Plant species diversity impacts nitrogen removal and nitrous oxide emissions as much as carbon addition in constructed wetland microcosms

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Abstract

With the aim to develop an approach for treating wastewater with low carbon (C) to nitrogen (N) ratio in constructed wetlands (CWs), we compared the effects of C addition and species diversity on N removal and nitrous oxide (N₂O) emissions. Compared with the monocultures without C addition, C addition significantly increased N removal efficiency in monocultures (reached 71% on average); increasing species richness impacted N removal as much as C addition, and the N removal efficiency in the four-species mixture reached 75%. Both C addition and species richness level significantly increased N₂O emission rates (reached 1.26 and 1.40 mg m⁻² d⁻¹, respectively), but had no significant effect on N₂O emissions per unit N removal. The effects of species richness on N removal and N₂O emissions were stronger than species identity. Compared with C addition, assembling plant communities with high diversity in actual applications could be an effective and more economical approach for treating wastewater with low C/N ratio in CWs.

Keywords: Species richness; Species identity; Ecosystem function; Carbon source.

1. Introduction

With the rapid growth of population and urbanization, the increasing production of wastewater with high-strength nitrogen (N) has become a serious problem impacting water quality, air quality and human health (Galloway et al., 2008; Schwarzenbach et al., 2010). A large amount of nitrous oxide (N_2O) is emitted during wastewater processing (Kampschreur et al., 2009; Huang et al., 2013). Thus, it is of great significance to develop technologies to treat wastewater that has high N removal and low N_2O emissions.

Constructed wetlands (CWs) have been widely used for treating various wastewaters with the benefit of low operation and maintenance requirements (Vymazal et al., 2007; Liu et al., 2012). Yet, there are great differences in the ratio of C to N (C/N) among different sources of wastewater (Wu et al., 2014, Vymazal, 2014). Wastewater from some high-tech industry sources, such as semiconductors and optoelectronics, has a very low C/N ratio (Kumar et al., 2012). The influent C/N ratio, representing the relative amount of C source available in a system, plays a crucial role in N removal and N_2O emissions in CWs (Huang et al., 2013). Lack of available organic carbon could inhibit biological N removal in CWs, cause incomplete denitrification and increase N_2O emissions in CWs at the same time (Kampschreur et al., 2009; Huang et al., 2013). Finding ways to improve the performance of CWs for treating wastewater with a low C/N ratio has been an active area of research.

Adding labile C to wastewater to enhance the denitrification is an approach that can be used to improve the N removal in CWs for treating wastewater with a low C/N ratio (Sirivedhin and Gray, 2006; Wu et al., 2014). However, excessive C availability in

wastewater often competes for limited oxygen supply and then inhibits nitrification (Saeed and Sun, 2011). In addition, as C addition obviously increases the cost (Wu et al., 2014), finding the low-cost alternative approaches are needed.

Plants in CWs can provide organic matter as the C sources for denitrification (Picek et al., 2007). The soil C pool in different systems varies with the plant productivity, chemical composition of root exudates, turnover rate of the root, and composition and activity of microbial community in the rooting zones. (De Deyn et al., 2008). Increasing species richness not only increases the soil C pool (Chang et al. 2014; Lange et al., 2015), but also increases plant productivity and plant N uptake, and then increases the N removal (Ge et al., 2015). In addition, assembling plant communities with several species almost does not raise the cost than monoculture, so increasing plant diversity should be an economical approach for substituting C addition to improving N removal in CWs.

Nitrous oxide is a byproduct of nitrification and an intermediate product of denitrification (Canfield et al., 2010). Regulating the C/N ratio by C addition may be an approach that can be employed to control N₂O emissions. Wu et al. (2009) reported that when influent C/N ratio is 5, N₂O emission from CWs was the minimum. Plant species richness may also affect N₂O emissions by influencing biotic and abiotic factors, such as microbial communities, N concentrations, and availability of C sources (Thomson et al., 2012). In grassland ecosystem, N₂O emissions decreased with species richness (Niklaus et al., 2006) or no response (Abalos et al., 2014) or. In CWs for treating wastewater without extra C input, N₂O emissions increase with increasing species richness (Sun et al. 2013; Chang et al., 2014). In order to determine whether assembling

high species richness could replace C addition when treating wastewater with low C/N ratio in CWs, it is important to compare the effects of these two approaches on N₂O emissions.

