



Treatment of secondary effluent by a novel tidal-integrated vertical flow constructed wetland using raw sewage as a carbon source: Contribution of partial denitrification-anammox



Xuan Zhan^{a,b}, Yongqiang Yang^{a,*}, Fanrong Chen^a, Shijun Wu^a, Runliang Zhu^a

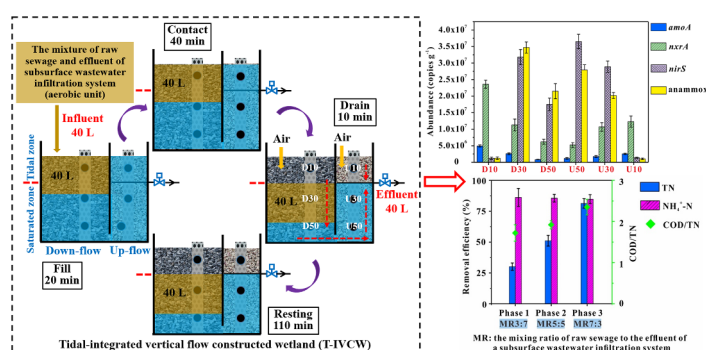
^a CAS Key Laboratory of Mineralogy and Metallogeny/Guangdong Provincial Key Laboratory of Mineral Physics and Materials, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, 511 Kehua Street, Guangzhou 510640, China

^b University of Chinese Academy of Sciences, 19 Yuquan Road, Beijing 100049, China

HIGHLIGHTS

- A T-IVCW was developed to treat secondary effluent using a cost-saving electron donor.
- Efficient $\text{NH}_4^+\text{-N}$ and TN removal rates were achieved with the C/N ratio of 2.35.
- Partial denitrification-anammox (PDN/AMX) played a vital role in nitrogen removal.
- *Denitratisoma* and *Candidatus Brocadia* were responsible for PDN/AMX in the saturated zone.
- The T-IVCW is an alternative strategy for the treatment of low C/N ratio sewage.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:

Tidal-integrated vertical flow constructed wetland
Low C/N ratio
Mixing ratio
Partial denitrification-anammox
Microbial community
Functional genes

ABSTRACT

Nitrogen removal in constructed wetlands (CWs) is often challenged by limited nitrification due to low oxygen transfer and/or limited denitrification due to the lack of carbon source. In this study, a novel tidal-integrated vertical flow constructed wetland (T-IVCW) was developed to treat secondary effluent with low chemical oxygen demand/total nitrogen (C/N) ratio raw sewage as a carbon source. Three different mixing ratios of raw sewage to secondary effluent (3:7, 5:5, and 7:3) on nitrogen removal performance and microbial community were investigated. The results showed that mixing ratios slightly affected $\text{NH}_4^+\text{-N}$ removal, but significantly affected TN removal. When the mixing ratio was 7:3 with influent C/N ratio of 2.35, high removal efficiencies of $\text{NH}_4^+\text{-N}$ (85.08%) and TN (81.18%) were obtained. Cluster analysis revealed that mixing ratios and vertical variation of local redox conditions were the main drivers of the microbial community. The tidal zone was characterized by the presence of unclassified Xanthomonadaceae, *Nitrospira*, and *Rhodanobacter*, while *Candidatus Brocadia* and *Denitratisoma*, which involves in anammox and denitrification, dominated the community composition in the saturated zone. These results were further confirmed by the corresponding functional genes (*amoA*, *nxrA*, *nirS*, and *anammox*). Thus, the partial denitrification-anammox (PDN/AMX) and denitrification were proposed as the major pathways related to nitrogen removal. Furthermore, with the increase of mixing ratio, the PDN/AMX played an increasingly important role in nitrogen removal and accounted for at least 52.91% when the mixing ratio was 7:3. This study provides an alternative strategy for the treatment of low C/N ratio sewage and new insights into nitrogen transformation pathways in CWs.

* Corresponding author.

E-mail address: yqyang@gig.ac.cn (Y. Yang).

<https://doi.org/10.1016/j.cej.2020.125165>

Received 3 March 2020; Received in revised form 15 April 2020; Accepted 19 April 2020

Available online 22 April 2020

1385-8947/ © 2020 Elsevier B.V. All rights reserved.

1. Introduction

Nitrogen is one of the nutrients that induce eutrophication in water bodies. In China, almost 90% of wastewater treatment plants have problems with nutrient removal, especially total nitrogen (TN), thereby leading to a high TN concentration ($> 15 \text{ mg}\cdot\text{L}^{-1}$) in the secondary effluent [1], which is an important source of nitrogen in rivers and lakes. Additionally, decentralized systems such as subsurface wastewater infiltration systems (SWISs) cannot remove nitrogen effectively, thereby resulting in a high NO_3^- content in the effluent [2,3]. Therefore, improving the TN removal from secondary effluent has become an urgent need throughout China [4].

