
| | 5 | ↗ | ↔ | ↔ | ↗ |
| Denitrification | 1 | ↘ | ↔ | ↔ | ↘ |
| | 5 | ↗ | ↔ | ↔ | ↘ |
| Substrate adsorption | 1 | ↔ | ↔ | ↔ | ↔ |
| | 5 | ↔ | ↔ | ↔ | ↘ |

Notes: Arrows indicate a significant increase (↗), decrease (↘), or no effect (↔). A value of "1" indicates a C/N ratio of 1, while "5" indicates a C/N ratio of 5.

Similarly, increasing species richness reduced $CH_4$ emissions at a C/N ratio of 1, but not 5 (Fig. 2c). Regardless of whether the C/N ratio was 1 or 5, the $CH_4$ emissions were lowest for the mixture of *O. javanica × A. donax × R. carnea*, while those of the *R. carnea* monoculture were the highest ($P < 0.05$; Fig.

2d). To allocate *O. javanica* and *A. donax* in the community reduced $CH_4$ emissions (Table 2). For the *R. carnea* monoculture, the $CH_4$ emissions was declined when the C/N ratio was increased to 5 ($P < 0.01$; Fig. 2d).

# 3.2 Effects of plant diversity on GWP under two C/N ratios

For comprehensively evaluate GHG emissions, the $N_2O$ and $CH_4$ emissions were integrated into the GWP. When the C/N ratio was 1, the GWP negatively responded to species richness ($P < 0.05$; Fig. 3a). The GWP of the microcosms with the *T. fluminensis × R. carnea × A. donax* mixture was the lowest, and that of the *T. fluminensis* monoculture was the highest ($P < 0.05$; Fig. 3b). Configuring the *A. donax* and *R. carnea* in community reduced the GWP when the C/N ratio was 1 (Table 2). The $N_2O$ emissions composed the major part of GWP at a C/N ratio of 1, while $CH_4$ emissions were the driver at a C/N ratio of 5 (Fig. 3b). The GWPs of all the combinations was decreased as the C/N ratio increasing to 5, and the lowest GWP appeared in the *O. javanica × A. donax × R. carnea* mixture ($P < 0.05$; Fig. 3b).

The trend of GWP per g N removal was similar to that of GWP. The GWP per g N removal negatively responded to species richness only when the C/N ratio was 1 ($P < 0.05$; Fig. 3c). The GWP per g N removal of the microcosms with the *T. fluminensis × R. carnea × A. donax* mixture was the lowest ($P < 0.05$; Fig. 3d). Increasing the C/N ratio to 5 decreased the GWPs of the combinations except for the mixture of *T. fluminensis × R. carnea × A. donax*. The GWP per g N removal of the *T. fluminensis × R. carnea × A. donax* mixture under a C/N ratio of 1 was as low as the average value of monocultures under a C/N ratio of 5 ($P > 0.05$; Fig. 3d).

# 3.3 Effects of plant diversity on N removal and pathways under two C/N ratios

When the C/N ratio was 1, the TN mass removal rate was slightly improved with increasing species richness only when the C/N ratio was 5 ($P < 0.001$; Fig. 4a). The TN mass removal rate of *O. javanica* was lower than that of the other plant combinations ($P < 0.05$, Fig. 5a). Increasing the C/N ratio to 5 reduced the TN mass removal rate in each combination ($P < 0.05$, Fig. 5a). When the C/N ratio was 5, the monoculture of *A. donax* and the mixtures containing *A. donax* had the highest TN mass removal rates ($P < 0.05$; Fig. 5a). Regardless of whether the C/N ratio was 1 or 5, the presence of *A. donax* increased the TN mass removal rate (Table 2).

Plant uptake, denitrification, and substrate adsorption are three major N removal pathways. Species richness was positively correlated with plant uptake under the two C/N ratios ($P < 0.001$; Fig. 4b). When the C/N ratio was 1, the plant uptake was highest in the *A. donax* monoculture and the mixtures containing *A. donax* ($P < 0.05$; Fig. 5b). Increasing the C/N ratio to 5 resulted in a decrease in plant uptake in the *A. donax* monoculture and all the mixtures containing *A. donax* ($P < 0.05$; Fig. 5b). When the C/N ratio was 5, the *A. donax × T. fluminensis × O. javanica* mixture had the highest plant uptake ($P < 0.05$; Fig. 5b). Regardless of whether the C/N ratio was 1 or 5, the presence of *A. donax* increased plant uptake but inhibited denitrification (Table 2). Under the two C/N ratios, increasing species richness decreased denitrification ($P < 0.001$; Fig. 4c). Increasing the C/N ratio from 1 to 5 decreased denitrification in each combination ($P < 0.01$; Fig. 5c). Regardless of whether the C/N ratio was 1 or 5, the highest levels of denitrification occurred in the monocultures of *T. fluminensis* and *R. carnea* ($P < 0.05$; Fig. 5c). Substrate adsorption slightly decreased with increasing species richness only when the C/N ratio was 5 ($P < 0.05$; Fig. 4d), and increasing the C/N ratio to 5 led to a reduction in substrate adsorption ($P < 0.01$; Fig. 5d).

## 4 Discussion

# 4.1 Increasing plant richness alleviates GHG emissions under a low C/N ratio

Low C/N ratio in wastewater, theoretically defined as lower than 2.86, usually leads to high $N_2O$ emissions (Itokawa et al. 2001; Duan et al. 2021). In this study, when the C/N ratio was 1, the average $N_2O$ emissions of monoculture microcosms accounted for 5% of the inflow TN mass (Table S2). This contribution is slightly higher than the scope of previous reports (0.001 ~ 4%; Table S2). Fortunately, increasing plant richness in CWs can make the mean $N_2O$ emissions of mixtures (species richness at 3 or 4) 70% lower than that of monoculture microcosms when the C/N ratio is 1 (Fig. 2a). This result is different from the fact that increasing plant richness in CWs from 1 to 4 increased $N_2O$ emissions by 68 ~ 267% when treating C-free wastewater (Fig. S4), but is consistent with the trend reported by Du et al. (2020) that the mean $N_2O$ emissions of mixtures were 46% lower than those of monocultures at a C/N ratio of 1. Community denitrification is one of the important factors affecting $N_2O$ emissions (Maucieri et al. 2017). In this study, increasing plant richness reduced the denitrifying N indirectly by promoting plant uptake by 2-fold compared with that of monocultures and then reduced $N_2O$ emissions (Fig. 3c, d; Fig. S5). Furthermore, it was found that the higher the N in the ecosystem was, the stronger the effect of improving plant richness on reducing $N_2O$ in the CW ecosystem (Fig. S4). The above results show that when CWs are used for treating wastewater with a low C/N ratio, improving plant species richness has great potential for $N_2O$ emissions reduction.