Species identity (species composition) is an important component of species diversity. In fact, species identity can play a greater role in controlling ecosystem productivity than species richness in grassland and marine communities (Hooper and Vitousek, 1998; Bruno et al., 2005). Abalos et al. (2014) demonstrated that species identity surpasses species richness as a key driver of N₂O emissions in a grassland ecosystem. In CWs, species identity can significantly affect ecosystem productivity (Sun et al., 2013; Ge et al., 2015), N removal (Read et al., 2008; Ge et al., 2015) and N₂O emissions (Sun et al., 2013). As such, it is key to further understand the relative importance of species identity and species richness on N removal and N₂O emissions in CWs.

In this study, microcosms simulating vertical flow CWs were established. These microcosms included monoculture and mixture of plant species. All plant communities were supplied with a simulated wastewater with or without C (sucrose) addition. The objectives of this study were to (1) investigate the effects of C addition on N removal and N₂O emissions; (2) study the effects of species richness and identity on N removal and N₂O emissions; (3) compare the effects of plant diversity and C addition on N removal and N₂O emissions.

2. Materials and methods

2.1 Experimental design

The study was conducted in 50 simulated vertical flow CWs microcosms in an open field at Zhejiang University (30°18' N, 120°05' E, Hangzhou City, Southeast China). The microcosms were constructed using ceramic tubs (53 cm long × 17.5 cm wide × 18.5 cm high) and filled with coarse sand (0.5-3 mm) to a depth of about 12 cm.

On the basis of the physiological and morphological traits, which influence the resource requirements and growth traits, four common local plant species, *Juncus effusus* L., *Oenanthe javanica* (Blume) DC, *Phalaris arundinacea* L. and *Rumex japonicus* Houtt. were selected for the experiment. The seedlings were transplanted into microcosms with a density of 12 individuals per microcosm in March 2013.

In this study, the simulated wastewater was based on the Hoagland nutrient solution (Hoagland and Arnon, 1950) with minor modifications. Nitrate-N was the sole N source, and the concentration (336 mg L⁻¹) was three times higher than that of Hoagland. Four treatments were used here: (1) monocultures supplied with simulated wastewater without C addition was set as the control; (2) monocultures supplied with simulated wastewater with C addition; (3) high species richness (four-species mixture) supplied with simulated wastewater without C addition; and (4) four-species mixture supplied with simulated wastewater with C addition. As shown in Fig. 1, the experiment was arranged in a completely random block design with five replicates laid out in five blocks, totally 50 microcosms.

For the experiment supplied with simulated wastewater with C addition, sucrose was added to create chemical oxygen demand (COD) to N ratio (1:1). The simulated wastewater was supplied once every ten days, for a total of eight times. During the experiment, each microcosm received 18.816 g total inorganic N (TIN). The water level

was kept at 1.5 cm above the sand surface of each microcosm, and each microcosm contained 7 L of simulated wastewater. The microcosms were supplied daily with tap water to compensate for evaporation loss.

2.2 Sampling and measurements

Water samples were taken on the eighth day after the last supply of simulated wastewater and then were stored in a freezer at -20 °C. Each water sample was filtered using a membrane syringe filter (pore size 0.45 µm) before analysis. The total organic C concentration (TOC) in water was measured by the non-dispersion infrared method, the nitrate-N and ammonium-N concentrations were measured by spectrophotometric method (Clescerl et al., 1999), although nitrate-N was the sole N source, the ammonium-N may come from the organic N released by plants (Chang et al., 2014). The sum of nitrate-N and ammonium-N concentrations is the TIN concentration. The N removal efficiency (NRE) in the last batch of supplying simulated wastewater was calculated based on mass balance:

$$\text{NRE}(\%) = (N_i - N_e)/N_e \times 100$$

where N_i is the amount of influent TIN of each microcosm (mg); N_e is the amount of effluent TIN of each microcosm (mg).

