**Decreases in ammonia volatilization in response to greater plant diversity in
microcosms of constructed wetlands**

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Abstract

Ammonia volatilization from wastewaters with a high concentration of ammonium is a serious environmental and health problem. Constructed wetlands (CWs) are widely used for treating wastewater, and plant diversity clearly improves some functions of ecosystem such as nitrogen removal. However, whether plant diversity can affect ammonia volatilization from wastewater is still unknown. In this study, we conducted a microcosm experiment with different plant diversity treatments using four plant species. Results showed that, (1) ammonia volatilization decreased with increasing plant species richness; (2) ammonia volatilization from systems containing *Rumex japonicus* was lower than other systems; and (3) ammonia volatilization was affected more by species composition than species richness. This paper is the first to report that ammonia volatilization is reduced by plant diversity, and that some plant species combinations are important to reduce ammonia volatilization from CWs when treating wastewater.

Keywords: Ammonia reduction; Ammonium concentration; Species richness; Species composition.

1. Introduction

Ammonia (NH_3) volatilization causes broad concern due to its environmental and health impacts (Sutton et al., 2008; Gu et al., 2014), especially its contribution to haze (Kirkby et al., 2011; Shen et al., 2014). Wastewaters with a high concentration of ammonium nitrogen ($\text{NH}_4^+\text{-N}$, those from livestock production and agricultural fertilizer application) are the important source of NH_3 volatilization (Reis et al., 2009; Huang et al., 2012). How to deal with wastewaters with a high concentration of $\text{NH}_4^+\text{-N}$ and reduce NH_3 volatilization has become an urgent challenge.

Constructed wetlands (CWs) have been widely applied to treat wastewater pollution around the world (Liu et al., 2009; Vymazal, 2014). CWs, as an alternative to the wastewater treatment plant, are feasible and suitable to treat the decentralized agricultural wastewaters with low cost (Liu et al., 2012). However, problems still exist in the large amount of NH_3 that volatilizes from wastewater in the treatment process. While there are some abatement methods for reducing NH_3 from wastewater, such as acidification and rapid nitrification by artificial means, these methods are expensive and energy consuming, and therefore are not feasible (Lin et al., 2005; VanderZaag et al., 2015). There is still a lack of appropriate abatement methods, while alternative ecological approach like plants receives less attention.

Plants play an important role in nutrient cycle and physiochemical condition of substrate (Vymazal, 2007) and can affect NH_3 volatilization in wastewater treatment processes in CWs (Mattsson and Schjoerring, 2002; Poach et al., 2002). Some studies have found that *Bromus erectus* monocultures emitted more than twice the NH_3 than *Lolium perenne* monocultures (Mattsson and Schjoerring, 2002), while the *Scirpus* sp. and *Typha* sp. monocultures had no difference in mean NH_3 volatilization (Poach et al., 2002). The effect of plant communities with diverse species on NH_3 volatilization has not yet been reported to our knowledge.

Plant diversity (species richness and composition) is a major determinant of ecosystem processes and functions, such as productivity, nutrient retention, microbial activity, greenhouse gas emissions, nitrogen removal (Tilman et al., 1996; Zhang et al., 2012; Chang et al., 2014; Ge et al., 2015; Grace et al., 2016). In this study, we

conducted a microcosm experiment to simulate CWs. We chose four species planted in a gradient of increasing richness from 1 to 4 species and assembled 15 possible species combinations. We measured the NH_3 volatilization flux from each microcosm. The aim of this study was to explore in the wastewater treatment process, (i) the relationship between NH_3 volatilization and plant species richness; (ii) the relationship between NH_3 volatilization and plant species composition; and (iii) the relative importance of the effects of species richness versus species composition on NH_3 volatilization.

2. Materials and methods

2.1. Microcosm design, planting pattern and wastewater irrigation

We set up a microcosm experiment to test the effect of plant diversity on NH_3 volatilization. The study site, located at the campus of Zhejiang University in Hangzhou City (120°05' E, 30°18' N), Southeast China, has a humid subtropical climate with an average annual temperature of 18.7 °C and rainfall of 1,350 mm. The microcosm simulated a constructed wetland and consisted of a porcelain column (length × width × height = 51 cm × 38 cm × 18 cm) filled with sand (particle diameter = 0.2 mm - 3 mm) to a depth of 15 cm. Prior to the experiment, the sand was washed with tap water to ensure there was no plant propagule left.

