

1 **The Intensified Constructed Wetlands Are Promising for Treatment of**  
2 **Ammonia Stripped Effluent: Nitrogen Transformations and Removal**  
3 **Pathways**

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13 **Abstract**

14 This study investigated the treatment performance and nitrogen removal  
15 mechanism of highly alkaline ammonia-stripped digestate effluent in horizontal  
16 subsurface flow constructed wetlands (CWs). A promising nitrogen removal performance  
17 (up to 91%) was observed in CWs coupled with intensified configurations, i.e., aeration  
18 and effluent recirculation. The results clearly supported that the higher aeration ratio and  
19 presence of effluent recirculation are important to improve the alkalinity and pollutant  
20 removal in CWs. The influent pH (>10) was significantly decreased to 8.2–8.8 under the

21 volumetric hydraulic loading rates of 0.105 and 0.21 d<sup>-1</sup> in the CWs. Simultaneously, up to  
22 91% of NH<sub>4</sub><sup>+</sup>-N removal was achieved under the operation of a higher aeration ratio and  
23 effluent recirculation. Biological nitrogen transformations accounted for 94% of the  
24 consumption of alkalinity in the CWs. The significant enrichment of δ<sup>15</sup>N-NH<sub>4</sub><sup>+</sup> in the  
25 effluent (47–58‰) strongly supports the occurrence of microbial transformations for  
26 NH<sub>4</sub><sup>+</sup>-N removal. However, relatively lower enrichment factors of δ<sup>15</sup>N-NH<sub>4</sub><sup>+</sup> (–1.8 ‰ –  
27 –11.6‰) compared to the values reported in previous studies reflected the inhibition  
28 effect of the high pH alkaline environment on nitrifiers in these CWs.

## 29 **Capsule**

30 Intensified horizontal subsurface flow constructed wetlands is promising for the treatment  
31 of ammonia stripped effluent with high alkalinity consuming capacity.

32 **Keywords:** Alkaline wastewater; constructed wetlands; effluent recirculation; microbial  
33 community; isotopic composition; intermittent aeration

## 34 **1. Introduction**

35 Anaerobic digestion (AD) technology has been widely used in livestock waste  
36 treatment as a result of the benefits to environmental protection and bioenergy  
37 generation (Mao et al., 2015; Sakar et al., 2009). The number of AD plants has dramatically  
38 increased in recent years. In Europe, the total number of AD plants increased by 10% in  
39 comparison to the previous year and reached 14,572 in 2013 (Torrijos, 2016). In China, the  
40 number of large scale anaerobic digestion plants with monomer volumes of more than 300 m<sup>3</sup>

41 rapidly increased to 15,531 at the end of 2013; these plants can process approximately 42%  
42 of the 3.8 billion ton livestock manure (NDRC, 2014). The generated biogas from these  
43 intensive scaled AD plants can then be further purified and/or upgraded to cooking gas or  
44 converted to electricity (Mao et al., 2015). However, if the surrounded arable land is not  
45 sufficient to consume digestate as fertilizer, the largely generated digestate will cause  
46 potential negative impacts on the environment . Therefore, the adequate management or  
47 disposal of the surplus digestate has been recently discussed in order to avoid to the  
48 drawbacks of the sustainable development of the AD technology (Sheets et al., 2015).

49 Nitrogen (mainly in the form of ammonium) concentrations in the surplus digested  
50 effluent from the AD process of the animal slurry are generally far over the treatment  
51 capacity of the conventional biological treatment technology (Lei et al., 2007). Moreover,  
52 the relatively low chemical oxygen demand/total nitrogen (COD/TN) ratio (1–3) of the  
53 digested effluent is also insufficient to facilitate comprehensive TN removal through  
54 heterotrophic denitrification (Li et al., 2016). However, instead of being converted to  
55 dinitrogen gas, this high nitrogen content effluent could also be sustainably reused in  
56 agriculture (Kizito et al., 2015).

57 For the recovery of nitrogen from waste effluents, several techniques have been  
58 suggested, including microbial fuel cells (Kuntke et al., 2012), reverse osmosis (Mondor et  
59 al., 2008), electrodialysis (Mondor et al., 2008), and adsorption (Tada et al., 2005).  
60 Ammonia stripping, which transfers the ammonia from the liquid into an air stream  
61 through pH adjustment and aeration, is recognized as an effective technology for

62 ammonia recovery/removal from ammonia-rich wastewater due to its low cost, easy  
63 installation, and high ammonia removal ability (Yuan et al., 2016). For the treatment of  
64 digested effluents or for the recovery of nitrogen from digested effluents, the ammonia  
65 stripping technique has already been well studied by scientists (Jiang et al., 2014) and  
66 implemented by engineers (Zeng et al., 2006). However, the discharged effluent of this  
67 stripping process often has a high alkalinity with  $\text{pH} > 10$ , which may give rise to another  
68 environmental issue by adversely affecting aquatic ecosystems (Mayes et al., 2009).  
69 Serna-Maza et al., 2015) proposed using this alkaline effluent to upgrade methane content  
70 of biogas by consuming  $\text{CO}_2$  from the biogas and  $\text{OH}^-$  from the alkaline effluent  
71 simultaneously while pumping the biogas through this solution. The pH of the effluent  
72 after this step is still normally higher than 9. The most conventional management option  
73 for high alkaline wastewater is confined to direct chemical neutralization as the priority  
74 treatment. However, this requires a sustained capital input that may not be suitable for  
75 low-income farmers and/or agricultural industries in developing countries.

76 Constructed wetlands (CWs) are man-made systems that have already been  
77 acknowledged in recent years for the treatment of polluted waters, owing to their cost-  
78 effectiveness, easy maintenance, and efficient performance (Wu et al., 2014). Some  
79 previous studies have preliminarily reported the potential of traditional horizontal  
80 subsurface flow CWs to buffer high alkaline ( $\text{pH} > 10$ ) industry wastewaters, such as cement  
81 and lime drainages (Mayes et al., 2005), bauxite residue leachate (Higgins et al., 2017),  
82 and steel slag drainage (Mayes et al., 2009). Most of the published work, however, only

83 focuses on the alkalinity buffering potential of these wetlands and ignores the removal  
84 performance of associated pollutants and degradation pathways (Buckley et al., 2016;  
85 Mayes et al., 2009). Only a few studies examined the precipitation of divalent metals, e.g.,  
86 Co, Mn, Ni, and Zn, in these alkaline environments (Higgins et al., 2017; Higgins et al.,  
87 2016). Nitrogen transformation dynamics and respective pathways could be strongly  
88 influenced by and interact with highly alkaline conditions, but knowledge in this field is  
89 still insufficient.

90 To improve the treatment performance and minimize the occupied land area, some  
91 intensive operational strategies with new configurations (e.g., bottom aeration and  
92 effluent recirculation) were gradually integrated into the traditional CW beds (Wu et al.,  
93 2014). In alkaline conditions, the artificial bottom aeration can improve the biological  
94 degradation of organics into CO<sub>2</sub> that can theoretically be easily dissolved to neutralize  
95 alkalinity. Moreover, the aeration by introducing air into the bed will also physically supply  
96 more CO<sub>2</sub> dissolved in water to neutralize alkalinity (Summerfelt et al., 2015). The aeration  
97 is also closely linked to nitrogen removal, by not only effectively intensifying the  
98 nitrification process with extra oxygen into the bed, but also benefiting simultaneous  
99 alkalinity consumption due to the generation of protons in nitrification (Li & Irvin, 2007).  
100 Another commonly used operation is the effluent recirculation, which could additionally  
101 increase the water/substrate contact time. The effluent recirculation was demonstrated  
102 to improve denitrification by utilizing the numerous NO<sub>3</sub><sup>-</sup>-N accumulated in the effluent  
103 (Wu et al., 2017). Due to the aforementioned nitrogen removal pathways through

104 microbial activities, the contribution of nitrogen removal through NH<sub>3</sub> volatilization should  
105 be studied. The higher hydraulic loading rate (HLR), which induces more pollutants into  
106 CWs, may cause negative effect on the microbial community and plant growth and  
107 decrease the treatment performance. Associated with such various nitrogen  
108 transformations, the knowledge on how the abundance and functioning of the microbial  
109 community response to the alkaline environment and operational strategies should be  
110 investigated.