Gas samples were collected on the eighth day after the last supply of simulated wastewater using the static chamber method described in Chang et al. (2014). The PVC static chambers (24 L) were placed on the ground surface of microcosms. The gas collection was carried out from 8:00 to 10:00 am at 30 min intervals. Gas samples were collected in 100 mL gas sampling bags (Plastic gas, Delin Company, China) using 50 mL polyurethane syringes. The air temperature inside the chamber was monitored

during gas collection. The N_2O concentration was determined using a gas chromatograph (Agilent - 7820, USA). The N_2O emission rate ($\text{mg m}^{-2} \text{d}^{-1}$) was calculated according to the equation described in Cheng et al. (2007). Then $\text{N}_2\text{O-N}$ emission per unit N removal (mg g^{-1}) was calculated as the $\text{N}_2\text{O-N}$ emission per day divided by TIN removal per day in the last batch of supplying simulated wastewater.

At the end of the experiment, all plants were harvested by species, cleaned and measured for above- and below-ground biomass after the samples were oven dried at 65°C for 72 hr. Dry plant tissue materials were ground to the fine powders. Then plant tissue N concentrations and foliar $\delta^{15}\text{N}$ value were measured using an isotope ratio mass spectrometer linked to an elemental analyzer (Flash HT2000, Thermo Finnigan, Bremen, Germany). Plant tissue N pools were calculated by multiplying tissue N concentration per species by its tissue biomass. Above- and below-ground N pools were summed to obtain the total N pool. The contribution of plant N uptake to TIN removal was calculated as total N pool divided by TIN removal across the experiment based on the mass balance.

2.3 Statistical analysis

In order to compare the effects of C addition and increasing species richness on N removal and N_2O emissions, one-way ANOVA was used to test for treatment effects and Least Significant Difference (LSD) contrasts to correct for multiple comparisons (Tilman et al., 2012). Student's t-test was used to detect significant differences in N removal, N_2O emissions, biomass, N pool and foliar $\delta^{15}\text{N}$ of four monocultures between the treatments with and without C addition. If necessary, the data were log-transformed to meet the statistical assumptions of the tests. To investigate the effects of species

richness and identity on N removal, N₂O emissions, biomass and N pool, one-way ANOVA was performed for each experiment, including all monocultures and the most diverse mixture (four-species mixture). The treatment sum of squares was partitioned into a priori contrast (richness effect) between the highest diverse mixture and the monocultures (Bruno et al., 2005). The residual sum of squares reflected the variation among the monocultures, thus representing the identity effect. Both effects were tested using the error mean square as the denominator in the F-test. We calculated the magnitude of effect (ω^2 , the relative contribution expressed as the percentage of the total variance) for the species richness and identity effects (Graham and Edwards, 2001). All statistical analyses were conducted using the SPSS software (SPSS 20.0, SPSS Inc, Chicago, USA). Statistical significance level was set at $\alpha = 0.05$. All values were expressed as means $\pm$ standard error (S.E.).

3 Results and discussion

3.1 Effects of carbon addition or plant species diversity on nitrogen removal efficiency

Low C availability is one of the factors limiting denitrification process, and then affects N removal efficiency (Ding et al., 2012). In this study, C addition significantly increased the N removal efficiency in monocultures, with the average N removal increased from 56% to 70% (Fig. 2a). Our results are similar to previous studies that reported the N removal efficiency raised to 64% (Wu et al., 2009) and 62% (Zhao et al., 2010) with C addition, respectively. The responses of the four monocultures to C addition were different: C addition significantly increased N removal efficiency in monocultures of *O. javanica* and *P. arundinacea* (from 42% to 70% and from 54% to

67%, respectively), whereas it had no effect on N removal efficiency in the monocultures of *R. japonicus* and *J. effusus*. In fact, the N removal efficiency in the monocultures of *R. japonicus* and *J. effusus* without C addition (70% and 65%, respectively) was similar to those of the monocultures of *O. javanica* and *P. arundinacea* with C addition (Fig. 2d, Table 1). These results indicate that there is no need to add C in CWs planted with *R. japonicas* or *J. effusus*.