Four common early-spring species, *Cichorium intybus* L., *Lolium perenne* L., *Medicago sativa* L. and *Rumex japonicus* Houtt., with similar size and vitality were transplanted into the microcosms in March, 2014. We planted these species to establish 15 species combination treatments that ranged from a richness gradient of 1 to 4 species: monocultures of the four species, all possible combinations of two-species, all possible combinations of three-species, and a combination with all species. The experiment was arranged in a complete block design with 6 blocks, ensuring that each block contained one microcosm from each treatment and excluding the occurrence of identical replicates in the same block. Therefore, each treatment had 6 replicates and there were a total of 90 microcosms. Each microcosm had 12 individuals with an equal number of individuals assigned to each species. Weeds were removed by hand during the experiment to maintain the original designed species composition.

The simulated wastewater was the Hoagland nutrient solution (Hoagland and Arnon, 1950) with a minor modification. $\text{NH}_4^+\text{-N}$ was the sole nitrogen source in the influent, and its concentration was 336 mg L^{-1} (Table 1). The simulated wastewater was supplied once every 10 days, from late March to late June, 2014. Each microcosm received 7 L simulated wastewater, and the water level was kept 3 cm below the sand surface.

2.2. Sampling methods

The volatilization of NH_3 was measured once using the static chamber technique (Van der Stelt et al., 2007) one day after wastewater feeding at the end of experiment. The chamber was a square polyvinyl chloride chamber (68 L), and was placed to cover a microcosm, with its opening edge overlapping with the edge of porcelain column. A small flask, containing 20 mL 3.2 M H_2SO_4 , was placed on the surface of the sand in advance, in order to trap the volatilized NH_3 . The gas absorption continued for 16 hours and was conducted simultaneously for each microcosm in the same block in order to detect the effect of plant diversity. Immediately after gas collection, the absorption liquid was put into a 100 mL polyethylene plastic bottle, and stored temporarily in a refrigerator at 4 °C. Before gas sampling, 100 mL wastewater was sampled in each microcosm and stored in a refrigerator at -20 °C. The plants were harvested by species after gas sampling. When conducting the harvest, we added water into the microcosms to loosen the sand, so that the whole plants including the aboveground and belowground parts could be taken out of the sand easily.

2.3. Parameter analyses

When the gas was collected, the concentration of $\text{NH}_4^+\text{-N}$ in the absorption liquid was detected by Nessler's reagent colorimetry. The NH_3 volatilization flux (F , $\mu\text{g N m}^{-2} \text{ h}^{-1}$) in the CWs was,

$$F = \frac{C \times V}{T \times S} \quad (1)$$

where C is the concentration of $\text{NH}_4^+\text{-N}$ in the absorption liquid ($\mu\text{g N mL}^{-1}$); V is the volume of absorption liquid (mL); T is absorption time (16 h); S is the trapping area of the chamber (0.194 m^2).

The water samples were filtered by membrane (pore size, 0.45 μm) prior to the analysis for $\text{NH}_4^+\text{-N}$ concentration in wastewater. Plant total biomass was calculated as the dry weight per square meter after the plant samples were oven-dried at 65 $^\circ\text{C}$ for 72 hours and then weighed.

2.4. Statistical analyses

In order to detect the effect of species richness, the simple linear regression was used to test the relationships between plant species richness and the response variables (NH_3 volatilization flux, wastewater $\text{NH}_4^+\text{-N}$ concentration, and plant biomass). In order to detect the effect of species composition, the independent-samples t-test was applied to find differences between means of the response variables when a species was present and when it was absent from the system. One-way ANOVAs tested for differences in the response variables among the 15 plant combination treatments. Where treatment effects proved to be significant, a least significant difference (LSD) test was used as a *post hoc* test to ascertain differences among treatment levels. When species richness and composition both showed effects in above separate analyses, the multiple linear regression was used to account concurrently for the effects of species richness and species composition on the response variables (Engelhardt and Ritchie, 2002). In a multiple regression, “composition” effect was described as the presence or absence of the four species, therefore there were four multiple regressions for each response variable (NH_3 volatilization, wastewater $\text{NH}_4^+\text{-N}$ concentration). All analyses were performed in SPSS (SPSS Inc., Chicago, IL, USA; Version 20). We set the level of significance at $\alpha = 0.05$. The data were presented as means $\pm$ 1 standard error.