111 In this study, three lab-scale horizontal subsurface flow CWs have been established  
112 to 1) investigate the treatment feasibility of highly alkaline ammonia-stripped digestate  
113 effluent in CWs; 2) determine the influence of volumetric hydraulic loading rates (HLR<sub>v</sub>)  
114 and intensifying strategies (bottom aeration and effluent recirculation) on pollutant  
115 removal under the highly alkaline conditions; and 3) discuss the nitrogen transformation  
116 dynamics and removal pathways by N-isotopic fractionation assessment and microbial  
117 community response.

## 118 **2. Materials and methods**

### 119 **2.1 Experimental setup and operational conditions**

120 Three laboratory-scale horizontal subsurface flow CWs (CW1, CW2, and CW3) were  
121 established in this study. They weremade of Plexiglass containers with a length, width, and  
122 height of 100 cm, 15 cm, and 50 cm, respectively. Each container was filled with a 45-cm  
123 layer of sand (Ø 0.2 to 0.6 cm, with average porosity of 35%) as the main CW media. The

124 water height in each CW was approximately 40 cm. Two perforated plastic boards were  
125 placed at a distance of 3 cm in front of the influent and effluent openings to create small  
126 water distribution and collection zones, respectively. These zones were designed to  
127 ensure the equal distribution of horizontal-flow influent in the main CW bed and were  
128 filled with large gravel pieces with diameters of 2-4 cm. The CWs shared a 150-L influent  
129 tank, constantly mixed by a submerged centrifugal pump placed at the bottom. The pump  
130 was controlled by a timer and was switched on for 5 mins in every 30 mins with an air flow  
131 rate of 50 L/h. Three individual peristaltic pumps were used to pump the wastewater from  
132 the influent tank to the three CWs separately. All CWs were planted with *Juncus effuses*,  
133 at a density of 70-80 stalks per CW. Experimental CWs were placed in a greenhouse and  
134 operated under defined environmental conditions simulating an average summer day  
135 under moderate climatic conditions. The conditions were controlled at 22°C during the  
136 day (from 6 A.M. to 9 P.M) and 16°C at night (from 9 P.M to 6 A.M.). Lamps (Master SON-  
137 PIA 400 W; Phillips, Shanghai, China) were switched on during the day as an artificial light  
138 source when natural illumination was below 60 klx.

139 The experiment was conducted for 200 d under two continuous phases at different  
140 influent volumetric hydraulic loading rates ( $HLR_v$ ) (Table 1). To evaluate the operational  
141 conditions of CWs treating the ammonia-stripped digestate effluent, the  $HLR_v$  was set at  
142 0.105 and 0.21  $d^{-1}$  for all CWs in phase I and II, respectively. To decrease the toxic effect of  
143 alkalinity on microbial communities and enhance  $NH_4^+$ -N removal under intensified  
144 operations, different artificial aeration ratios and effluent recirculation rates were set up

145 in the CWs. An air bar connected to an air pump (ACO-818, Aiseng, Guangdong, China)  
146 intermittently supplied air to the bottom of the CWs with a flow rate of about 180 L/h.  
147 The pump was actuated via a timer and kept the aeration ratios (on : off) at 1h:5h, 1h:1h,  
148 and 1h:5h for CW1, CW2, and CW3, respectively. The effluent of CW2 and CW3 were  
149 recirculated to the influent collection area of the CWs controlled by a peristaltic pump (BT  
150 100-2J, Longer Precision Pump Co. Ltd., Baoding, China) with effluent recirculation ratio of  
151 1:1 and CW1, as the control, had no effluent recirculation.

152 All CWs were fed by ammonia-stripped digestate effluent. The original anaerobic  
153 digestate was collected from a mesophilic biogas plant located in the Dong Hua Shan  
154 Village, Shunyi District, Beijing, China. The concentration of COD, BOD<sub>5</sub> and NH<sub>4</sub><sup>+</sup>-N in the  
155 original anaerobic digestate was around 19410, 10800 and 3810 mg/L, respectively. The  
156 anaerobic digestate was then treated by ammonia stripping by adding lime and by  
157 aeration for 14 h. The final supernatant, with alkalinity of around 3,000 mg/L, was used as  
158 the influent of the CWs. The pH and dissolved oxygen (DO) of the influent were 10.2 and  
159 0.01 mg/L through the whole experiment (Table 2). The average concentrations of COD,  
160 TN, and residual PO<sub>4</sub><sup>3-</sup>-P were approximately 1,300 mg/L, 270 mg/L, and 7 mg/L,  
161 respectively.

## 162 **2.2 Sampling and analysis**

### 163 **2.2.1 Water quality**

164 Both influent and effluent were collected three times a week for water quality  
165 analysis. Three replicates of all parameters were measured for each sample. The pH value

166 and DO were measured in-situ using a portable Orion 5-Star multimeter with a pH  
167 electrode (9172BNWP; THERMO, USA) and DO electrode (086030MD; THERMO, USA).  
168 Alkalinity was analyzed by the titration method (CDJ-5 Automatic Titrator, China).  
169 Chemical oxygen demand (COD) was measured using a Hach DR5000 spectrophotometer  
170 calibrated and operated according to the manufacturer's instructions (Method 8000).  
171 Biological oxygen demand (5 d, BOD<sub>5</sub>) was determined by measuring the amount of  
172 oxygen absorbed by a sample in the presence of microorganisms for five days at 20°C with  
173 nitrification inhibitors addition (APAH, 2012). Standard pre-treatment and reagent  
174 addition according to the standard methods (APAH, 2012) were used to determine the  
175 concentrations of PO<sub>4</sub><sup>3-</sup>-P (Mo-Sb Anti Spectrophotometric Method), NH<sub>4</sub><sup>+</sup>-N (NH<sub>4</sub><sup>+</sup>-  
176 N/4500-NH<sub>3</sub> F; phenate method), and NO<sub>2</sub><sup>-</sup>-N (NO<sub>2</sub><sup>-</sup>-N/4500-NO<sub>2</sub><sup>-</sup> B; colorimetric method)  
177 using a spectrophotometer (Gold S54T; Lengguang Tech, China). NO<sub>3</sub><sup>-</sup>-N was analyzed by  
178 continuous flow colorimetry (SEAL AutoAnalyzer 3, UK). Total nitrogen (TN) was measured  
179 with a TOC-TN analyzer (Shimadzu TOC-VCSN, Kyoto, Japan). To compare the nitrogen  
180 removal performance, both removal efficiency (Rem, Equation 1) and mass removal rate  
181 (MRR, Equation 2) were calculated according to the following equations:

182 
$$\text{Rem (\%)} = \frac{C_{in} - C_{out}}{C_{in}} \times 100 \quad (1)$$

183 
$$\text{MRR (mg/d)} = (C_{inf} - C_{eff}) \times \text{HLR}_v \quad (2)$$

184 where C<sub>inf</sub> and C<sub>eff</sub> are the concentrations of the influent and effluent of each CW,  
185 respectively, in mg/L and HLR<sub>v</sub> is the volumetric hydraulic loading rate of each CW in d<sup>-1</sup>.

### 186 **2.2.2 NH<sub>3</sub> volatilization**

187 NH<sub>3</sub> volatilization may play an important role in nitrogen removal during high pH  
188 conditions (Vymazal, 2007). Therefore, NH<sub>3</sub> volatilization from the three experimental  
189 CWs was measured by the venting method that used a traditional enclosure with sponge-  
190 trapping and KCl extraction methods (Cabrera et al., 1994). Six replicates for each CW  
191 were collected at day 75 and 175 to represent the NH<sub>3</sub> volatilization performance in  
192 experiment phases I and II, respectively. The contribution of NH<sub>3</sub> volatilization to the NH<sub>4</sub><sup>+</sup>-  
193 N removal was calculated as follows (Equation (3)):

$$194 \quad \text{Contribution (\%)} = \frac{V_{\text{NH}_3} \times A}{(C_{\text{inf}} - C_{\text{eff}}) \times \text{HLR}_v} \times 100 \quad (3)$$

195 where  $V_{\text{NH}_3}$  is the NH<sub>3</sub>-N volatilization rate in mg/m<sup>2</sup>/d, A is the surface area of each CW in  
196 m<sup>2</sup>,  $C_{\text{inf}}$  and  $C_{\text{eff}}$  are influent and effluent concentration of NH<sub>4</sub><sup>+</sup>-N in mg/L, and  $\text{HLR}_v$  is the  
197 volumetric hydraulic loading rate of each CW in d<sup>-1</sup>.