In this study, the $N_2O$ emissions of CWs account for 90% of GWP (calculated as the combination of $N_2O$ and $CH_4$ emissions) when the C/N ratio is 1 (Fig. 3b). This result is similar to the values of 84% reported by Du et al. (2020) and 88% reported by Luo et al. (2020). Another study pointed out that $N_2O$ emissions can account for up to 75% of the C footprint in wastewater treatment plants (Daelman et al. 2013), indicating that controlling $N_2O$ emissions is the key factor in reducing GWP. However, there is often a trade-off between $N_2O$ and $CH_4$ emissions (Saha et al. 2017), so reducing the emissions of one gas may be at the cost of adding another one. For example, Yao et al. (2012) found that although the application of N fertilizer in rice fields reduced $CH_4$ emissions by 53%, it increased $N_2O$ emissions by 6-fold and was not conducive to reducing GWP. However, in this study, increasing species richness reduced $CH_4$ emissions by 56% on the premise of reducing $N_2O$ emissions at a C/N ratio of 1 in the CW microcosms

(Fig. 2c). Therefore, a simultaneous reduction in the emissions of the two GHGs was realized, so the system has great potential for a final reduction in GWP.

### 4.2 Species identity surpasses species richness in terms of the effect on GHG mitigation under a low C/N ratio

The plant species also affects community GHG emissions in CWs (Maucieri et al. 2017). *A. donax* was the most conducive to GHG emissions reduction among the species in this study (Table 2, Table S3). Compared with the microcosms without this species, the presence of *A. donax* in microcosms reduced $N_2O$ by 71% under a low C/N ratio (Table 2, Table S3). The allocation of *A. donax* in the community also reduced $N_2O$ emissions by 42% in the treatment of C-free wastewater (Luo et al. 2020). Configuring species with high plant uptake in the community is conducive to $N_2O$ emissions reduction (Oram et al. 2020). *A. donax* has a high plant N uptake capacity that is 40% (Kouki et al. 2012) or 77% (Meng et al. 2015) higher than that of *Typha latifolia*, and its productivity is higher than that of 19 other species (Liu et al. 2012). The plant N uptake of *A. donax* was 2- to 23-fold higher than that of the other species in this study (Fig. 4b; Fig. S6). The allocation of *A. donax* in the community increased plant N uptake by 200% (Table S3) and indirectly reduced the $N_2O$ emissions reflected by structural equation modeling (Fig. S5). In addition, *A. donax* has developed roots and a high oxygen secretion capacity, which is conducive to reducing denitrification and reducing $N_2O$ (Lai et al. 2011; Kuypers et al. 2018). In this study, *A. donax* had the largest underground biomass production, and the presence of *A. donax* improved the DO concentration while reducing denitrification (Fig. S1; Table S3). An increase in DO may promote $CH_4$ oxidation (Bhullar et al. 2013), and the presence of *A. donax* reduced $CH_4$ emissions by 63% (Table 2; Table S3). In conclusion, *A. donax* is an excellent species for practical use since it has a high plant N uptake capacity and mitigates the emissions of two GHGs in the plant community.

It is worth noting that the total biomass production of a single plant of *A. donax* in mixtures was always higher than that in monoculture (Fig. S7, S8). This result means that *A. donax* can play a greater role in GHG emissions reduction in mixed communities than in monocultures. In fact, *A. donax* is also recommended as a high-yield biofuel production species to further reduce the GWP worldwide (Corno et al. 2014). Moreover, the presence of *A. donax* enhanced the TN mass removal by 8 mg $m^{-2}$ $day^{-1}$ in community (Fig. 4a; Table 2). Therefore, the combination containing *A. donax* is suitable for the wastewater treatment with a low C/N ratio to improve N removal while reducing GWP.

GHG emissions vary greatly among plant species, and monocultures increase the risk of high emissions. When the C/N ratio was 1, the $N_2O$ emissions of the *T. fluminensis* monoculture were higher than those of other species in previous studies (1.3 ~ 293.8 mg $m^{-2}$ $day^{-1}$, Fig. S3, Table S2). In this study, $NO_2^-$-N was positively correlated with $N_2O$ emissions (Fig. 6a). The accumulation of $NO_2^-$-N in this configuration (4.6 mg $L^{-1}$, Fig. 6b) indicated incomplete denitrification so that N was released in the form of $N_2O$ (Itokawa et al. 2001; Pan et al. 2013). Regarding $CH_4$ emissions, the monoculture of *R. carnea* yielded a high level in previous studies (-3.3 ~ 110.6 mg $m^{-2}$ $day^{-1}$, Table S2). However, no high emissions were observed in mixed communities containing these two species (Fig. 2b, c; Fig. 3b). Du et al. (2020) reported that species with a small biomass proportion in a mixture had little effect on community functioning, supporting the mass ratio hypothesis (Grime 1998). Similarly, in this study, the biomass proportions of *T. fluminensis* and *R. carnea* in mixtures only accounted for 8% and 3% on average, respectively (Fig. S7). Mixing plant species eliminated the disadvantage of high GHG emissions of *T. fluminensis* and *R. carnea* and was conducive to the ornamental value of the plant community (Hu and Gill 2015; Table S1). Therefore, although monocultures of *T. fluminensis* and *R. carnea* are not recommended for treating low-C/N ratio wastewater, they can be used in mixtures to ensure clean treatment.

Interestingly, *A. donax* combined with two high-GHG-emissions species (*T. fluminensis* and *R. carnea*) resulted in the most effective plant combination when treating a low-C/N wastewater (Fig. 2b, d). The plant species composition had a greater effect than plant species richness on GHG emissions reduction (Table 3; Table S6). This result is similar to the findings of Abalos et al. (2014) in grassland; that is, plant species composition explained a greater proportion of $N_2O$ emissions. In this study, when the C/N ratio was 1, compared with the average GWP of monocultures, the mixture of *T. fluminensis × R. carnea × A. donax* reduced the GWP of the microcosm by 92% (Fig. 3b). The $N_2O$ emissions of *T. fluminensis × R. carnea × A. donax* were 76% lower than those of the *A. donax* monoculture, leading to transgressive under-depletion of GWP in this community (Fig. 6c, d). Similarly, the $NH_3$ volatilization of *Rumex japonicus* × *Cichorium intybus* × *Lolium perenne* was found to be 60% lower than that in the monoculture with the lowest value (Luo et al. 2016). In general, allocating appropriate species combinations, rather than only improving richness, has a higher potential for GWP reduction.