The aerobic unit and secondary effluent are characterized by high contents of NO_3^- with low chemical oxygen demand (COD), which are not conducive to denitrification as $2.85 \text{ g COD}\cdot\text{g}^{-1}\text{N}$ is theoretically needed for denitrification via NO_3^- reduction without assimilation [5]. Post-denitrification generally requires an external carbon source because the internal carbon source is almost depleted in the aerobic process. A variety of external carbon sources, including methanol, sodium acetate, corncob, and plant tissues [6–8], have been used to enhance the denitrification efficiency. Compared with other carbon sources, raw sewage with a high COD/TN (C/N) ratio is an ideal internal carbon source if used properly because it is cost free, eco-friendly, easily obtained, and low maintenance. In recent years, organic carbon in raw sewage has been efficiently used for denitrification in hybrid constructed wetlands (CWs) with step-feeding [9]. However, there is a paradox in the step-feeding in that higher NO_3^- removal requires a higher step-feeding rate to increase the C/N ratio in the post anoxic unit, thereby resulting in a higher NH_4^+ -N content in the final effluent [10]. In order to meet the NH_4^+ -N discharge limit, the step-feeding rate is usually below 50% of the total influent, which inevitably deteriorates the NO_3^- -N removal efficiency, especially for sewage with a low C/N ratio. This highlights the importance of the simultaneous removal of both NO_3^- -N and the introduced NH_4^+ -N by the post-denitrification unit.

Tidal-flow CWs (TFCWs) are a relatively new technology that utilize a novel oxygen transfer method [11]. TFCWs generate a rhythmic

sequential cycle of a flood/wet phase and a drain/dry phase, which increases both the nitrification and denitrification in a single reactor [12]. High TN removal rates were achieved in TFCWs when the C/N ratios were higher than 10 [12,13]. However, a number of laboratory studies showed limited denitrification process in TFCWs, thereby resulting in NO_3^- -N and/or NO_2^- -N accumulation due to the degradation of organic matter during the aerobic stage [14–16]. The flood and drain (F/D) cycle of TFCW is a crucial parameter affecting the hydraulic loading rate (HLR) and aerobic/anoxic conditions for maximum nitrogen removal. In previous studies, long cycles of $> 8 \text{ h}$ were usually adopted [12,15]. Nevertheless, the knowledge of the influence of short cycles on nitrogen removal in TFCW is scarce, and further investigation is required.

Integrated vertical flow CWs (IVCWs), which are composed of a down-flow cell and an up-flow cell in series, have become one of the main types of CWs in China owing to their high purification efficiency [17]. High TN removal efficiency ($\sim 90\%$) was achieved in IVCWs at low HLR ($< 5 \text{ cm}\cdot\text{d}^{-1}$) [17,18]. However, the IVCW did not perform well in terms of TN removal efficiency (13.9%) at a high HLR ($25 \text{ cm}\cdot\text{d}^{-1}$), and NH_4^+ -N was the predominant nitrogen form in the effluent owing to insufficient dissolved oxygen (DO) [19]. Similarly, the removal rate of NH_4^+ -N deteriorated from 99% to 74% when the hydraulic retention time (HRT) declined from 5 d to 2 d in a baffled subsurface-flow CW (multi-stage IVCW) [20]. Generally, the insufficient oxygen supply in permanently saturated CWs creates predominantly anoxic conditions. Recent research on vertical flow CWs showed that partial saturation conditions achieve a significantly higher TN removal [21], suggesting that the co-occurrence of aerobic and anoxic conditions within a single CW is the key to achieve good nitrogen removal. Therefore, it seems reasonable to infer that the oxygen supply would be greatly improved if the upper part of the IVCW were converted into a tidal flow pattern, which can be called a tidal-integrated vertical flow constructed wetland (T-IVCW). Accordingly, various redox conditions could be shaped, which might result in the enhancement of nitrogen removal at a high HLR.