### 198 **2.2.3 Nitrogen isotopic composition**

199 While microbial transformation processes can produce isotope fractionation, other  
200 processes such as microbial immobilization, mineralization, and plant uptake as well as  
201 dilution and hydrological transport do not cause any changes in stable isotope abundances  
202 (Coban et al., 2015). Thus, nitrogen isotope fractionation was used as a tool to determine  
203 the biodegradation of nitrogen through nitrification and denitrification processes. The  
204 <sup>15</sup>N/<sup>14</sup>N composition of NH<sub>4</sub><sup>+</sup>-N and NO<sub>3</sub><sup>-</sup>-N in water samples were measured by an  
205 elemental analyzer (vario PYRO cube, Elementar, Hanau, Germany) combined with a

206 stable isotope mass spectrometer (Isoprime 100, Isoprime Ltd., UK). The isotope analyses  
207 were conducted on day 75 and 175 with six replicates of each measurement to represent  
208 the performance in phases I and II, respectively. The  $^{15}\text{N}/^{14}\text{N}$  isotope ratio of  $\text{NH}_4^+\text{-N}$  or  
209  $\text{NO}_3^-\text{-N}$  are expressed as delta ( $\delta$ )-notation in per mil (‰) relative to the standard air and  
210 calculated using Equation (4):

$$211 \quad \delta^{15}\text{N} (\text{‰}) = \frac{R_{\text{sample}} - R_{\text{air}}}{R_{\text{air}}} \times 1000 \quad (4)$$

212 where  $R_{\text{sample}}$  and  $R_{\text{air}}$  are the  $^{15}\text{N}/^{14}\text{N}$  ratios for the sample (in our case  $\text{NH}_4^+\text{-N}$  or  $\text{NO}_3^-\text{-N}$ )  
213 and the atmospheric nitrogen (international standard), respectively.

214 The enrichment factor in ‰,  $\epsilon$ , representing the difference in  $\delta^{15}\text{N}$  (‰) of  $\text{NH}_4^+\text{-N}$   
215 or  $\text{NO}_3^-\text{-N}$  between effluent and influent samples, was calculated based on the study by  
216 (Mariotti et al., 1981) (Equation (5)):

$$217 \quad \delta = \delta_0 + \epsilon \times \ln f \quad (5)$$

218 where  $\delta$  and  $\delta_0$  are effluent and influent  $\delta^{15}\text{N}$  (‰) values of the  $\text{NH}_4^+\text{-N}$  or  $\text{NO}_3^-\text{-N}$ ,  $f$  is the  
219 concentration fraction between effluent and influent corresponding to  $\text{NH}_4^+\text{-N}$  or  $\text{NO}_3^-\text{-N}$ ,  
220 expressed as  $C(\text{NH}_4^+\text{-N})/C_0(\text{NH}_4^+\text{-N})$  or  $C(\text{NO}_3^-\text{-N})/C_0(\text{NO}_3^-\text{-N})$ .

#### 221 **2.2.4 Microbial community analysis**

222 In order to investigate the differences in the functions and responses of microbial  
223 communities in CWs when treating ammonia-stripped digestate effluent, quantitative  
224 real-time PCR was used to analyze the nitrogen-transforming microbial communities in  
225 each CW at the end of each experimental phase. During sampling, 10 g of substrate

226 samples (in triplicate) were collected from the middle layer (25 cm in depth) of each CW.  
 227 The substrate samples were vacuum dried for DNA extraction through the Omega soil  
 228 DNA extraction kit followed by the manure. The gene copy numbers of 16S rDNA,  
 229 ammonia oxidizing bacteria (AOB), ammonia oxidizing archaea (AOA), nitrogen reductase  
 230 bacteria (*nirK* and *nirS*), and anaerobic ammonium oxidation bacteria (anammox) were  
 231 quantified using a real-time PCR system (Applied Biosystem 7500, USA) and SYBR Green  
 232 qPCR mix (Takara Bio Inc., Japan). The primer sequences, target gene lengths, and  
 233 annealing temperatures are shown in Table S1. All PCR reactions were performed in  
 234 triplicate and product specificity was tested through gel electrophoresis and melting curve  
 235 analysis. The correlation coefficients ( $R^2$ ) for standard curves were larger than 0.99. The  
 236 values were expressed as gene copies per gram substrate.

### 237 **2.2.5 Alkalinity consumption associated with nitrogen removal**

238 Alkalinity is consumed during nitrification and generated during denitrification in  
 239 CWs. Theoretically, the alkalinity consumption through nitrification and denitrification was  
 240 3.57 mg/L alkalinity per mg/L of N removed, which can be calculated using Equation (6) (Li  
 241 & Irvin, 2007):

$$242 \quad \Delta \text{Alk}_{\text{theory}} = 3.57 \times ([\text{NH}_4^+\text{-N}]_{\text{inf.}} + [\text{NO}_3^-\text{-N}]_{\text{inf.}} + [\text{NO}_2^-\text{-N}]_{\text{inf.}} \\ 243 \quad - [\text{NH}_4^+\text{-N}]_{\text{eff.}} - [\text{NO}_3^-\text{-N}]_{\text{eff.}} - [\text{NO}_2^-\text{-N}]_{\text{eff.}}) \text{ (mg/L)} \quad (6)$$

244 Moreover, the contribution of alkalinity consumption to nitrogen removal can be  
 245 calculated by Equation (7):

$$246 \quad \text{Contribution (\%)} = \frac{\Delta \text{Alk}_{\text{theory}}}{\text{Alk}_{\text{inf.}} - \text{Alk}_{\text{eff.}}} \times 100 \quad (7)$$

247 where  $Alk_{inf.}$  and  $Alk_{eff.}$  represent the alkalinity of the influent and effluent of the CWs.

## 248 **2.3 Statistical analysis**

249 Statistical analyses were conducted using XLStat Pro® statistical software (XLStat,  
250 Paris, France). The pH, DO, alkalinity, pollutant removal performance/MRR,  $NH_3$   
251 volatilization, nitrogen isotope composition, and microbial community abundance in  
252 different CWs at the same experiment phase were tested by one-way ANOVA. The  
253 differences between experiment phases I and II were tested by Student's t-test. Significant  
254 differences in all comparisons were set at  $p < 0.05$ . Principal Component Analysis (PCA) was  
255 conducted using all measured parameters, including pH, DO, the MRR of alkalinity,  $PO_4^{3-}$ -P,  
256 COD, BOD,  $NH_4^+$ -N, TN, and the accumulated efficiency of  $NO_3^-$ -N and  $NO_2^-$ -N. For PCA,  
257 data was standardized to a Z-score with mean = 0 and standard deviation = 1 to ensure  
258 that each variable had the same influence in the analysis.

## 259 **3. Results**

260 Visually, the plants were healthy during the experiment which may be because  
261 *Juncus effusus* has been demonstrated to be an alkaline-tolerant wetland plant (Mayes et  
262 al., 2005). The pH value of the influent was constant at around  $10.2 \pm 0.1$  (Table 2). After  
263 the water flowed through the wetland beds, the pH decreased to less than 9.0 in the  
264 effluent of all experimental CWs. The pH of the effluent of CW1 ( $8.81 \pm 0.11$ ) was  
265 significantly higher than that of the other two CWs ( $p < 0.05$ ), which might be linked to the  
266 operational strategy of this CW with a lower aeration ratio and without effluent

267 recirculation. However, the increase in the volumetric hydraulic loading rate with shorter  
268 retention time from the experimental phase I to phase II did not show a significant effect  
269 on pH ( $p>0.05$ ) in the CWs. The DO content in the influent was  $0.01 \pm 0.01$  mg/L and  
270 increased to  $0.26 \pm 0.06$ ,  $1.66 \pm 0.09$ , and  $1.22 \pm 0.13$  mg/L in the effluent of CW1, CW2,  
271 and CW3 during experimental phase I, respectively (Table 2 and Fig. S1b). Even though the  
272 effluent DO content for all CWs decreased slightly with an increase in volumetric hydraulic  
273 loading rate from phase I to phase II, the effluent DO in CW2 was still significantly higher  
274 ( $p<0.05$ ) than that in CW1 and CW3. After the stabilization period (around 50 d), the  
275 removal efficiencies of organics, including both COD and BOD<sub>5</sub>, achieved more than 40% in  
276 all CWs. The values were similar during the two experiment phases, however, significantly  
277 higher MRR in phase II (5.3-9.6 g/d) compared with phase I (2.9-4.8 g/d) were observed  
278 along with the increased HLR<sub>v</sub> in all CWs.