Table 3
Results of two-way ANOVA of the effects of and interactions between plant species richness (SR) or species composition (SC) and C/N ratio on $N_2O$ and $CH_4$ emissions (mg $m^{-2}$ $day^{-1}$), GWP (g $CO_2$ eq $m^{-2}$ $day^{-1}$), and GWP per g N removal (GWP/NR) (g $CO_2$-eq g $N^{-1}$).

| Source of effect | df | SS | MS | SS% | *P* |
|---|---|---|---|---|---|
| **$N_2O$ emissions** | | | | | |
| Block | 5 | 3.60 | 0.72 | 0.95 | 0.308 |
| C/N | 1 | 274.75 | 274.75 | 72.12 | < 0.001 |
| SR | 1 | 4.45 | 4.45 | 1.17 | 0.007 |
| SC | 7 | 22.48 | 3.21 | 5.90 | < 0.001 |
| C/N×SR | 1 | 5.81 | 5.81 | 1.53 | 0.002 |
| C/N×SC | 7 | 19.55 | 2.79 | 5.13 | < 0.001 |
| **$CH_4$ emissions** | | | | | |
| Block | 5 | 2.30 | 0.46 | 3.04 | 0.312 |
| C/N | 1 | 2.26 | 2.26 | 2.99 | 0.017 |
| SR | 1 | 0.33 | 0.33 | 0.44 | 0.355 |
| SC | 7 | 29.64 | 4.23 | 39.21 | < 0.001 |
| C/N×SR | 1 | 1.70 | 1.70 | 2.25 | 0.037 |
| C/N×SC | 7 | 7.03 | 1.00 | 9.30 | 0.016 |
| **GWP** | | | | | |
| Block | 5 | 4.45 | 0.89 | 1.12 | 0.237 |
| C/N | 1 | 284.39 | 284.39 | 71.54 | < 0.001 |
| SR | 1 | 3.59 | 3.59 | 0.90 | 0.020 |
| SC | 7 | 15.84 | 2.26 | 3.98 | 0.002 |
| C/N×SR | 1 | 7.80 | 7.80 | 1.96 | < 0.001 |
| C/N×SC | 7 | 26.96 | 3.85 | 6.78 | < 0.001 |
| **GWP/NR** | | | | | |
| Block | 5 | 4.43 | 0.89 | 1.79 | 0.247 |

| Source of effect | df | SS | MS | SS% | *P* |
|---|---|---|---|---|---|
| C/N | 1 | 133.40 | 133.40 | 53.81 | < 0.001 |
| SR | 1 | 3.60 | 3.60 | 1.45 | 0.021 |
| SC | 7 | 15.95 | 2.28 | 6.43 | 0.002 |
| C/N×SR | 1 | 7.89 | 7.89 | 3.18 | < 0.001 |
| C/N×SC | 7 | 27.26 | 3.90 | 11.00 | < 0.001 |

# 4.3 Manipulating plant combinations is a win-win measure compared to increasing the C/N ratio

Increasing the C/N ratio was found to reduce the $N_2O$ emissions of monoculture CWs by 28 ~ 85% (Table S2). In this study, when the C/N ratio was increased from 1 to 5, $N_2O$ emissions of monoculture CWs decreased by 1-fold in average (Fig. 2b). The $N_2O$ emissions of each plant microcosm decreased to the same low level when the C/N ratio was 5 (0.28 mg $m^{-2}$ $day^{-1}$, Fig. 2b), and further increasing species richness no longer affected $N_2O$ emissions under a high C/N ratio (Fig. 2a). However, in this study, the mass of N released in the form of $N_2O$ from microcosms with a high C/N ratio was lower than 0.4%, which is at the low discharge level of CWs (0.001 ~ 4%, Table S2). Moreover, on the basis of reducing $N_2O$ emissions, raising the C/N ratio further reduced the average $CH_4$ emissions of monocultures by 60% and finally reduced GWP by 98%. This effect is almost equivalent to the optimal emissions reduction obtained with plant combinations. The GWP per unit N removal is a suitable index to comprehensively measure the treatment performance of CWs (Du et al. 2020). The *T. fluminensis × R. carnea × A. donax* mixture reduced the GWP per unit N removal in the treatment of wastewater with a C/N ratio of 1 by 96%, and the value was as low as that of the monocultures with a C/N ratio of 5 (Fig. 3d). This mixture also provided a win-win combination of efficient N removal and low emissions under a low C/N ratio (Fig. 7a). The N removal efficiency of this combination was higher than 90%, which was higher than the average value of previous studies in CWs (55%, Table S4). These results showed that for the treatment of wastewater with a low C/N ratio, the configuration of an appropriate species combination has great potential and can be comparable to increasing the C/N ratio.

### 4.4 Plant diversity is a low-cost route to treat wastewater with a low C/N ratio in CWs

In the practical application of CWs, the trade-off between strengthening water treatment efficiency, reducing environmental impact, and increasing cost must be considered (Resende et al. 2019). The cost of wastewater treatment will rise when increasing the influent C/N ratio, which is generally achieved by adding external C sources (Sun et al. 2010). In wastewater treatment, the commonly used C sources include methanol, sucrose, glucose, and acetic acid, and their costs vary greatly (Table S5). Methanol is the cheapest (0.29 USD per $m^3$ wastewater treated; Table S5) among the commonly used C sources; however, it is toxic (Ramírez et al. 2006). Sucrose is nontoxic and relatively economical (0.45 USD per $m^3$

wastewater treated; Table S5). Under the same infrastructure, energy, and labor costs, when the C/N ratio was increased from 1 to 5 by adding C, the cost (seedlings, planting labor, and sucrose cost) per $m^3$ wastewater treated reached 0.55 USD, while the cost (seedlings and planting labor) for assembling the best plant composition was only 0.15 USD per $m^3$ wastewater treated (Table S5). This means that increasing plant diversity can save 73% of the cost compared with adding sucrose or save 62% of the cost compared with adding methanol (Table 4, Table S5). In addition, perennial plants can operate continuously in CWs (Table S1), and the harvested plant biomass can further effectively produce bioenergy (Liu et al. 2012; Tanaka et al. 2017). Therefore, assembling plant combinations can be more economical in the long terms. However, adding C requires continuous investment (Hussain et al. 2019). These findings showed that optimizing plant diversity will solve the problems, which raised by the low C/N ratio in wastewater, more economically than increasing C/N ratio.