Denitrification and plant N uptake are two of the main paths of nitrate-N removal (Vymzal et al., 2007). C addition significantly increased N removal efficiency in monocultures of *O. javanica* and *P. arundinacea*. As C addition had no effect on the plant N pool of *O. javanica* and *P. arundinacea* monocultures (Fig. 3c), it is speculated that C addition enhanced denitrification in the monocultures of these two species. The foliar $\delta^{15}\text{N}$ of *O. javanica* and *P. arundinacea* monocultures with C addition were higher than those without C addition confirming this speculation (Fig. 4a), since the increase in foliar $\delta^{15}\text{N}$ indicated enhancement of denitrification in the substrate (Vitousek et al., 1989). There was no significant difference in N removal in the monoculture of *J. effusus* with and without C addition (Fig. 2b). Although C addition significantly increased foliar $\delta^{15}\text{N}$ of *J. effusus* (Fig. 4), the plant N pool of *J. effusus* in treatments without C addition was higher than that in treatments with C addition (Fig. 3b). In treatments without C addition, the foliar ^{15}N of monoculture of *R. japonicus* was significant higher than that of other three monocultures, indicated high denitrification in sustrate in the monoculture of *R. japonicus*. C addition had no significant effect on the plant N pool and foliar $\delta^{15}\text{N}$ in the monoculture with *R. japonicus* between the treatments with and without C addition (Fig. 3c and 4a). These results indicate that C addition does not increase the

denitrification and N pool in the monoculture of *R. japonicus*. Overall, the effect of C addition on N removal efficiency depends on the species planted in CWs.

Increasing the species richness significantly increased N removal efficiency from 56% in monocultures on average to 75% in the mixture treatments without C addition (Fig. 2a, Table 1). One of the reasons may be high species richness increased plant C release. In this study, in the treatments without C addition, although there was not C in the supplied simulated wastewater, the C was test out and the average effluent TOC concentration reached 42 mg L⁻¹, indicating that the plants had released C into the microcosms. Previous studies reported that higher productive plant can release more C into the system (Kuzyakov et al., 2001; Cong et al., 2014). The biomass in the four-species mixture was higher than the monoculture on average in both the treatments with and without C addition (Fig. 5), it is surmised that the mixture produced more C for denitrification than the monocultures.

Another reason may be that increasing the species richness increases plant N uptake (Ge et al., 2015). The contribution of plant N uptake to N removal ranges from 5-50% in various types of CWs (Tanner, 2001; Ge et al., 2015). In this study, plant N uptake significantly increased with increases in species richness (Fig. 3a, Table 2), while the contribution of plant uptake to N removal averaged 22% and 42% in monocultures and four-species mixture without C addition, respectively (Fig. 3b). Overall, higher species richness increases N removal efficiency by providing more C for denitrification and promoting plant N uptake than monoculture.

In this study, the positive effect on N removal was stronger by species richness than by C addition. Compared to the control (monocultures without C addition), only

increasing the species richness (four-species mixture) increased the N removal by 34% on average, and only adding C increased the N removal by 25% on average (Fig. 2a). C addition only enhanced denitrification, while increasing the species richness not only enhanced denitrification but also promoted the plant N pool. Compared to the control, four-species mixture with C addition also increased N removal, but there was no significant difference between four-species mixture with C addition and without C addition (Fig. 2a). Assembling community with high species richness as a low-cost approach for increasing N removal efficiency can replace C addition in CWs in some extent.

3.2 Effects of carbon addition or plant species diversity on N₂O emissions

The proportion of loaded N emitted as N₂O-N can range from 0.001-3.010% in CWs for wastewater treatment (Mander et al., 2014). In this study, the proportion was 0.001-0.250%. The C availability in wastewater may affect aerobic and anaerobic conditions in CWs and consequently may influence N₂O emissions from treating wastewater (Kampschreur et al., 2009; Huang et al., 2013). In this study, C addition significantly increased N₂O emissions rate in monocultures, with the average N₂O emissions rate increased from 0.38 to 1.26 mg m⁻² d⁻¹ (Fig. 2b). Previous studies have shown that the N₂O emission rate from *Phragmites australis* monoculture is at a minimum (3.68 mg m⁻² d⁻¹) when the C/N ratio = 5 (Wu et al., 2009). Although the amount of C addition was relatively low (C/N = 1) in this study, the N₂O emission rates were still lower than the N₂O emission rate under optimal C/N in available literature (e.g., Wu et al., 2009). The responses of the four species monocultures to C

addition were different. In the monoculture of *J. effusus*, C addition significantly increased N₂O emission rates from 0.41 to 2.28 mg m⁻² d⁻¹, but this addition had no significant effect on other three species (Fig. 2e).