3. Results

3.1. Relationship of plant species richness to NH_3 volatilization, $\text{NH}_4^+\text{-N}$ concentration and plant biomass

According to the regression analysis, the NH_3 volatilization decreased with the increase of species richness (Fig. 1A, $P < 0.05$). However, according to the ANOVA, the microcosms with three plant species had the lowest mean NH_3 volatilization flux ($P <$

0.05). The NH_4^+ -N concentration in wastewater also decreased with the increase of species richness (Fig. 1B, $P = 0.07$). However, no significant relationship was found between species richness and total biomass production (Fig. 1C, $P > 0.05$).

3.2. Relationship of species composition to NH_3 volatilization, NH_4^+ -N concentration and plant biomass

There were significant differences in NH_3 volatilization among the 15 species combination treatments (Fig. 2A, $P < 0.05$). The NH_3 volatilization from *R. japonicus* monoculture was the lowest among the four monocultures, followed by the *M. sativa* monoculture, *L. perenne* monoculture, and *C. intybus* monoculture (Fig. 2A). The presence of *R. japonicus* in the microcosms reduced NH_3 volatilization by 63% compared with its absence (Table 2, $P < 0.05$). The presence of the other three species had no significant influence on NH_3 volatilization (Table 2). Furthermore, NH_3 volatilization from three-species polycultures containing *R. japonicus* was significantly lower than that from the lowest volatile monoculture, *R. japonicus* monoculture (Fig. 2A).

There were significant differences in wastewater NH_4^+ -N concentration among the 15 species combination treatments (Fig. 2B, $P < 0.05$). The NH_4^+ -N concentration in wastewater in *L. perenne* monoculture was the lowest among the four monocultures, followed by *R. japonicus* monoculture, *C. intybus* monoculture, and *M. sativa* monoculture (Fig. 2B). The presence of *L. perenne* in the microcosms reduced the NH_4^+ -N concentration in wastewater by 16% compared with its absence (Table 2, $P < 0.05$). The presence of *R. japonicus* in the microcosms reduced the NH_4^+ -N concentration by 22% compared with its absence (Table 2, $P < 0.05$). Overall, the microcosms with *R. japonicus* or *L. perenne* had higher nitrogen removal rate than other microcosms.

Plant biomass production was significantly different among the 15 species combination treatments (Fig. 2C, $P < 0.05$). The *L. perenne* monoculture produced the largest total biomass among the four monocultures, followed by the *R. japonicus* monoculture, *C. intybus* monoculture, and *M. sativa* monoculture (Fig. 2C). The

presence of *C. intybus* in the microcosms reduced the total biomass production by 48% compared with its absence (Table 2, $P < 0.05$). The presence of *L. perenne* in the microcosms increased total biomass production by 66% compared with its absence (Table 2, $P < 0.05$). The presence of *R. japonicus* in the microcosms increased total biomass production by 50% compared with its absence (Table 2, $P < 0.05$).

3.3 Relationship of NH₃ volatilization to NH₄⁺-N concentration in wastewater and plant biomass

The volatilization of NH₃ was positively related with the concentration of NH₄⁺-N in wastewater among most plant combinations, including all the polycultures and the *R. japonicus* monoculture (Fig. 3A). However, other monocultures produced either low NH₃ volatilization with high NH₄⁺-N concentration, or high NH₃ volatilization with low NH₄⁺-N concentration (Fig. 3A). The volatilization of NH₃ was negatively related with biomass production in all plant combinations except for the *L. perenne* monoculture, which had high plant biomass and high NH₃ volatilization (Fig. 3B).

4. Discussion

4.1. The volatilization of NH₃ in response to plant species richness

To our knowledge, this is the first study to report the response of NH₃ volatilization to plant diversity. We found that species richness reduced the NH₃ volatilization from systems by 25% on average through increasing one more plant species across the richness gradient ($y = -3.96x + 24.31$, Fig. 1A). In other words, NH₃ volatilization in four-species polycultures could be reduced by 58% compared to that of monocultures ($1 - (1-25\%) \times (1-25\%) \times (1-25\%) = 58\%$) according to the equation. The relationship between biodiversity and ecosystems functioning indicated by NH₃ volatilization is similar to previous studies that plant species richness has significant effects on ecosystem functioning (Hooper et al., 2005; Chang et al., 2014; Ge et al., 2015). Our finding suggests that increasing the species richness is effective to reduce NH₃ volatilization from CWs. The current NH₃ abatement methods, which are physical or chemical (Ndegwa et al., 2008; Wagner et al., 2011; VanderZaag et al., 2015), are more

expensive and have a higher carbon footprint than the ecological method provided by this study. We confirm that assembling plant communities with high species richness is an alternative approach for NH_3 volatilization reduction.