Table 4
Cost analysis of increasing the C/N ratio or species diversity.

| Cost (USD $m^{-3}$ wastewater) | Increasing diversity | Increasing C/N ratio | Data source |
|---|---|---|---|
| *Construction* | | | |
| Infrastructure | 104.00 | 104.00 | Gu et al. 2016 |
| Seedlings | 0.08 | 0.06 | Our data |
| Planting labor | 0.07 | 0.04 | Our data |
| *Operation and maintenance* | | | |
| Energy and labor | 0.01 | 0.01 | Gu et al. 2016 |
| Sucrose | 0.00 | 0.45 | Our data |

Notes: The unit is US$ $m^{-3}$ wastewater treated; the exchange rate from 1st November 2021 of 6.435 was used to convert US$ to RMB.

# 5 Conclusions

When treating wastewater with a C/N ratio as low as 1, both increasing the C/N ratio and assembling plant communities can make CWs efficient and green. Reasonable species combinations can mitigate the GWP of wastewater treatment by mitigating $N_2O$, and the reduction in GWP per g N removal achieved by this method is almost equivalent to that achieved by increasing the C/N ratio to 5. The excellent practical species *A. donax* has a high plant uptake ability, which provides an opportunity to improve efficiency while mitigating the environmental impact of treating wastewater with a low C/N ratio. Considering the economic costs of CWs, assembling optimal plant combinations, such as *T. fluminensis × R. carnea × A. donax*, is more cost-effective than increasing the C/N ratio. The biomass of high-yielding *A. donax* can be further considered to produce bioenergy to enhance the benefits of CWs. Future studies should expand the C/N ratio gradient, consider the time dynamics of GHG emissions, and explore species combinations suitable for different C/N ratios to optimize the treatment effect of CWs.

# Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

### Conflict of interest

The authors declare that they have no conflict of interest.


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### Authors' contributions

Jie Chang, Ying Ge, and Yuanyuan Du designed the experiments. Hang Jiang, Lichunxiao Wang, and Chenxu Xiang performed the experiments. Hang Jiang analyzed the data. Hang Jiang, Jie Chang, and Ying Ge wrote the first manuscript. Wenjuan Han and Yuanyuan Du provided editorial advice in the process of writing the article, and all authors provided insights throughout the completion of the manuscript.


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# Figures

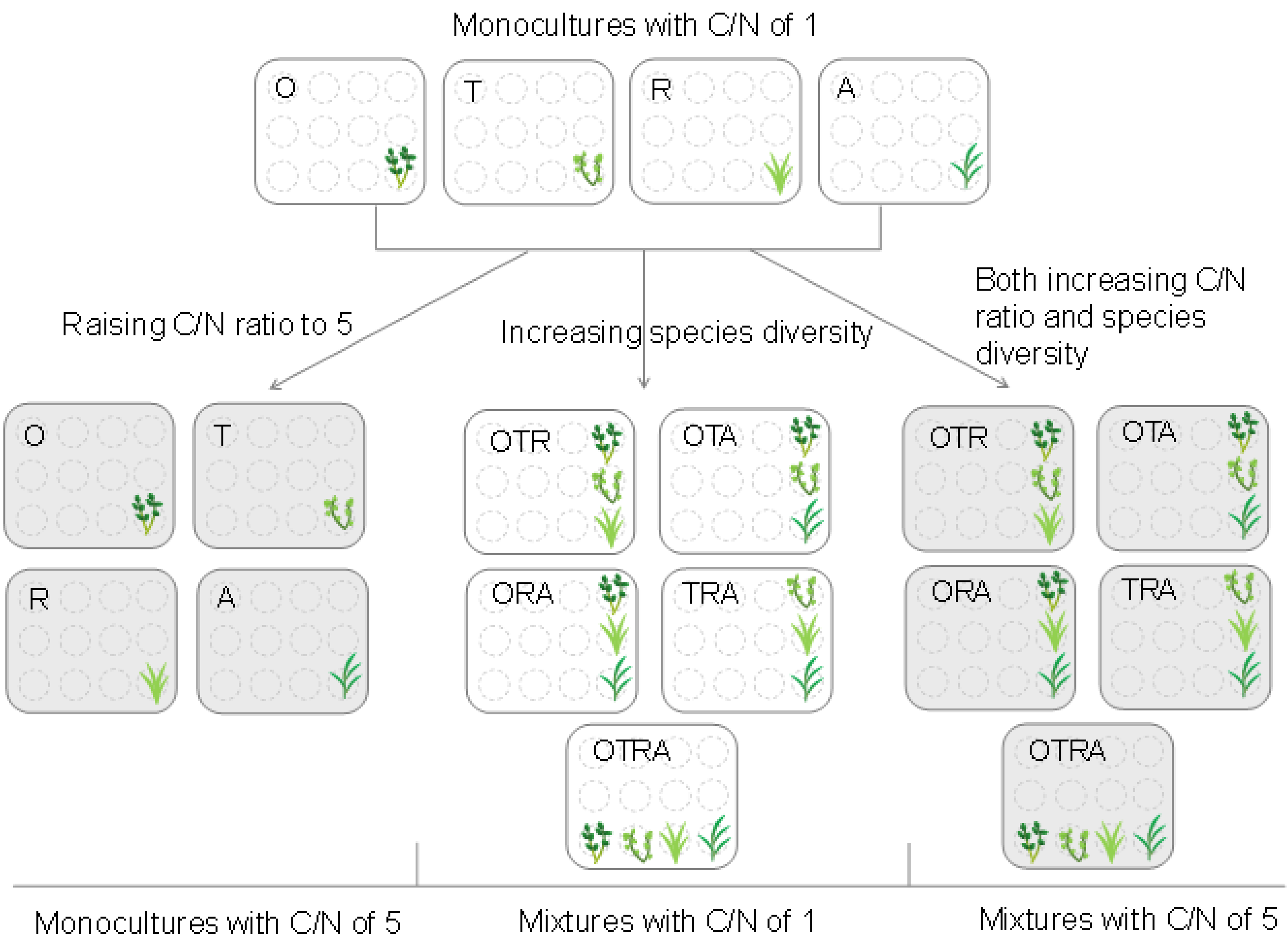



**Figure 1**

Experimental manipulation of species diversity and the C/N ratio. White background: C/N = 1; gray background: C/N ratio = 5. The abbreviations of the plant species are as follows: O, *O. javanica*; T, *T. fluminensis*; R, *R. carnea*; and A, *A. donax*. Each square frame represents a plant combination, and the dashed gray circles represent planting locations; the total number of plants in each combination is 12.