Increasing species richness significantly increased N₂O emission rates from 0.41 mg m⁻² d⁻¹ in monocultures to 1.4 mg m⁻² d⁻¹ in four-species mixture without C addition (Fig. 2b, Table 1). This result was consistent with that of Sun et al. (2013) and Chang et al. (2014), which reported that higher species richness increased N₂O emission rate in CW microcosms. One of the reasons may be that higher species richness produced more C (as discussed above) and then enhanced denitrification and increased the N₂O emission rate. Another reason may be increasing the species richness increased the capability of transferring N₂O from roots up to the atmosphere (Jørgensen et al., 2012).

Comparing the effects of C addition and species richness on N₂O emission rate, C addition increased N₂O emission rate by 231% on average, while the species richness increased N₂O emission rate by 269% on average compared to control; also, there was no significant difference between the treatments of C addition and increasing species richness (Fig. 2b). The results indicate that the positive effect of species richness on N₂O emission rate is equal to the positive effect of C addition.

The responses of the N₂O-N emissions per unit N removal in the four monocultures to C addition varied in our study. C addition significantly increased the N₂O-N emissions per unit N removal in *P. arundinacea* and *J. effusus* monocultures, but other two species monocultures had no significant response to C addition (Fig. 2f). Compared to the control, the mixture without C addition and the mixture with C addition both had

no significant effect on N₂O-N emissions per unit N removal (Fig. 2c, Table 1). In sum, assembling communities with high species richness can improve N removal and does not increase the N₂O-N emissions per unit N removal.

3.3 Relative importance of the effects of species richness and species identity

Most previous experiments in grassland and marine communities have shown that species identity played a more important role than species richness in controlling ecosystem processes (e.g., Hooper and Vitousek, 1998; Bruno et al., 2005). In this study, species richness had a stronger influence on aboveground biomass than species identity in treatments with or without C addition. In this case, species richness explained more than 50% of the variation, whereas species identity only accounted for about 30% of the variation (Table 2). However, species identity influenced below-ground biomass more strongly than species richness. Species identity explained more than 70% of the variation, whereas species richness only explained less than 20% of the variation (Table 2). Species identity had no effect on N removal in treatments with and without C addition (Table 1), although species identity significantly affected the plant N pool (Table 2), which was one of the main pathways for NO₃⁻-N removal. The difference in the plant N pool may be offset by the difference in denitrification, which was the other main pathway for NO₃⁻-N removal (Vymzal et al., 2007). However, species richness significantly affected N removal, explaining 31% of the variation in N removal in treatments without C addition (Table 1). The results demonstrate that species richness influence the N removal more strongly than species identity.

Species identity surpassing species richness as a key driver of N₂O emissions in

grassland has been reported by Abalos et al. (2014). In this study, species richness significantly affected N₂O emissions from CWs where there was no C addition, whereas species identity had no effect. Species richness and species identity both had no significant effect on N₂O emissions from CWs with C addition (Table 1). In treatments without C addition, the available C amount may be the main factor affecting N₂O emissions, and species richness was determined to be the key driver of N₂O emissions by producing more C. However, in treatments with C addition, the N₂O emissions may be affected by other relevant factors, such as N concentration. Overall, our results suggest that the effects of species richness on the N removal and N₂O emissions are both stronger than the effects of species identity.

4 Conclusions

Overall, this study showed that higher species richness and C addition both increased N removal efficiency and N₂O emission rate, but had no effect on the N₂O-N emission per unit N removal. The effects of species richness on N removal efficiency and N₂O emission were greater than species identity. Assembling communities with high species richness as an alternative approach to C addition could be an effective and more economical approach to improving the N removal efficiency and would not increase N₂O-N emission per unit N removal in CWs for treating low C/N ratio wastewater.

Acknowledgements

This work was funded by the National Science Foundation of China (No. 31270377, 31170305 and 51279121).

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Figure captions

Fig. 1 Experimental design. White background: treatments without carbon addition; grey background: treatments with carbon addition. Different shapes represent different species.

Fig. 2 Effects of C addition and plant species diversity on (a, d) nitrogen removal efficiency, (b, e) N₂O emission rate and (c, f) N₂O-N emission per unit N removal. White background: treatments without carbon addition; grey background: treatments with carbon addition. Rj: *R. japonicus*; Oj: *O. javanica*; Pa: *P. arundinacea*; Je: *J. effuses*. Different letters denote significant difference among treatments. Asterisks denote significant difference between treatments with carbon addition and without carbon addition ($P < 0.05$). There was no significant difference among four monocultures in both treatments with and without carbon addition. Mean $\pm$ S.E. Results on species richness and identity effects are in Table 1.