4.2. The volatilization of NH_3 in response to plant species composition

The volatilization of NH_3 was significantly influenced by the species composition (Fig. 2, Table 2). The communities with *R. japonicus* had lower NH_3 volatilization than the communities without this species (Table 2). The reason may be that *R. japonicus* has a well-developed root system, thus producing high biomass, consuming more nitrogen (Table 2), and then reducing NH_3 volatilization, since NH_3 volatilization positively responds to NH_4^+ -N concentration in wastewater (Fig. 3A; also indicated by previous studies, e.g. Zhang et al., 2014) while negatively responding to plant biomass production (Fig. 3B). Furthermore, the interaction between *R. japonicus* and some other species gives rise to the “under-emission reduction effect” (a phenomenon where the polyculture emits less NH_3 than the lowest volatile monoculture) of NH_3 . The effect is similar to the under-depletion of nitrogen (i.e. the polyculture depletes soil nitrogen pool to lower levels than the lowest monoculture nitrogen pool) found by previous study (Palmborg et al., 2005). It indicates that the species are complementary and perform a nonlinear behavior in the polycultures (Crawley, 2007; Tilman et al., 2014). For example, the polyculture of *R. japonicus* $\times$ *C. intybus* $\times$ *L. perenne* emitted 60% less NH_3 than the lowest volatile monoculture (Fig. 2A). In sum, assembling specific plant species combinations can reduce the volatilization of NH_3 from CWs.

4.3. Relative contribution of plant species richness and composition to the biodiversity effect on NH_3 volatilization

Several studies have indicated that species composition showed greater impacts than species richness on ecosystem functions, such as ecosystem productivity and N_2O emissions (Bruno et al., 2005; Abalos et al., 2014; Avolio et al., 2014). Our study had a similar result that NH_3 volatilization was driven more strongly by species composition than richness. When simultaneously accounting for the effects of species richness and

composition (the presence of particular species, *R. japonicus* in this study) on NH_3 volatilization in a multiple linear regression, the effect of species richness became insignificant while the composition exerted a greater influence (Table 3). The response of wastewater NH_4^+ -N concentration to species richness or composition is similar to NH_3 volatilization. When accounting for the effects of species richness and species composition (the presence of *L. perenne* or *R. japonicas* in community) simultaneously in a multiple linear regression, the NH_4^+ -N concentration did not decrease as species richness increased (Table 3). Furthermore, only species composition had an effect on biomass when separately considering the effects of species richness and composition (Fig. 1C, Fig. 2C, Table 2). As a whole, these results suggest that assembling proper plant combinations are more important than simply increasing species richness to reduce NH_3 volatilization, meanwhile improving nitrogen removal and biomass production in CWs.

5. Conclusions

This study provided an alternative method for reducing potential NH_3 volatilization from wastewater with a high NH_4^+ -N concentration by assembling plant diversity in CWs. We explored the relationship between NH_3 volatilization and plant diversity for the first time, extending the content of biodiversity-ecosystem functioning research. The results showed that high species richness reduced the NH_3 volatilization in CWs, while species composition showed greater effect than species richness. We found that *R. japonicus* is an effective tool species, and assembling plant communities with this species can achieve the synergy between NH_3 volatilization reduction and nitrogen removal. The synergy between the functions in response to biodiversity means that the biodiversity affects these functions of ecosystem in the same direction. Further studies should investigate more plant species to find more appropriate species and combinations for NH_3 volatilization reduction, and also consider other factors, such as pH and microorganisms, to explore the mechanisms by which plant diversity affects NH_3 volatilization and other functions of ecosystems.

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Figure Captions

Fig. 1. Plant species richness vs. NH_3 volatilization (A), wastewater NH_4^+ -N concentration (B) and plant total biomass (C). Solid line is a regression curve under $P < 0.05$; dashed line is a regression curve under $P < 0.1$.

Fig. 2. NH_3 volatilization (A), wastewater NH_4^+ -N concentration (B) and plant total biomass (C) in relation to plant combination treatments. Species abbreviations are: Ci- *C. intybus*, Lp- *L. perenne*, Ms- *M. sativa*, Rj- *R. japonicus*. Error bars indicate standard errors. Different lower case letters above bars indicate significant differences at $P < 0.05$.