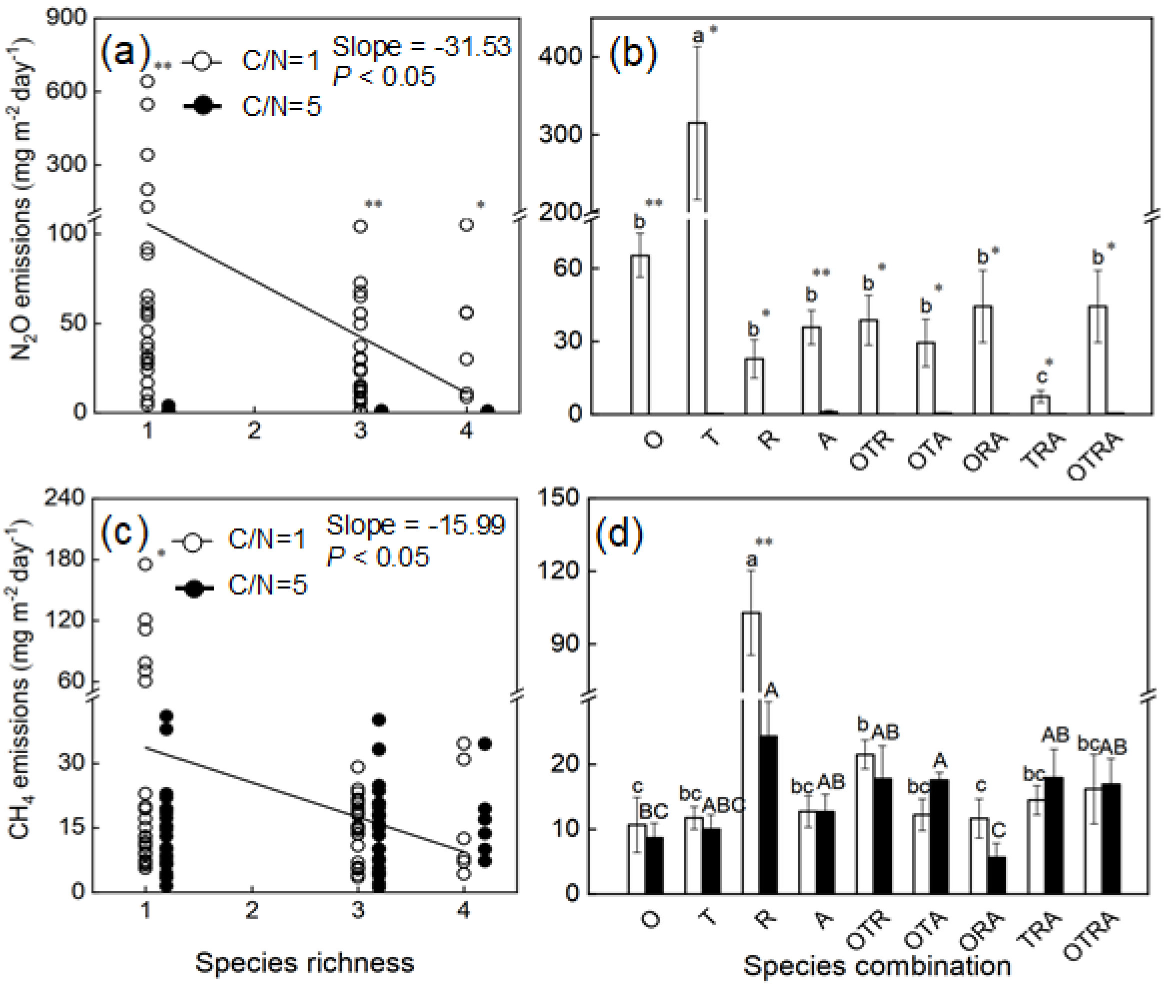
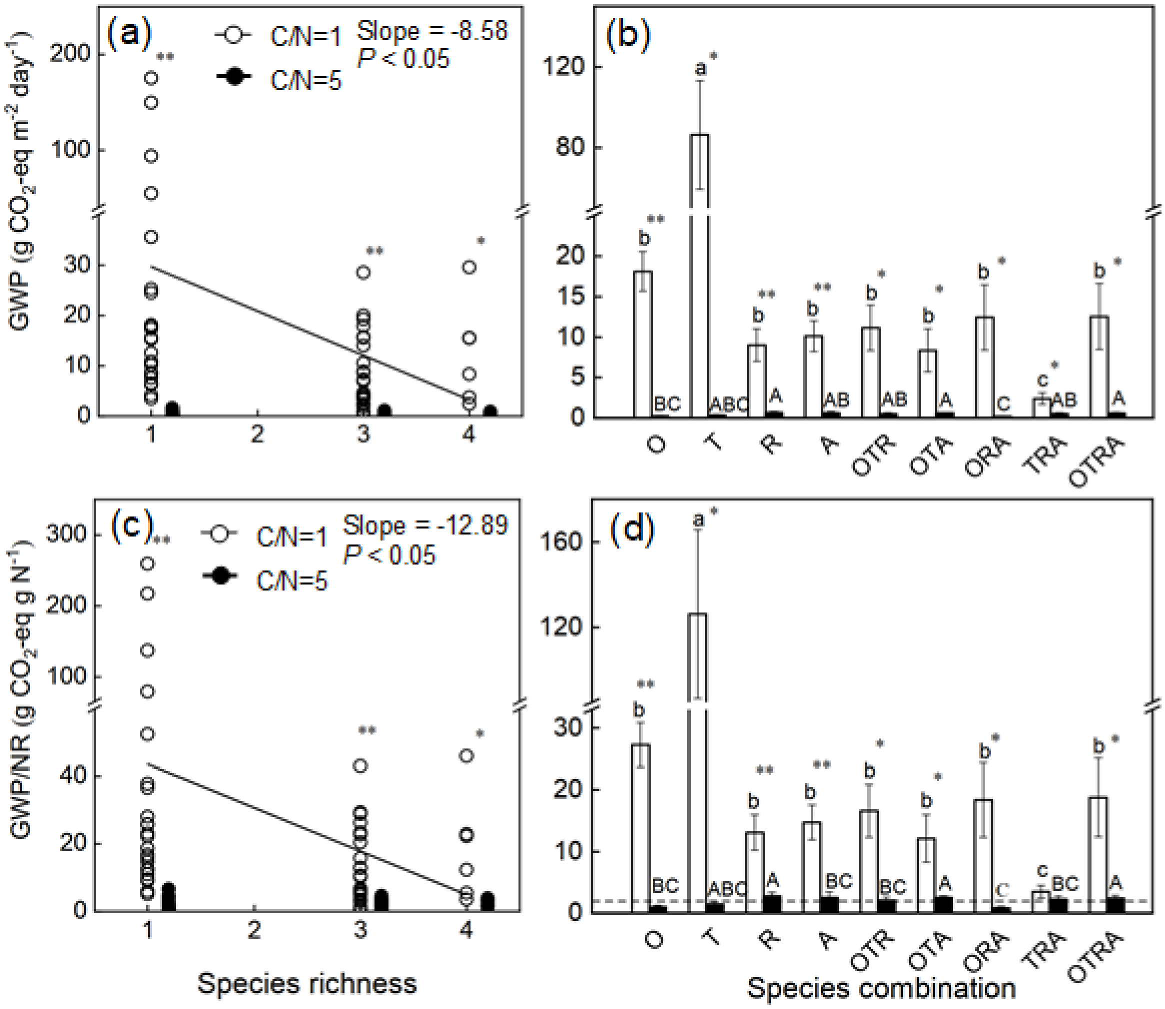