279 The alkalinity measured in the influent was relatively stable at  $3,000 \pm 200$  mg/L for  
280 all CWs in both experimental phases (Table 2). After water flowed through the wetland  
281 bed, the alkalinity values became  $2,111 \pm 169$  mg/L ( $28 \pm 2\%$  of reduction),  $1,697 \pm 103$   
282 mg/L ( $42 \pm 5\%$  of reduction), and  $1,998 \pm 151$  mg/L ( $34 \pm 3\%$  of reduction) in CW1, CW2,  
283 and CW3, respectively (Table 3 and Fig. S2a) during phase I. With the increase in HLR<sub>v</sub> from  
284 phase I to phase II, the consumption of alkalinity decreased by  $15 \pm 1\%$ ,  $30 \pm 2\%$  and  $21 \pm 5\%$   
285 in CW1, CW2, and CW3, respectively.

286  $\text{NH}_4^+\text{-N}$  was the main nitrogen form in the present alkaline influent with a  
287 concentration of  $260 \pm 25$  mg/L (Table 2 and Fig. 1). The measured  $\text{NO}_3^-\text{-N}$  and  $\text{NO}_2^-\text{-N}$

288 were constantly below 1 mg/L in the influent. Under the HLR<sub>v</sub> of 52.5 d<sup>-1</sup> during phase I,  
289 the NH<sub>4</sub><sup>+</sup>-N concentration in the effluents of CW1, CW2, and CW3 were 123 ± 15, 23 ± 5,  
290 and 42 ± 4 mg/L, respectively (Table 3 and Fig. 1) with removal efficiencies of 53 ± 6%, 91  
291 ± 9%, and 84 ± 8%. Following the increase in HLR<sub>v</sub> to 0.21 d<sup>-1</sup> in phase II, NH<sub>4</sub><sup>+</sup>-N removal  
292 efficiencies in all CWs were not significantly different (*p*>0.05) compared to phase I. In  
293 addition, a clear accumulation of NO<sub>3</sub><sup>-</sup>-N (28 – 45 mg/L) and NO<sub>2</sub><sup>-</sup>-N (8–14 mg/L) was  
294 observed for CW2 and CW3. However, no significant accumulation of NO<sub>3</sub><sup>-</sup>-N and NO<sub>2</sub><sup>-</sup>-N  
295 (less than 3 mg/L) was observed in the effluent of CW1. The volatilized ammonia was  
296 determined to be 11.8 ± 0.1 mg/m<sup>2</sup>/d in CW1, 13.5 ± 0.5 mg/m<sup>2</sup>/d in CW2, and 12.4 ± 0.6  
297 mg/m<sup>2</sup>/d in CW3 during phase I and increased to 14 ± 0.2 mg/m<sup>2</sup>/d in CW1, 16 ± 0.1  
298 mg/m<sup>2</sup>/d in CW2, and 13.9 ± 0.2 mg/m<sup>2</sup>/d in CW3, respectively during phase II. However,  
299 the percentage of NH<sub>3</sub> volatilization to total NH<sub>4</sub><sup>+</sup>-N removal kept less than 5% for all CWs  
300 in both experimental phases.

301 The isotopic composition of δ<sup>15</sup>N for NH<sub>4</sub><sup>+</sup>-N and NO<sub>3</sub><sup>-</sup>-N were analysed and shown in  
302 Fig. 3a and b. The enrichment factors were simulated and calculated in CWs for alkaline  
303 digestate effluent treatment in CWs (Fig. 3c and d). The average value of <sup>15</sup>N-NH<sub>4</sub><sup>+</sup> in the  
304 influent is constantly of 44.95%. The significantly higher values of <sup>15</sup>N-NH<sub>4</sub><sup>+</sup> composition  
305 were found in the effluent of all three CWs in phase I, which were 47%, 53% and 48% for  
306 CW1, CW2 and CW3, respectively. In phase II, the average <sup>15</sup>N-NH<sub>4</sub><sup>+</sup> composition  
307 significantly increased to 53%, 58% and 54% for CW1, CW2 and CW3, respectively. The  
308 average enrichment factor was significantly higher in CW2 (-3.3) than CW1 (-1.9) and CW2

309 (-1.8) in phase I. In phase II, the values significantly improved to -11.6 and -6.6 and -6.2 in  
310 CW1, CW2 and CW3, respectively. The influent  $\text{NO}_3^-$ -N concentration is below the limit  
311 detection, thus, we can only observe the  $\delta^{15}\text{N}$ - $\text{NO}_3^-$  composition in the effluent. They did  
312 not show as significantly different for all CWs during the two experimental phases, which  
313 was in a range of 34-42%.

314 The gene copy number of 16S rDNA was not significantly different between all CWs  
315 in both experimental phases (Fig. 4a). Clearly, the abundance of anammox was detected in  
316 all CWs and the values are significantly higher in CW3 for both experiment phases (Fig. 4b).  
317 Generally, it can be observed that significantly higher magnitude levels of AOA and AOB  
318 were found in CW2, followed by CW3 and CW1 (Fig. 4 c and d). However, the abundance  
319 of nirK and nirS were significantly higher in CW1 and CW3 compared with CW2 (Fig. 4e  
320 and f). The abundance of all functional nitrogen-transforming bacteria were generally  
321 significantly higher in phase II than phase I for all CWs.

#### 322 **4. Discussion**

323 Nitrogen (N) is one of the important pollutants in CWs for wastewater treatment  
324 (Wu et al., 2014). The N transformation in wetlands has been well investigated and  
325 reported to include various pathways such as physical adsorption, volatilization, and  
326 biological transformation (nitrification, denitrification, and anammox) when treating  
327 conventional wastewater such as domestic and municipal effluents with neutral pH  
328 (Vymazal, 2013; Wu et al., 2014). However, information on how these pathways are

329 altered in CWs when treating a highly alkaline digested effluent is still insufficient.  
330 Moreover, whether the volatilization of ammonia plays a significant role and whether  
331 nitrogen-transforming bacteria might be restricted under this high pH environment is also  
332 not clear, which is the main driver of this investigation.

333 It has been well recognized that the loss of  $\text{NH}_4^+\text{-N}$  through free ammonia ( $\text{NH}_3$ )  
334 volatilization is significant at pH above 9.3 (Vymazal, 2007). In the current study, the  
335 ammonia volatilization did not draw a significant contribution even though the pH in the  
336 influent is consistently higher than 10. The aeration at the bottom of the experimental  
337 CWs as a performance intensifying strategy can also enhance the turbulence of the water  
338 in the wetland bed and improve the mass transfer of ammonia from the dissolved form in  
339 water to air. Therefore, during the two experimental phases, we respectively measured  
340 the volatilization rate of ammonia from three lab-scale CWs by using sponge-trapping and  
341 the KCl extraction method. The results shown in Fig. 2 clearly indicate that the volatilized  
342 ammonia in CW2 (13.5 and 16  $\text{mg/m}^2/\text{d}$  in phase I and II, respectively) is significantly  
343 higher than that of CW1 (11.8 and 14  $\text{mg/m}^2/\text{d}$  in phase I and II, respectively) and CW3  
344 (12.4 and 13.9  $\text{mg/m}^2/\text{d}$  in phase I and II, respectively), regardless of volumetric hydraulic  
345 loadings in the two different experimental phases. This higher  $\text{NH}_3$  volatilization rate in  
346 CW2 might be closely related to the longer aeration ratio and effluent recirculation that  
347 provides a higher opportunity of water perturbation and ammonia diffusion transfer to air  
348 bubbles. The increase in  $\text{HLR}_v$  from phase I to phase II caused a higher loss of nitrogen by  
349 free ammonia volatilization. However, the calculated contribution of  $\text{NH}_3$  volatilization to

350 total  $\text{NH}_4^+\text{-N}$  removal using Equation 3 was still less than 5%, indicating alternative main  
351 transformation pathways in these three lab-scale CWs.