In recent years, anammox (AMX) has been widely studied owing to its significant advantages (requires less aeration and no organic carbon)

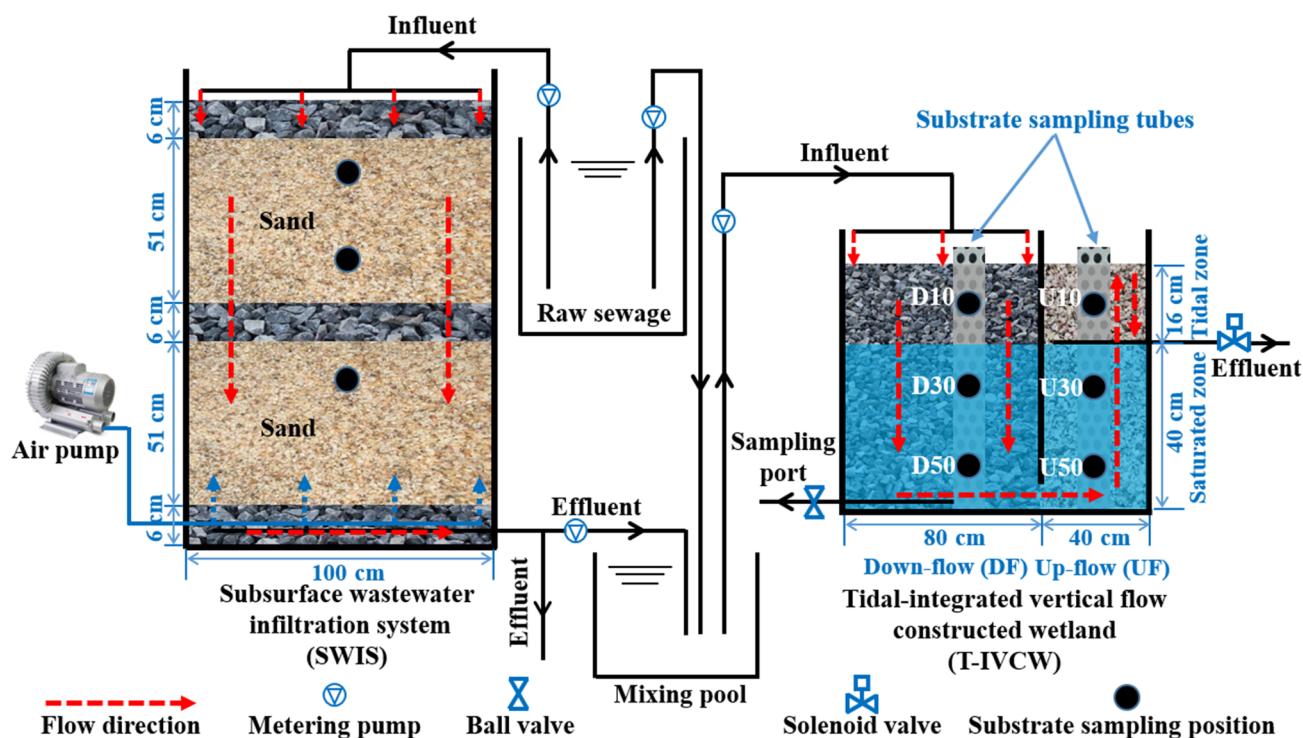


Fig. 1. The schematic diagram of the subsurface wastewater infiltration system (SWIS) and tidal-integrated vertical flow constructed wetland (T-IVCW).

compared to classical nitrification-denitrification [22]. AMX has been successfully applied for the treatment of wastewaters with high nitrogen concentrations, whereas the direct treatment of domestic sewage is still a great challenge owing to unstable partial nitrification (PN) [22–24]. More recently, the partial denitrification-AMX (PDN/AMX) process has been developed for simultaneously treating NH_4^+ and NO_3^- containing wastewaters [25]. The C/N ratio was proposed as the vital factor determining the NO_2^- production in PDN/AMX, and the suitable C/N ratios are 2–3 [26]. Therefore, PDN/AMX might be a feasible pathway for simultaneous removal of NH_4^+ and NO_3^- in CWs when treating low C/N ratio sewage. However, whether the co-existence of NH_4^+ and NO_3^- and appropriate C/N ratios in the T-IVCW will contribute to achieve PDN/AMX remains unclear.

In view of this, a novel T-IVCW system was developed to treat secondary effluent with raw sewage as a carbon source. A SWIS was employed to supply secondary effluent. The COD and nitrogen removal performances were investigated under three mixing ratios in the T-IVCW. Additionally, the microbial communities and functional microbes of the T-IVCW were elucidated using 454 high-throughput sequencing and quantitative polymerase chain reaction (qPCR) technology, thereby providing further insight into the mechanism of nitrogen removal in the system.

2. Materials and methods

2.1. Experimental apparatus and operating conditions

The experimental apparatus was located on the Guangzhou Institute of Geochemistry campus. It consisted of an SWIS as the aerobic unit and a T-IVCW (Fig. 1). The structure and operating conditions of the SWIS are described in Text S1 and Table S1.