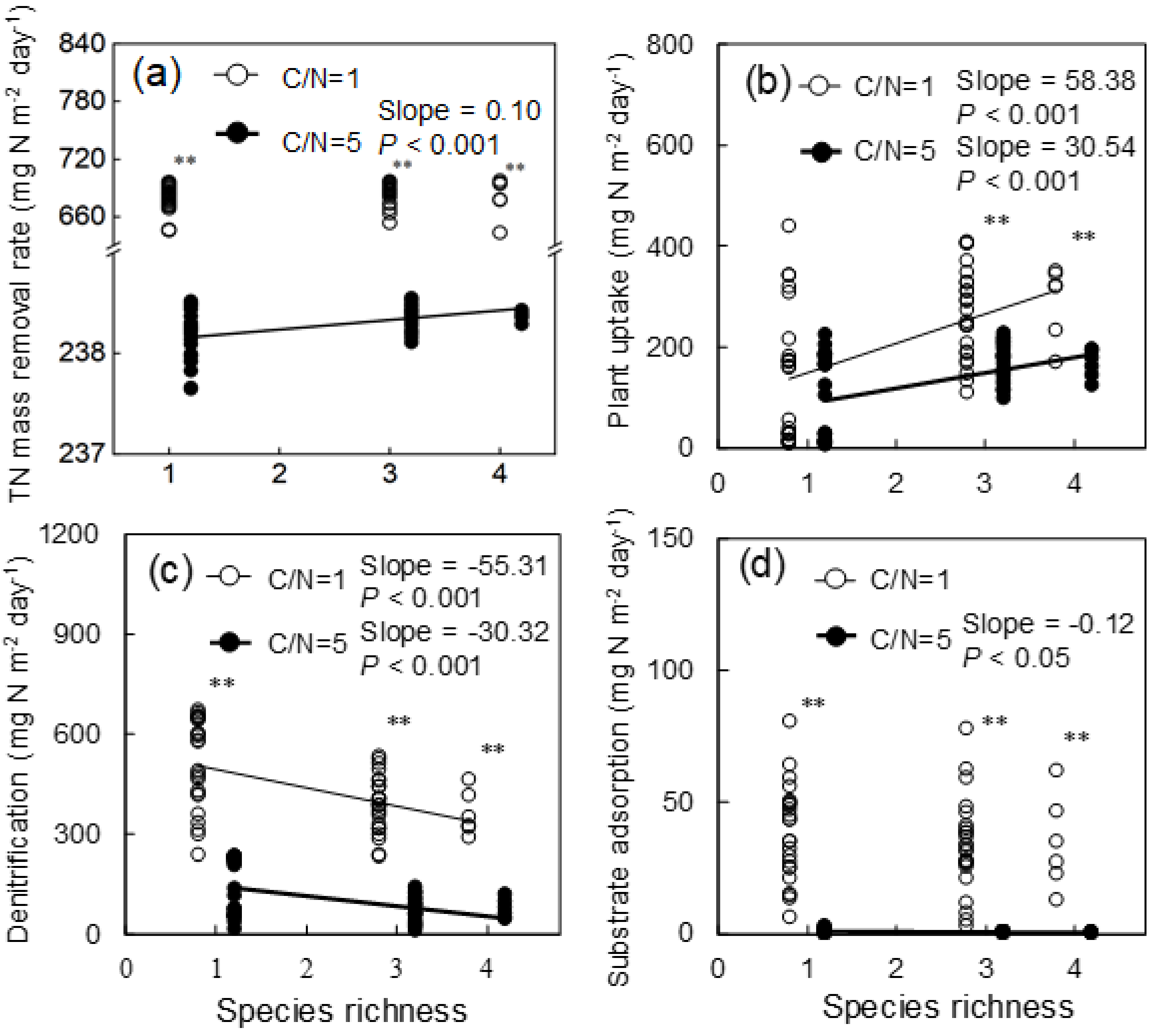

**Figure 2**

Effects of plant diversity on GHG emissions in CWs. (a) Relationship between $N_2O$ emissions and species richness; (b) $N_2O$ emissions among species combinations; (c) relationship between $CH_4$ emissions and species richness; (d) $CH_4$ emissions among species combinations. The abbreviations of the plant species are as follows: O, *O. javanica*; T, *T. fluminensis*; R, *R. carnea*; and A, *A. donax*. The open points, open bars, and thin lines indicate the treatments with a C/N ratio of 1, while the closed points, closed bars, and bold lines indicate the treatments with a C/N ratio of 5. Significant differences among combinations (at $P < 0.05$) are shown by letters above the bars: lowercase letters indicate a C/N of 1, and uppercase letters indicate a C/N of 5. The error bars mean the standard errors, and asterisks show significant differences between the two C/N ratios (* $P < 0.05$; ** $P < 0.01$).