Fig. 3 Effects of C addition and plant species diversity on (a, c) total plant N pool and (b, d) the contribution of plant N uptake on TIN removal. Background and species abbreviations are the same as in Fig.2. Different letters denote significant difference among treatments. There was no significant difference between the treatments without carbon addition and with carbon addition. Mean $\pm$ S.E. Results on species richness and identity effects are in Table 2.

Fig. 4 Foliar $\delta^{15}\text{N}$ in monocultures. Background and species abbreviations are the same as in Fig.2. Asterisks denote significant difference between treatments with and without carbon addition ($P < 0.05$). Mean $\pm$ S.E.

Fig. 5 Effects of C addition and plant species diversity on (a, c) aboveground biomass and (b, d) belowground biomass. Background and species abbreviations are the same in Fig.2. Different letters denote significant difference among treatments. There was no significant difference between the treatments with and without carbon addition. Mean $\pm$ S.E. Results on species richness and identity effects are in Table 2.

FIGURES

Fig. 2

Fig.3

Fig. 4

Fig. 5

Table 1 Analysis of variance (ANOVA) data on the effects of plant species richness and identity on N removal efficiency (%), N₂O emission rate (mg m⁻² d⁻¹) and N₂O-N emission /TIN removal (mg g⁻¹).

Variables	Treatments	Sources of effect	SS	d.f.	F	P	ω^2
N removal efficiency	without C addition	Richness	1208.07	1	4.56	0.045	30.25
		Identity	2355.97	3	2.97	0.057	16.69
	with C addition	Richness	400.01	1	1.63	0.216	9.10
		Identity	325.17	3	0.44	0.727	0.00
N ₂ O emission rate	without C addition	Richness	4.19	1	14.79	0.001	68.16
		Identity	1.01	3	1.19	0.339	0.94
	with C addition	Richness	1.20	1	0.82	0.377	0.00
		Identity	8.49	3	1.92	0.159	13.22
N ₂ O-N /TIN removal	without C addition	Richness	0.70	1	3.62	0.072	29.10
		Identity	0.66	3	1.15	0.353	1.64
	with C addition	Richness	1.29	1	2.12	0.161	10.54
		Identity	4.74	3	2.60	0.079	30.84

Richness and identity effects were tested with orthogonal planned contrasts. Effect sizes were estimated as ω^2 (Graham and Edwards, 2001), with negative estimates reported as zero. Significant *P* values (*P* < 0.05) are highlighted.

Table 2 Analysis of variance (ANOVA) data on the effects of plant species richness and identity on biomass (g m^{-2}), plant N pool (g m^{-2}) and plant N pool /TIN removal (%).

Variables	Treatments	Sources of effect	SS	d.f.	F	P	ω^2
Aboveground biomass	without	Richness	593719.47	1	17.78	<0.001	50.46
	C addition	Identity	1124218.91	3	11.22	<0.001	30.74
	with	Richness	987137.27	1	46.80	<0.001	60.07
	C addition	Identity	1594181.54	3	25.19	<0.001	31.73
Belowground biomass	without	Richness	96648.55	1	8.80	0.008	14.6
	C addition	Identity	1329607.57	3	40.33	<0.001	73.69
	with	Richness	63465.16	1	3.465	0.077	6.57
	C addition	Identity	1638154.61	3	29.82	<0.001	76.78
Plant N pool	without	Richness	1068.455	1	27.59	<0.001	52.83
	C addition	Identity	2148.456	3	18.50	<0.001	34.75
	with	Richness	626.061	1	9.24	0.006	34.66
	C addition	Identity	2090.899	3	10.29	<0.001	39.06
Plant N pool/ TIN removal	without	Richness	0.149	1	24.83	<0.001	48.72
	C addition	Identity	0.357	3	19.83	<0.001	38.50
	with	Richness	0.087	1	8.70	0.008	32.79
	C addition	Identity	0.316	3	10.53	0.001	40.60

Richness and identity effects were tested with orthogonal planned contrasts. Effect

sizes were estimated as ω^2 (Graham and Edwards, 2001). Significant P values ($P < 0.05$) are highlighted.