As illustrated in Fig. 1, the T-IVCW, which was made from stainless steel plates (1.2 m × 0.8 m × 0.6 m), was divided into two parts by a baffle plate. A plughole ($\Phi = 4.0$ cm) was equipped in the bottom of

the baffle plate to allow the wastewater to flow from one side to the other. The down-flow chamber (DF) (0.8 m × 0.8 m × 0.6 m) was filled with gravel (particle size of 5–12 mm and initial porosity of 34%), followed by the up-flow chamber (UF) (0.4 m × 0.8 m × 0.6 m), which was filled with zeolite (particle size of 2–4 mm and initial porosity of 30%). Two stainless steel casing tubes (1.0 m height × 0.1 m diameter) filled with the same substrates were vertically pre-buried in the two chambers of the T-IVCW for microbial sampling (Fig. 1). The mesh on the casing tubes allowed the same wastewater–air contact and hydraulic conditions (wetting and drying cycle) for the overall wetland bed and the substrate in the columns [12]. Distribution pipes were placed on the DF surface, and outlet pipes were set at 40 cm height in the UF substrates, which divided the mesocosm into two sections (Fig. 1). The upper section was 16 cm in height and operated with a tidal strategy, while the lower section was 40 cm in height and maintained with constant water saturation. In addition, one sampling outlet was set at the bottom of the DF to explore the relative contribution of nitrogen removal of the two chambers. The tidal operation was initiated by a metering pump and a solenoid valve (normally open) controlled by a timer. The flood/drain (F/D) cycle occurred every 3 h with an F/D ratio of 1 h: 2 h and 8 cycles per day. For each cycle, approximately 40 L of mixed wastewater was pumped into the T-IVCW at an HLR of $33 \text{ cm}\cdot\text{d}^{-1}$. The daily operation schedule of the T-IVCW is shown in Table S2.

The characteristics of the raw sewage, influent, and applied nitrogen loading rate (NLR) of the two systems are summarized in Table 1. Prior to the start of the formal experiment, the T-IVCW was operated for almost 10 months with eight mixing ratios from 1:9 (the volume ratio of raw sewage to SWIS effluent) to 8:2 to determine the suitable mixing ratios. The pre-experiment also minimized the effect of substrate adsorption and developed suitable microbial communities in the system. The experiments were divided into 3 phases, with mixing ratios of 3:7, 5:5, and 7:3 respectively. The duration of each phase was nearly 75 d, and the experiments were conducted from March to October in 2016. In

Table 1

Water quality parameters of the influent and effluent of the SWIS and T-IVCW during the experimental period.

Parameters	SWIS			T-IVCW (Phase 1)			T-IVCW (Phase 2)			T-IVCW (Phase 3)		
	Influent (Raw sewage)	Effluent	RE ^a (%)	Influent (3:7) ^b	Effluent	RE (%)	Influent (5:5)	Effluent	RE (%)	Influent (7:3)	Effluent	RE (%)
pH	7.51 (0.12) ^c	3.94 (0.36)	–	6.63 (0.34)	6.59 (0.43)	–	7.01 (0.12)	6.70 (0.15)	–	7.11 (0.08)	6.81 (0.12)	–
DO ($\text{mg}\cdot\text{L}^{-1}$)	0.48 (0.23)	6.89 (0.83)	–	5.58 (1.37)	6.03 (1.43)	–	2.91 (0.66)	4.47 (0.62)	–	1.07 (0.86)	4.93 (0.82)	–
$\text{NH}_4^+\text{-N}$ ($\text{mg}\cdot\text{L}^{-1}$)	30.78 (4.81)	0.63 (0.33)	97.94	8.93 (3.12)	1.19 (0.62)	86.17	15.32 (1.48)	2.19 (0.37)	85.51	24.40 (4.37)	3.66 (1.07)	85.08
$\text{NO}_3^-\text{-N}$ ($\text{mg}\cdot\text{L}^{-1}$)	0.55 (0.29)	25.94 (4.10)	–	16.10 (4.55)	15.42 (3.33)	0.99	12.91 (1.43)	11.13 (1.42)	13.85	8.76 (2.11)	2.19 (1.12)	75.34
TN ($\text{mg}\cdot\text{L}^{-1}$)	34.23 (5.00)	28.84 (4.63)	15.90	25.73 (5.24)	18.03 (3.92)	30.07	28.70 (2.22)	14.03 (1.60)	51.10	34.63 (4.34)	6.50 (1.46)	81.18
COD ($\text{mg}\cdot\text{L}^{-1}$)	90.68 (12.98)	12.64 (2.90)	85.98	43.47 (5.65)	8.70 (2.20)	80.13	55.09 (3.30)	12.32 (1.39)	77.60	80.67 (5.32)	23.14 (2.43)	71.37
COD/TN	2.65	0.44	–	1.69	0.48	–	1.92	0.88	–	2.35	3.56	–
CLR ^d ($\text{g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$)	36.27	–	–	14.34	–	–	18.18	–	–	26.62	–	–
ALR ^e ($\text{g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$)	12.31	–	–	2.95	–	–	5.05	–	–	8.05	–	–
NLR ^f ($\text{g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$)	13.69	–	–	8.49	–	–	9.47	–	–	11.43	–	–
CRR ^g ($\text{g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$)	–	31.22	–	–	11.47	–	–	14.11	–	–	19.16	–
ARR ^h ($\text{g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$)	–	12.06	–	–	2.55	–	–	4.33	–	–	6.84	–
NRR ⁱ ($\text{g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$)	–	2.15	–	–	2.54	–	–	4.84	–	–	9.28	–

^a Removal efficiency.