352 The assessment of N-isotopic fractionation as a good technique to identify the  
353 predominant N removal pathway was used in this study, since microbial transformation  
354 processes normally produce significant isotopic fractionation, while other processes such  
355 as microbial immobilization, mineralization, and plant uptake as well as dilution and  
356 hydrological transport do not cause and/or cause insignificant changes in stable isotope  
357 abundances (Chen et al., 2014; Erler et al., 2010; Reinhardt et al., 2006). The results in Fig.  
358 3 show that the significant enrichment of  $\delta^{15}\text{N-NH}_4^+$  in the effluent of all experimental  
359 CWs (47–58‰) compared to the influent  $\delta^{15}\text{N-NH}_4^+$  (44.95‰) indicates the importance of  
360 microbial transformation for  $\text{NH}_4^+\text{-N}$  removal (Sjøvik & Mørkved, 2008). Moreover, the  
361 higher enrichment factor in the effluent of CW2 ( $\epsilon = -3.3\text{‰}$  in phase I and  $\epsilon = -6.6\text{‰}$ )  
362 compared to the other two CWs indicates the role of the aeration in increasing the  
363 nitrification capacity of the wetland by supplying more oxygen. This result is consistent  
364 with the ammonium removal and nitrate production performance results (Fig. 1).  
365 However, the enrichment factors of  $\delta^{15}\text{N-NH}_4^+$  obtained in this study ( $-1.8\text{‰}$ – $-11.6\text{‰}$ )  
366 were much lower than the range reported in previous studies ( $-14.2\text{‰}$ – $-38.2\text{‰}$ ) (Casciotti  
367 et al., 2003; Coban et al., 2015), reflecting the inhibition of nitrifiers in the CWs suffering  
368 from the high pH alkaline environment. The inhibition of nitrifiers cannot be detected by  
369 the ammonium removal performance characterized by physicochemical parameters.

370 Alkaline wastewater has been well recognized for its negative impact on microbial  
371 communities (Qu et al., 2011), macrophage growth, and biota survival (Mayes et al., 2005).  
372 Some studies tested the potential of CWs to treat alkaline industrial wastewater (Kadlec &  
373 Wallace, 2008; Mayes et al., 2009). But little information is available on the interaction of  
374 nitrogen transformation with the consumption of alkalinity during the treatment process,  
375 particularly under intensified operational strategies, such as aeration. In the present study,  
376 external aeration at the bottom of the wetland bed can not only pump more CO<sub>2</sub> from the  
377 atmosphere to neutralize the pH of the pore water (Table 3), but also supply more O<sub>2</sub> to  
378 activate the microbes. In this case, higher microbial respiration under aeration can  
379 generate more CO<sub>2</sub> from the degradation of organic matter and increase the partial  
380 pressure of CO<sub>2</sub> ( $p\text{CO}_2$ ) to neutralize the pH. Moreover, nitrification driven by aeration can  
381 also consume more alkalinity in the water system. Although the above-mentioned  
382 transformations often occur simultaneously, the results calculated by Equations 6 and 7  
383 strongly support that N transformation processes accounting for 94% of the contribution  
384 to alkalinity consumption play a key role in this study.

385 To better understand the effect of intensifying strategies such as aeration and  
386 recirculation on nitrogen transformation in CWs treating alkaline digestate effluent, the  
387 abundances of AOA, AOB, *nirK*, *nirS*, anammox, and 16S rDNA were determined for all  
388 CWs at different experimental phases by a molecular biology analysis (Fig. 4). Higher  
389 abundance of AOA and AOB (Fig. 4c and d) in CW2 is consistent with NH<sub>4</sub><sup>+</sup>-N removal  
390 performances (Table 3 and Figure 1), demonstrating the positive effect of aeration on

391 promoting nitrification (Wu et al., 2017). Less abundant *nirK* and *nirS* might be responsible  
392 for nitrate accumulation in the effluent. It is interesting that a comparative number of  
393 anammox was also found in all three lab-scale experimental wetlands. Anammox is a  
394 recently discovered nitrogen removal process that can remove ammonium from  
395 wastewater by consuming less oxygen and organic carbon (Zhu et al., 2013). The high  
396 abundance of anammox in the wetlands treating a highly alkaline digestate strongly  
397 indicates alternative nitrogen transformation pathways different from the classic  
398 nitrification-denitrification pathways. However, high gene abundance doesn't equal a high  
399 activity of functional microorganisms, thus the transcriptome information may need  
400 further investigation.

401 To fully understand the complex interaction between different factors, principal  
402 component analysis (PCA) was used to provide an overview of the system performance  
403 using a useful and friendly visualization method to normalize all parameters (Guo et al.,  
404 2017; Kizito et al., 2017). The pollutant removal performance of CWs was significantly  
405 influenced by HLR<sub>v</sub> and intensifying strategies (bottom aeration and effluent recirculation)  
406 under this highly alkaline influent condition (Fig. 5). The overlapping patterns of influent  
407 from phase I and II represented the stable influent conditions during the entire  
408 experiment. The patterns of CW2 and CW3 placed in the upper-right corner compared to  
409 CW1 in the plot indicated the benefits of aeration and effluent recirculation on pollutant  
410 biodegradation under aerobic conditions (Wu et al., 2014). This improved pollutant  
411 removal capacity clearly indicated the potential of intensified CWs by the treatment of

412 highly alkaline digestate. However, the higher aeration ratio and different effluent  
413 recirculation configurations need to be further studied in CWs to treat alkaline  
414 wastewater.

## 415 **5. Conclusions**

416 The intensified horizontal subsurface flow CWs coupled with aeration and effluent  
417 recirculation were effective in treating alkaline ammonia-stripped digestate effluent with  
418 high alkalinity consuming capacity. The higher aeration ratio and presence of effluent  
419 recirculation in the present study showed a positive effect on the alkalinity and pollutant  
420 removal. The N-isotopic fractionation assessment indicated the predominant role of  
421 microbial transformations in nitrogen removal, but lower enrichment factors reflected the  
422 inhibitory effect of nitrifiers in the high pH alkaline environment. Biological nitrogen  
423 transformations account for 94% of the alkalinity consumption and NH<sub>3</sub> volatilization  
424 contributes to a maximum of 5% of total ammonium removal.

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**Table 1**

Operation conditions of three constructed wetlands during entire experimental phase.

Phases	Time (d)	Wetlands	Aeration ratio (on: off)	Effluent Recirculation	HLR <sub>v</sub> (d <sup>-1</sup> )
I	110	CW1	1 h:5 h	---	0.105
		CW2	1 h:1 h	1:1	
		CW3	1 h:5 h	1:1	
II	90	CW1	1 h:5 h	---	0.21
		CW2	1 h:1 h	1:1	
		CW3	1 h:5 h	1:1	

HLR<sub>v</sub> represent volumetric hydraulic loading rate.

**Table 2**

Influent composition of three constructed wetlands during two experimental phases. Values are presented as mean  $\pm$  standard deviation (sampling number).