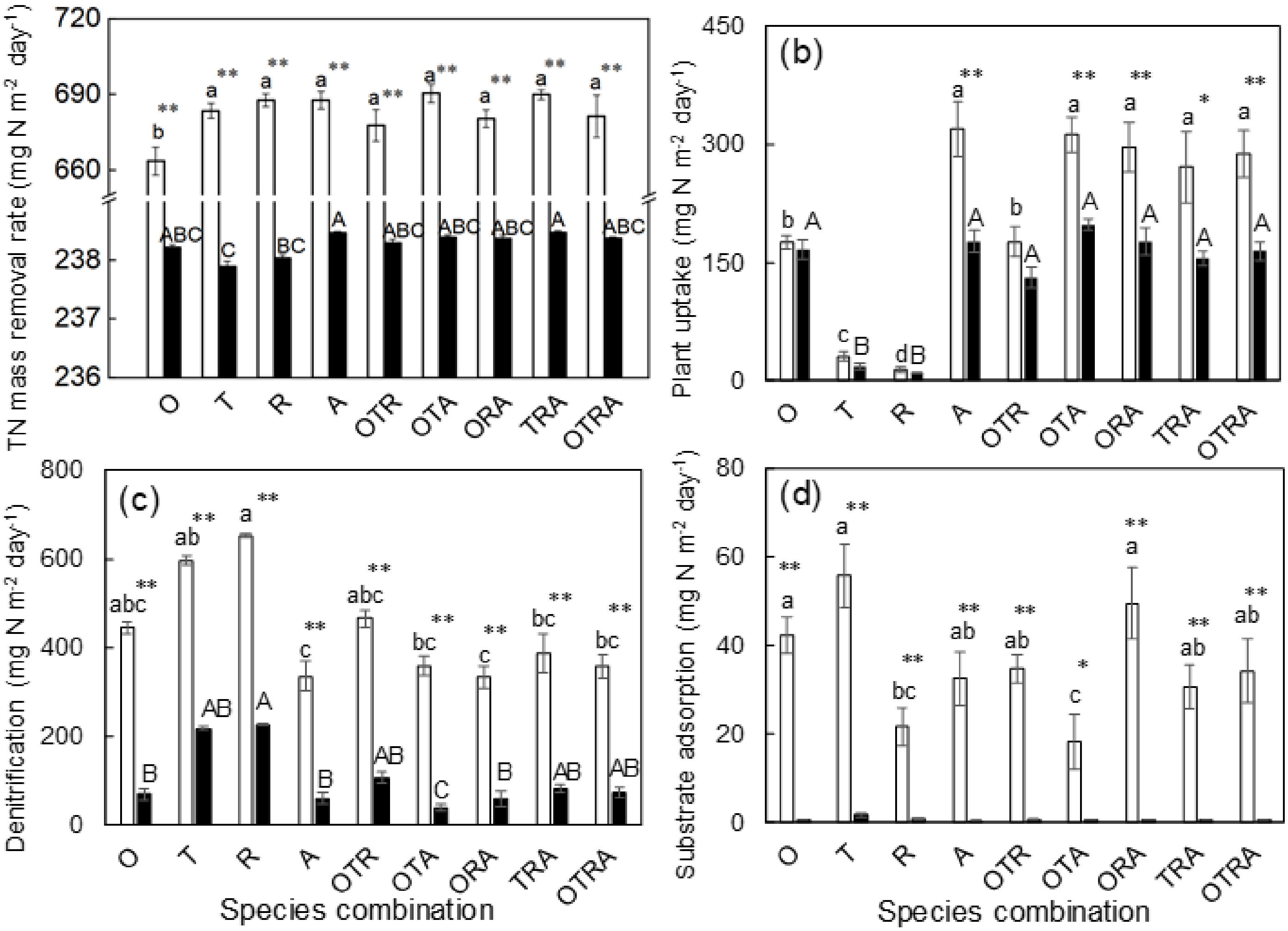

## Figure 3

Effects of plant diversity on GWP in CWs. (a) Relationship between GWP and richness; (b) GWP for species combinations, where the inset panel shows the contribution ratios of $N_2O$ and $CH_4$ to GWP under the two C/N ratios; (c) relationship between GWP per unit N mass removal (GWP/NR) and richness; (d) GWP/NR among species combinations under two C/N ratios, and the dash line represent the average of GWP/NR at the C/N ratio of 5. The abbreviations of plant species (O, T, R, and A), letters, points, and bars are the same as those in Fig. 2. The asterisks indicate significant differences between the two C/N ratios (* $P < 0.05$; ** $P < 0.01$).




## Figure 4

Effects of plant species richness on N removal under the two C/N ratios. (a) TN mass removal rate, (b) plant uptake, (c) denitrification, (d) and substrate adsorption in response to species richness under two C/N ratios. The points and lines are the same as those in Fig. 2. The asterisks indicate significant differences between the two C/N ratios (** *P* < 0.01).




## Figure 5

N mass removal rates among the plant species combinations. (a) TN mass removal rate, (b) plant uptake, (c) denitrification, and (d) substrate adsorption among species combinations under the two C/N ratios. The abbreviations of plant species (O, T, R, and A), letters, and bars are the same as those in Fig. 2. The asterisks indicate differences between the two C/N ratios (* *P* < 0.05, ** *P* < 0.01).