^b The mixing ratio of raw sewage to SWIS effluent.

^c The values are expressed as average (standard deviation).

^d COD loading rate.

^e $\text{NH}_4^+\text{-N}$ loading rate.

^f Total nitrogen loading rate.

^g COD removal rate.

^h $\text{NH}_4^+\text{-N}$ removal rate.

ⁱ Total nitrogen removal rate.

order to compare with previous studies, the mixing ratios of 3:7 (raw sewage: secondary effluent), 5:5, and 7:3 in this study could also be expressed as step-feeding ratios of 42.9%, 100.0%, and 233.3%, respectively.

Several studies indicated that endogenous organic carbon supply from plant biomass decay and root-zone exudation are insufficient to achieve full denitrification [10,27]. Plant root exudates might potentially fuel a denitrification rate of 0.026–0.073 $\text{g}\cdot\text{N}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ in sub-surface flow CWs [28], which is far below the NLR ($> 8 \text{ g}\cdot\text{N}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$) of this study. Therefore, plants were not involved in this study because of the short HRT and high NLR. More importantly, unplanted design can eliminate the influence of plants in different growth stages on nitrogen removal.

2.2. Samples and analytical methods

Once the mixing ratios were adjusted, the T-IVCW was acclimated for 2 weeks before sampling. Water samples were collected at regular intervals from the inlets and outlets of the aerobic SWIS and T-IVCW, respectively. The wastewater quality parameters, including COD, $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, and $\text{NO}_2^-\text{-N}$, were analyzed according to the Chinese standard methods [29]. TN was analyzed using a TOC/TN analyzer (TOC-V_{CPH}, Shimadzu). DO and pH were measured in situ using a portable multi-parameter meter (Orion 5-Star, Thermo Scientific).

To investigate the influence of the mixing ratios on the microbial community structure, substrate samples were collected from different layers (D10, D30, D50, U50, U30, and U10) at the end of each phase (Fig. 1). During each sampling, the pre-buried columns were taken out from the CW bed when the tidal zone was drained. The substrate samples obtained from phase 3 were also used to study the distribution of functional microbes in the T-IVCW. Furthermore, substrate samples

in the SWIS were also collected from layers S20, S40, and S70. The substrate samples were mixed well and stored at -20°C until further analysis.

2.3. Microbial analysis

2.3.1. DNA extraction

A PowerSoil DNA isolation kit (MoBio Laboratories, USA) was applied to extract DNA from the collected substrates according to the manufacturer's instructions and a previous study [30]. Then, DNA concentration and purity were measured using a UV spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific, USA).

2.3.2. High-throughput sequencing

The V3–V4 regions of the bacterial 16S rRNA gene were amplified by universal primers 338F (5'-ACTCTACGGGAGGAGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3'), and the detailed amplification procedures can be found in Liu et al. [31]. Illumina MiSeq sequencing was conducted by Majorbio (Shanghai, China) to analyze the structure of the microbial community. High-quality sequences were clustered into operational taxonomic units (OTUs) at 97% similarity using the MOTHUR program. Raw sequencing data obtained from this study were deposited into the NCBI Sequence Read Archive database with accession number SRP199027.

2.3.3. Quantitative polymerase chain reaction

Quantitative analysis of the AMX bacteria (AMX bacterial 16S rRNA) was conducted, and fragments of the following functional genes were targeted: ammonia monooxygenase (*amoA*), nitrite oxidoreductase (*nxrA*), and *cd1*-containing nitrite reductase (*nirS*). The primer sequences (Table S3) and PCR programs were cited from Hu et al. [17].