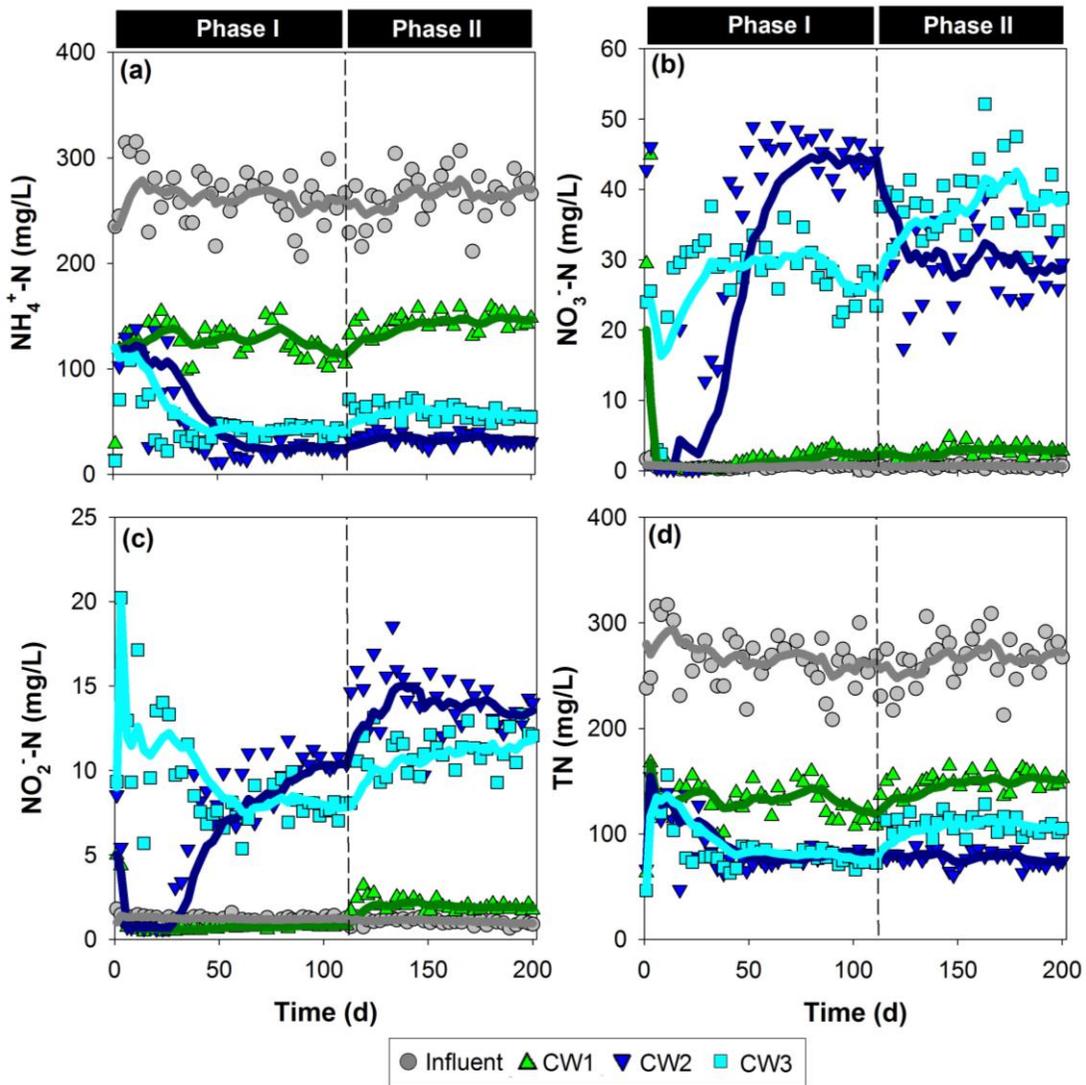
Parameters	Unit	Phase I (n=38)	Phase II (n=33)
pH		10.22 $\pm$ 0.16	10.17 $\pm$ 0.13
DO	mg/L	0.01 $\pm$ 0.01	0.01 $\pm$ 0.01
Alkalinity	mg/L	2986 $\pm$ 215	3021 $\pm$ 244
PO <sub>4</sub> <sup>3-</sup> -P	mg/L	7.89 $\pm$ 1.82	5.84 $\pm$ 1.32
COD	mg/L	1311 $\pm$ 105	1287 $\pm$ 94
BOD <sub>5</sub>	mg/L	689 $\pm$ 95	703 $\pm$ 64
NH <sub>4</sub> <sup>+</sup> -N	mg/L	263.7 $\pm$ 25.4	264.3 $\pm$ 22.9
NO <sub>3</sub> <sup>-</sup> -N	mg/L	0.5 $\pm$ 0.1	0.4 $\pm$ 0.1
NO <sub>2</sub> <sup>-</sup> -N	mg/L	0.2 $\pm$ 0.1	0.5 $\pm$ 0.1
TN	mg/L	260 $\pm$ 30	274 $\pm$ 29

**Table 3**

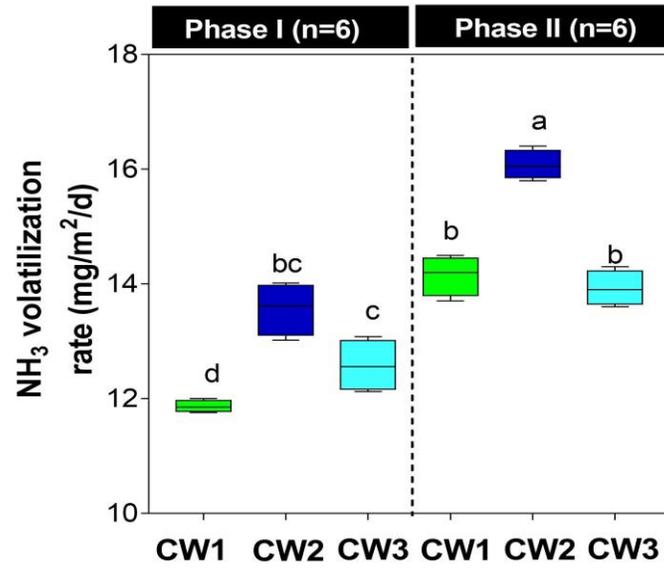
Effluent water quality, removal efficiency, and mass removal rate (MRR) for the three lab-scale horizontal-flow constructed wetlands (CWs) during the whole experiment. Values are presented as mean  $\pm$  standard deviation.