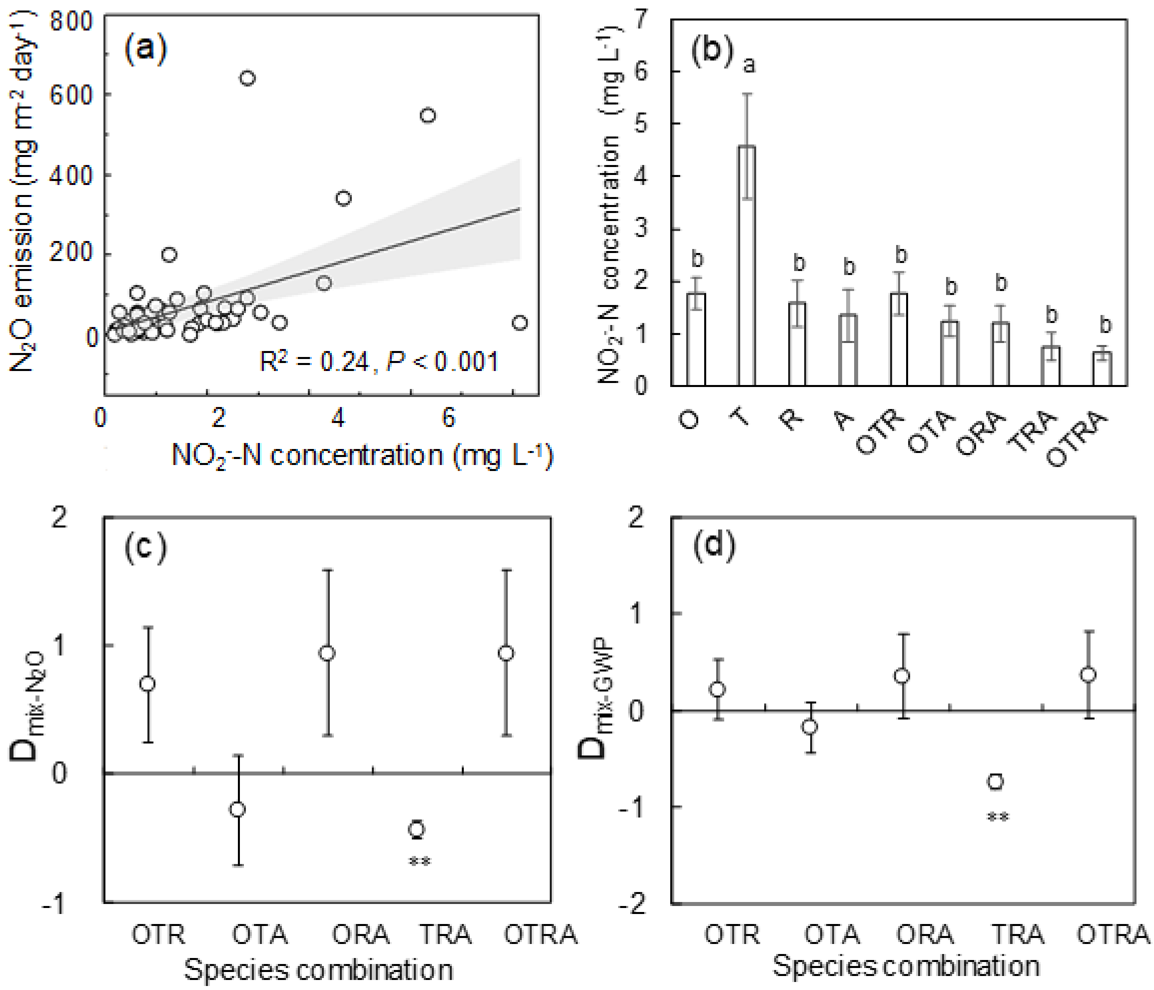



**Figure 6**

Relationships between $NO_2^-$-N concentration and $N_2O$ emissions and the biodiversity effects at a C/N ratio of 1. (a) Relationship between $N_2O$ emissions and $NO_2^-$-N concentration in the effluent, with the gray shaded area representing the 95% confidence interval of the fitting line; (b) $NO_2^-$-N concentrations among species combinations; (c) transgressive under-emission of $N_2O$ among mixtures; (d) transgressive under-emission of GWP among mixtures. The abbreviations of plant species (O, T, R, and A) are the same as in Fig. 2. When the C/N ratio was 5, the effluent $NO_2^-$-N concentration, $N_2O$ emissions, and GWP of the microcosms were nearly zero, so the analysis was not performed. The error bars mean the standard errors, and asterisks show the significant differences between the $D_{mix}$ value and zero (** $P < 0.01$).

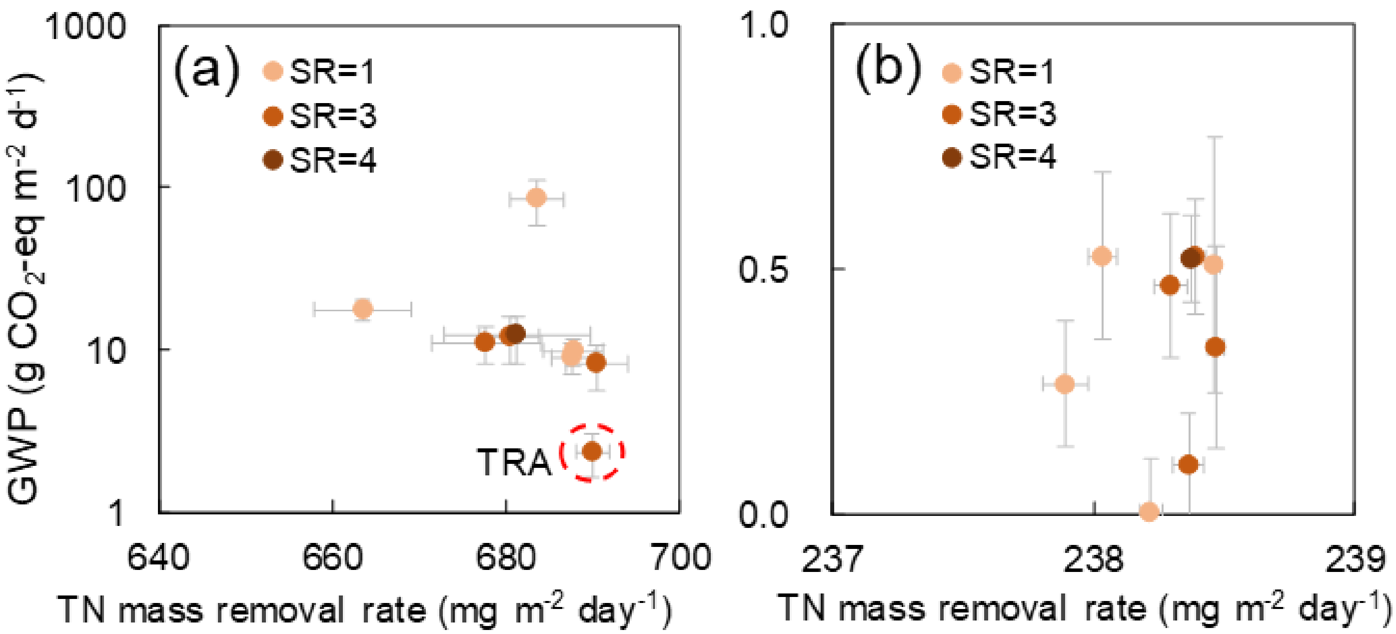



**Figure 7**

Synergy between mitigating GHG emissions and improving N removal when treating wastewater with two C/N ratios. (a) Relationship between GWP and TN mass removal rate at a C/N ratio of 1; the mixture of *T. fluminensis × R. carnea × A. donax* (in the red circle) had high N mass removal with low GWP; (b) relationship between GWP and TN mass removal rate at a C/N ratio of 5.

## Supplementary Files

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- JiangHCNratioSIOK.docx