Fig. 3. Relations of NH_3 volatilization to wastewater NH_4^+ -N concentration (A), and total biomass (B). Error bars indicate standard errors. Closed circles symbolize polyculture systems; open circles symbolize monoculture systems and the species names were indicated beside the circles: Ci- *C. intybus*, Lp- *L. perenne*, Ms- *M. sativa*, Rj- *R. japonicus*. Solid line is the regression curve for polyculture systems.

Table 1

Content of nutrient components in the simulated wastewater (modified Hoagland nutrient solution)

Macroelements		Microelements	
Nutrient components	Content (g L ⁻¹)	Nutrient components	Content (mg L ⁻¹)
(NH ₄) ₂ SO ₄	1.58	H ₃ BO ₃	2.86
CaCl ₂ ·2H ₂ O	0.74	MnCl ₂ ·4H ₂ O	1.81
KH ₂ PO ₄	0.14	ZnSO ₄ ·7H ₂ O	0.22
MgSO ₄ ·7H ₂ O	0.49	CuSO ₄ ·5H ₂ O	0.08
KCl	0.45	H ₂ MoO ₄ ·4H ₂ O	0.09
		FeSO ₄ ·7H ₂ O	5.56
		Na ₂ EDTA	7.44

Table 2

Means and standard errors of flux of NH₃ volatilization ($\mu\text{g N m}^{-2} \text{ h}^{-1}$), wastewater NH₄⁺-N concentration ($\mu\text{g N mL}^{-1}$) and biomass production (g m^{-2}) in the presence and absence of the four species

Parameter		NH ₃ volatilization	NH ₄ ⁺ -N concentration	Total biomass
<i>C. intybus</i>	Presence	17.75 $\pm$ 2.01	624.9 $\pm$ 26.3	520.0 $\pm$ 42.8*
	Absence	13.72 $\pm$ 1.80	586.9 $\pm$ 37.0	1003.8 $\pm$ 62.8
<i>L. perenne</i>	Presence	16.06 $\pm$ 1.82	556.9 $\pm$ 29.2*	876.3 $\pm$ 53.2*
	Absence	15.65 $\pm$ 2.09	664.6 $\pm$ 32.1	528.8 $\pm$ 63.3
<i>M. sativa</i>	Presence	14.94 $\pm$ 1.60	642.1 $\pm$ 34.1	644.8 $\pm$ 61.8
	Absence	16.98 $\pm$ 2.34	565.4 $\pm$ 25.7	814.0 $\pm$ 62.6
<i>R. japonicus</i>	Presence	8.80 $\pm$ 0.76*	535.9 $\pm$ 25.2*	852.7 $\pm$ 49.8*
	Absence	23.60 $\pm$ 2.22	685.1 $\pm$ 33.9	568.1 $\pm$ 71.9

Notes: Values are means $\pm$ 1 SE. Asterisks in the “presence” columns indicate statistically significant differences among means when a particular species is present vs. absent in the communities ($P < 0.05$).

Table 3

Effect of species richness and presence of individual species on ammonia volatilization and wastewater NH_4^+ -N concentration in multiple linear regressions.

Factor	Overall model			Species presence/absence		Species richness	
	Intercept	<i>P</i> value	<i>R</i> ²	Slope	<i>P</i> value	Slope	<i>P</i> value
NH_3							
<i>C. intybus</i>	24.31	< 0.001	0.17	8.88	0.002	-6.18	< 0.001
<i>L. perenne</i>	24.31	0.012	0.10	4.37	0.142	-5.05	0.003
<i>M. sativa</i>	24.26	0.034	0.08	1.07	0.717	-4.21	0.013
<i>R. japonicus</i>	23.64	< 0.001	0.33	-14.79	< 0.001	-0.02	0.989
NH_4^+ -N							
<i>C. intybus</i>	704.0	0.034	0.08	91.72	0.061	-68.33	0.014
<i>L. perenne</i>	704.0	0.037	0.07	-89.54	0.068	-23.02	0.401
<i>M. sativa</i>	698.0	0.004	0.12	133.26	0.005	-76.57	0.004
<i>R. japonicus</i>	697.5	0.003	0.13	-143.14	0.003	-7.31	0.787

Note: NA indicates that the statistical analysis does not apply to this factor. Variance Inflation Factors were tested to be < 1.3.

Fig. 1

Fig. 2

Fig. 3