Experimental Phases	Parameters	Phase I - Effluent			Reduction (%)			MRR (g/d)		
		CW 1	CW 2	CW 3	CW1	CW 2	CW3	CW1	CW 2	CW3
Phase I	pH	8.81 $\pm$ 0.11 <sup>a</sup>	8.28 $\pm$ 0.06 <sup>c</sup>	8.45 $\pm$ 0.07 <sup>b</sup>	/	/	/	/	/	/
	DO (mg/L)	0.26 $\pm$ 0.06 <sup>c</sup>	1.66 $\pm$ 0.09 <sup>a*</sup>	1.22 $\pm$ 0.13 <sup>b*</sup>	/	/	/	/	/	/
	Alkalinity (mg/L)	2122 $\pm$ 169 <sup>a*</sup>	1697 $\pm$ 103 <sup>b*</sup>	1998 $\pm$ 151 <sup>a*</sup>	28 $\pm$ 2	42 $\pm$ 5	34 $\pm$ 3	6.6 $\pm$ 0.3	9.9 $\pm$ 0.5	8.0 $\pm$ 0.3
	PO <sub>4</sub> <sup>3-</sup> -P (mg/L)	1.05 $\pm$ 0.57	1.64 $\pm$ 0.63	1.18 $\pm$ 0.51	87 $\pm$ 10	79 $\pm$ 6	85 $\pm$ 6	0.05 $\pm$ 0.01	0.05 $\pm$ 0.01	0.05 $\pm$ 0.01
	COD (mg/L)	821 $\pm$ 37 <sup>a</sup>	708 $\pm$ 41 <sup>b</sup>	739 $\pm$ 41 <sup>b</sup>	37 $\pm$ 2	46 $\pm$ 3	44 $\pm$ 2	3.9 $\pm$ 0.6	4.8 $\pm$ 1.1	4.5 $\pm$ 0.9
	BOD <sub>5</sub> (mg/L)	331 $\pm$ 27 <sup>a</sup>	210 $\pm$ 37 <sup>b</sup>	271 $\pm$ 39 <sup>a</sup>	52 $\pm$ 6	70 $\pm$ 6	61 $\pm$ 7	2.9 $\pm$ 0.6	3.8 $\pm$ 0.9	3.3 $\pm$ 0.6
	NH <sub>4</sub> <sup>+</sup> -N (mg/L)	123 $\pm$ 15 <sup>a</sup>	23 $\pm$ 5 <sup>c*</sup>	42 $\pm$ 4 <sup>b*</sup>	53 $\pm$ 6	91 $\pm$ 9	84 $\pm$ 8	1.1 $\pm$ 0.1	2.0 $\pm$ 0.5	1.8 $\pm$ 0.3
	NO <sub>3</sub> <sup>-</sup> -N (mg/L)	2.1 $\pm$ 0.7 <sup>c</sup>	45.3 $\pm$ 2.6 <sup>a*</sup>	28.1 $\pm$ 3.8 <sup>b*</sup>	/	/	/	/	/	/
	NO <sub>2</sub> <sup>-</sup> -N (mg/L)	0.8 $\pm$ 0.1 <sup>b*</sup>	9.9 $\pm$ 1.3 <sup>a*</sup>	7.9 $\pm$ 1.0 <sup>a*</sup>	/	/	/	/	/	/
TN (mg/L)	130 $\pm$ 16 <sup>a</sup>	78 $\pm$ 5 <sup>b</sup>	78 $\pm$ 6 <sup>b*</sup>	50 $\pm$ 6	70 $\pm$ 4	70 $\pm$ 5	3.5 $\pm$ 0.2	1.5 $\pm$ 1.3	1.5 $\pm$ 0.2	
Phase II	pH	8.76 $\pm$ 0.12 <sup>a</sup>	8.25 $\pm$ 0.10 <sup>b</sup>	8.51 $\pm$ 0.11 <sup>a</sup>	/	/	/	/	/	/
	DO (mg/L)	0.22 $\pm$ 0.05 <sup>c</sup>	1.17 $\pm$ 0.17 <sup>a</sup>	0.76 $\pm$ 0.13 <sup>b</sup>	/	/	/	/	/	/
	Alkalinity (mg/L)	2595 $\pm$ 169 <sup>a</sup>	2134 $\pm$ 142 <sup>b</sup>	2392 $\pm$ 145 <sup>ab</sup>	15 $\pm$ 1	30 $\pm$ 2	21 $\pm$ 5	24 $\pm$ 0.2	14.4 $\pm$ 0.1	10.1 $\pm$ 0.2
	PO <sub>4</sub> <sup>3-</sup> -P (mg/L)	2.21 $\pm$ 0.48	2.17 $\pm$ 0.65	1.25 $\pm$ 0.39 <sup>b</sup>	62 $\pm$ 8	62 $\pm$ 7	78 $\pm$ 9	0.06 $\pm$ 0.01	0.06 $\pm$ 0.01	0.07 $\pm$ 0.01
	COD (mg/L)	825 $\pm$ 56 <sup>a</sup>	679 $\pm$ 40 <sup>b</sup>	740 $\pm$ 40 <sup>b</sup>	36 $\pm$ 2	47 $\pm$ 3	43 $\pm$ 2	7.4 $\pm$ 0.5	9.6 $\pm$ 1.1	8.7 $\pm$ 0.6
	BOD <sub>5</sub> (mg/L)	387 $\pm$ 30 <sup>a</sup>	306 $\pm$ 28 <sup>b</sup>	352 $\pm$ 27 <sup>ab</sup>	47 $\pm$ 4	58 $\pm$ 4	52 $\pm$ 4	5.3 $\pm$ 0.8	6.5 $\pm$ 0.5	5.9 $\pm$ 0.5
	NH <sub>4</sub> <sup>+</sup> -N (mg/L)	144 $\pm$ 8 <sup>a</sup>	34 $\pm$ 5 <sup>c</sup>	59 $\pm$ 6 <sup>b</sup>	45 $\pm$ 3	87 $\pm$ 12	77 $\pm$ 9	2.0 $\pm$ 0.2	3.6 $\pm$ 0.5	3.2 $\pm$ 0.3
	NO <sub>3</sub> <sup>-</sup> -N (mg/L)	2.8 $\pm$ 0.8 <sup>c</sup>	28.8 $\pm$ 5.1 <sup>b</sup>	38.5 $\pm$ 4.6 <sup>a</sup>	/	/	/	/	/	/
	NO <sub>2</sub> <sup>-</sup> -N (mg/L)	2.0 $\pm$ 0.4 <sup>c</sup>	14.1 $\pm$ 1.7 <sup>a</sup>	11.1 $\pm$ 1.2 <sup>b</sup>	/	/	/	/	/	/
TN (mg/L)	149 $\pm$ 9 <sup>a</sup>	77 $\pm$ 7 <sup>c</sup>	109 $\pm$ 8 <sup>b</sup>	44 $\pm$ 3	71 $\pm$ 6	59 $\pm$ 4	2.0 $\pm$ 0.2	3 $\pm$ 0.3	2.6 $\pm$ 0.2	

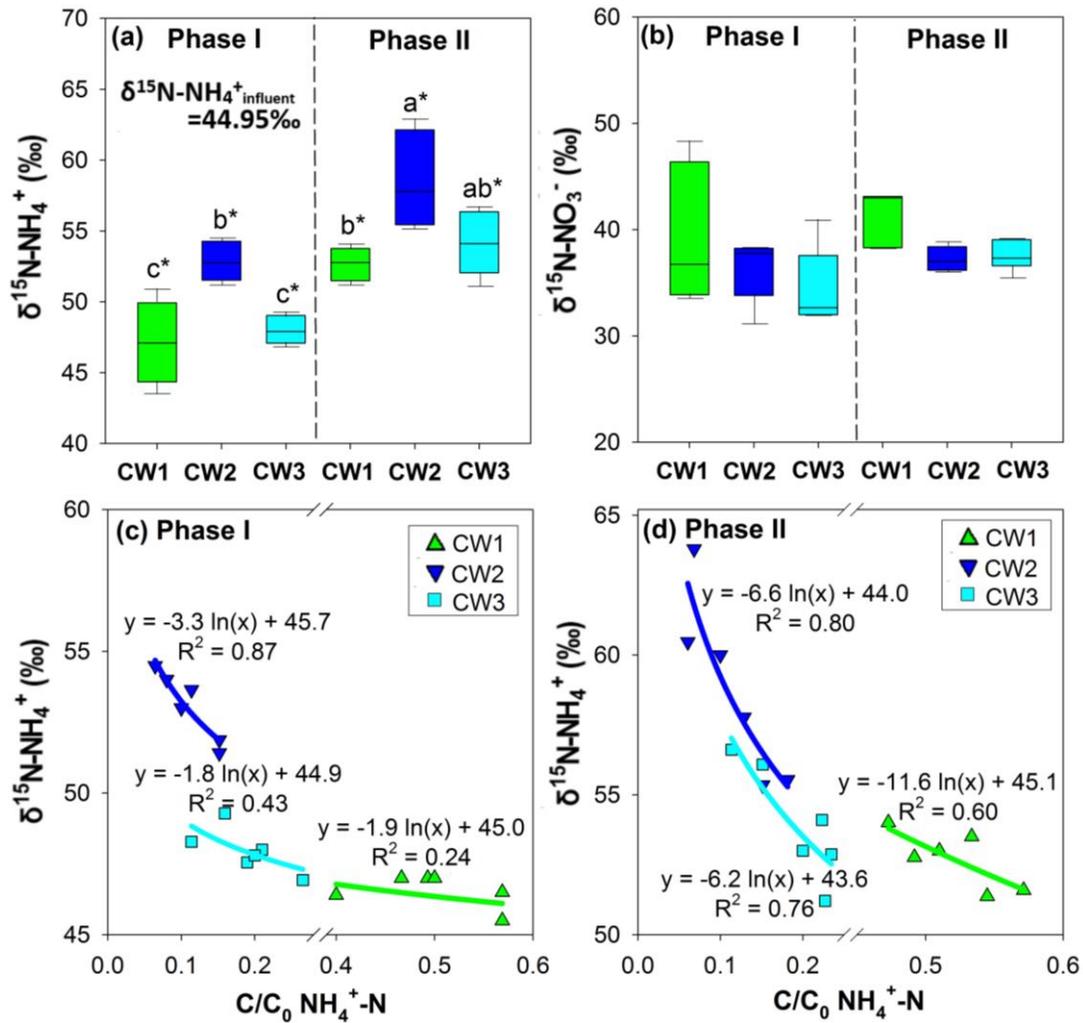
Different letter beside the value represent the significant difference between different CWs in the same experiment phase, \* beside the values represents the significant difference between two experiment phases for the same CW.