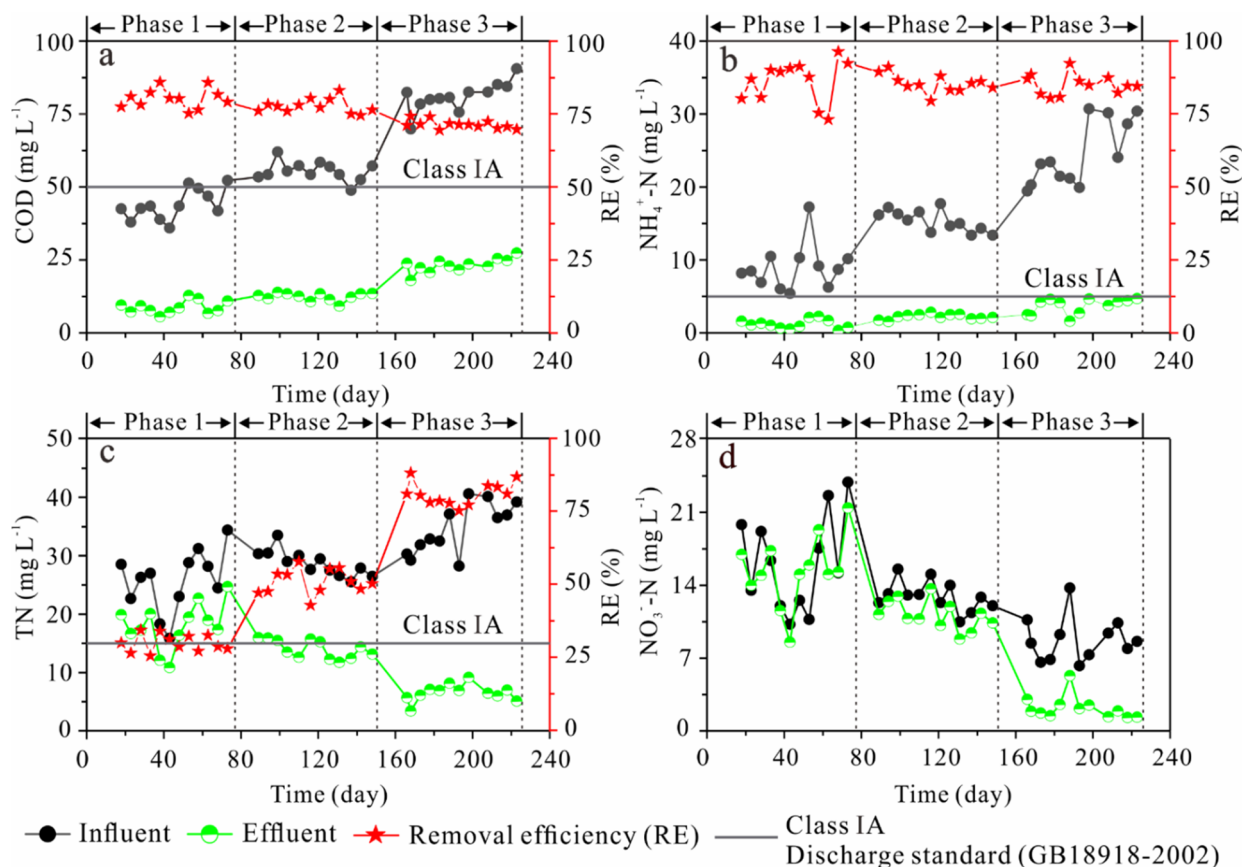


Fig. 2. Pollutants removal performance of the T-IVCW: (a) COD concentrations and removal efficiencies; (b) $\text{NH}_4^+\text{-N}$ concentrations and removal efficiencies; (c) TN concentrations and removal efficiencies; (d) $\text{NO}_3^-\text{-N}$ concentrations.

2.4. Statistical analysis

Analyses of the bacterial community data were conducted using the Majorbio cloud platform (<https://cloud.majorbio.com/>). Moreover, principal component analysis of MiSeq data (relative OTU distribution matrix) was conducted using XLSTAT 2016 software (Addinsoft, France). One-way analysis of variance was performed using SPSS software (version 18.0) to compare the differences between various samples, and the statistically significant level was set at $p < 0.05$.

3. Results and discussion

3.1. Overall performance of the SWIS

The dynamics of the influent and effluent parameters from the SWIS during the experiment are shown in Fig. S1, and the calculated removal efficiencies are listed in Table 1. The average influent concentrations of COD, $\text{NH}_4^+\text{-N}$, and TN were 90.68, 30.78, and 34.23 $\text{mg}\cdot\text{L}^{-1}$, respectively, with temporal fluctuations. Meanwhile, stable removal efficiencies of 85.98% and 97.94% for COD and $\text{NH}_4^+\text{-N}$ were achieved, respectively, which was in accordance with other SWISs [3,32]. However, most of the influent $\text{NH}_4^+\text{-N}$ was converted to $\text{NO}_3^-\text{-N}$ (25.94 $\text{mg}\cdot\text{L}^{-1}$), thereby resulting in a low removal efficiency of TN (15.26%) and low effluent pH (3.94) in the SWIS. Two protons will be generated when an NH_3 molecule is oxidized, thereby resulting in a substantial decrease in pH if the buffer capacity is low [33]. The elevated $\text{NO}_3^-\text{-N}$ effluent concentrations indicated that nitrification was favored within the SWIS while denitrification was limited. This was likely a result of the aerobic conditions and carbon source deficiency [3], which made the SWIS an ideal aerobic unit.