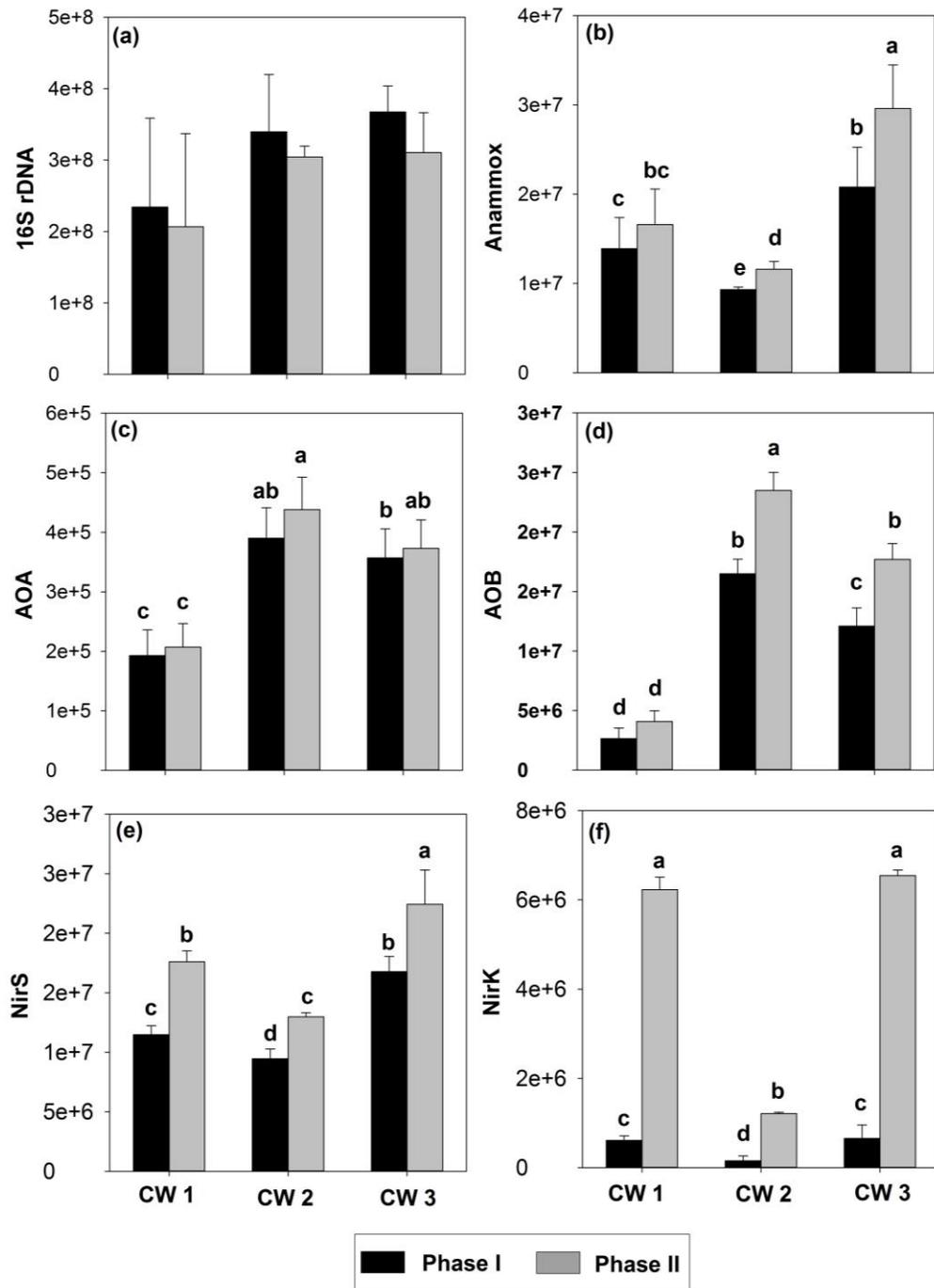
**Fig. 1** Dynamics of  $\text{NH}_4^+\text{-N}$  (a),  $\text{NO}_3^-\text{-N}$  (b),  $\text{NO}_2^-\text{-N}$  (c), and TN (d) in the influent and effluent of three lab-scale horizontal-flow constructed wetlands (CW1, 2, and 3) during the whole experiment.



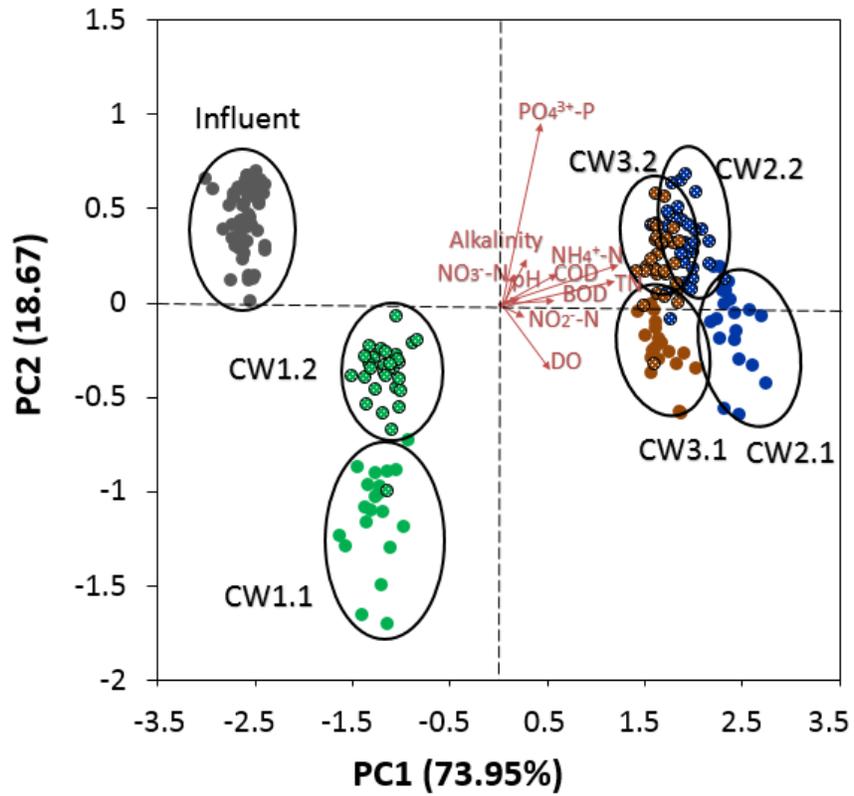
**Fig. 2** NH<sub>3</sub> volatilization rates in three lab-scale horizontal-flow constructed wetlands (CW1, 2, and 3) during the whole experiment. Different letters above the box plot represent the significant differences.



**Fig. 3** The composition of  $\delta^{15}\text{N-NH}_4^+$  (a) and  $\delta^{15}\text{N-NO}_3^-$  (b) composition and the enrichment changes in  $\text{NH}_4^+\text{-N}$  isotopic composition versus concentrations for determination of enrichment factor ( $\epsilon$ ) for nitrification in phase I (c) and phase II (d) in three horizontal-flow constructed wetlands (CW1, 2, and 3) during the whole experiment ( $n = 6$  for each measurement). Different letters above the box plot represent the significant differences.



**Fig. 4** Gene copy number (copies / g substrate) of 16S rDNA (a), Anammox (b), AOA (c), AOB (d), nirS (e), and nirK (f) in three horizontal-flow constructed wetlands (CW1, 2, and 3) at the end of each experiment phase. Different letters above the bar plot represent the significant differences.



**Fig. 5** Principal component analysis of the treatment performance patterns in three constructed wetlands during the two experiment phases. The first number (1–3) after CW represents three different CW types; the second number (1–2) represents experiment phase I and II.