3.2. Overall performance of the T-IVCW

As shown in Table 1, the aqueous pH returned to near neutral after mixing with raw sewage, although the pH of the SWIS effluent was very low (approximately 4.0), which could have been attributed to the strong pH buffering capacity of the raw sewage. Therefore, using raw sewage as a carbon source can not only increase the C/N ratio of the aerobic unit effluent, but also adjust its pH, thereby facilitating subsequent nitrogen removal. The pH in the T-IVCW effluent was slightly lower than that in the influent, while both were enhanced with the mixing ratio increase.

When the influent COD increased from 43.47 $\text{mg}\cdot\text{L}^{-1}$ (phase 1) to 80.67 $\text{mg}\cdot\text{L}^{-1}$ (phase 3), the corresponding COD removal rate (CRR) also increased from 11.47 $\text{g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ to 19.16 $\text{g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$, though the COD removal efficiency declined from 80.13% to 71.37%. Comparably, the T-IVCW achieved a much lower CRR than that of TFCWs ($> 100 \text{ g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$) [15,34] and an IVCW (44.3 $\text{g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$) [19] owing to the different COD loading rates.

Similarly, the influent $\text{NH}_4^+\text{-N}$ sharply increased from 8.93 $\text{mg}\cdot\text{L}^{-1}$ (phase 1) to 24.40 $\text{mg}\cdot\text{L}^{-1}$ (phase 3). The T-IVCW achieved stable $\text{NH}_4^+\text{-N}$ removal efficiencies (approximately 85%) during the three phases, with 1.19, 2.19, and 3.66 $\text{mg}\cdot\text{L}^{-1}$ of $\text{NH}_4^+\text{-N}$ in the effluent (Table 1), which complied with Chinese Class I(A) of the Wastewater Discharge Standard (GB18918-2002) (Fig. 2b). The $\text{NH}_4^+\text{-N}$ removal rates (ARRs) in the T-IVCW were 2.55, 4.33, and 6.84 $\text{g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$, thereby showing an increasing trend among the three phases. The ARR in phase 3 was significantly higher than those of IVCWs ($< 0.7 \text{ g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$) and various types of hybrid CWs (2.1–2.5 $\text{g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$) [10,17,19,35], because the tidal flow of the upper part in the T-IVCW increased the oxygen supply as evidenced by the high DO content in the effluent (Table 1). However, the ARR in the T-

Table 2

Nitrogen removal performance in various constructed wetlands with different enhanced measures.

System ^a	Wastewater type	Carbon source	Enhanced measure	Influent C/N ratio	HRT/HLR	CRR ^b	NRR ^c	Removal efficiency (%)			Reference
					$\text{h}/\text{m}\cdot\text{d}^{-1}$			$(\text{g}\cdot\text{m}^{-2}\cdot\text{d}^{-1})$	COD	$\text{NH}_4^+\text{-N}$	
T-IVCW	domestic	raw sewage	zeolite	2.35	10/0.33	19.16	9.28	71.37	85.08	81.18	This study
IVCW	synthetic	–	–	0.97	144/0.05 ^d	–	0.67 ^d	–	92.5	87.5	[17]
	synthetic	glucose	–	4 ^e	–/0.03	2.28 ^{d,f}	0.54 ^d	$> 95^g$	> 90	~ 94	[18]
	synthetic	sucrose	–	9.41	36/0.25	44.3	1.27	59.9	< 0	15	[19]
Multi-stage IVCW	domestic	raw sewage	rice husk	2.35	120/0.10	12.6 ^d	6.79 ^d	79	99	99.7	[20]
	synthetic	sodium acetate	alum sludge + woodchips	0.93	48/0.152 ^d	17.45 ^d	16.47 ^d	80.3	~ 56	74.42	[40]
VFCW	domestic	raw sewage	partially saturated	5.22 ^d	24 ^d /0.13	27.55	4.91	66.04	69.23	56.33	[21]
	synthetic	sucrose	wheat straw + intermittent aeration	5.24	72/0.05 ^d	10.88	1.39	92.71	98.87	63.02	[27]
	synthetic	glucose	intermittent aeration	9.39 ^d	72/0.07	34.46	3.64	94.71	99.11	93.91	[38]
	domestic	raw sewage	recirculation + intermittent aeration	6	6/0.179	67	8.6	91.6 ^d	79 ^d	75 ^d	[39]
	dairy	diluted dairy	slag + recirculation	–	168/0.025 ^d	–	12.1 ^h	–	80.1 ^d	75.7 ^d	[41]
TFCW	piggery	diluted piggery	multistage + step-feeding (25%)	7.31	57/0.29	$> 112^d$	15.85	> 80	96	83	[34]
	synthetic	glucose	–	12	24/0.33 ^d	105.7 ^d	8.12 ^d	95	80	82	[12]
	domestic	raw sewage	step-feeding (50%)	6.87	7.5/0.64	185.6 ^d	22.36 ^d	96.85	89.95	80.20	[15]
	domestic	raw sewage	biochar	14.19	4/0.47	580.1 ^d	35.07 ^d	96.2	89.8	82.5	[13]
	synthetic	glucose	active alumina	4.57	4/1.35	285 ^d	53.46 ^d	85.9	86.1	72.8	[36]
Hybrid CW	domestic	raw sewage	step-feeding (33.3%)	7.11 ^d	–/0.071	25.33 ^d	2.38 ^d	88.1	66.7 ^d	58.7	[10]
	domestic	raw sewage	step-feeding (35.6%)	7.11 ^d	–/0.126	43.5 ^d	3.20 ^d	85.2	26.9 ^d	44.6	[10]

^a IVCW: integrated vertical-flow CW; multi-stage IVCW: baffled subsurface-flow CW; VFCW: vertical flow CW; TFCW: tidal flow CW; Hybrid CW: hybrid vertical flow (VF) + horizontal flow (HF) CW.

^b COD remove rate.

^c Total nitrogen removal rate.

^d calculated according to the data in the articles.

^e TOC/TN.

^f TOC removal rate.

^g TOC removal efficiency.

^h Total inorganic nitrogen removal rate.

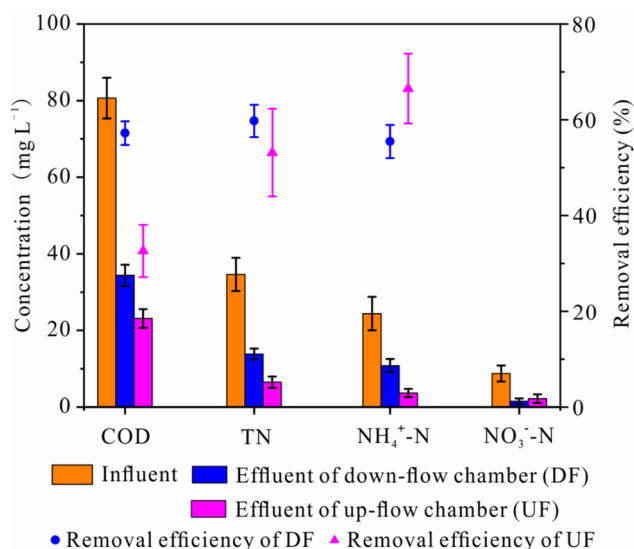


Fig. 3. Mean concentrations of COD, NH_4^+ -N, NO_3^- -N, and TN in the T-IVCW along the water flow direction and the removal efficiencies of the two chambers. The effluent of up-flow chamber was the final effluent of the T-IVCW.

IVCW were much lower than those in TFCWs [15,36] owing to the shallow depth of the tidal zone (16 cm) and the low influent NH_4^+ -N concentrations.

Meanwhile, the mixing ratio significantly affected the TN removal efficiency of the T-IVCWs (Fig. 2c). In phase 1, the average TN concentrations in the influent and effluent were $25.73 \text{ mg}\cdot\text{L}^{-1}$ and $18.03 \text{ mg}\cdot\text{L}^{-1}$, respectively, with a removal efficiency of 30.07% (Table 1). In phase 2, a higher TN removal efficiency (51.1%) was achieved with a lower effluent TN concentration ($14.03 \text{ mg}\cdot\text{L}^{-1}$). In these two phases, NO_3^- -N was dominant in the effluent while NH_4^+ -N could be neglected (Fig. S2), thereby suggesting that denitrification was limited owing to a lack of sufficient organic carbon (influent C/N ratios of 1.69 and 1.92, respectively). In phase 3, the T-IVCW achieved effective TN elimination (75.51%–88.32%) with 3.41 – $9.16 \text{ mg}\cdot\text{L}^{-1}$ of TN in the effluent, which was much lower than the limit value ($15.0 \text{ mg}\cdot\text{L}^{-1}$) of the Chinese Class I(A) standard (GB18918–2002). However, the C/N ratio of 2.35 in the T-IVCW clearly could not meet the theoretical requirement ($2.85 \text{ mg COD}\cdot\text{mg}^{-1} \text{ NO}_3^-$ -N) for denitrification to reduce NO_3^- -N to N_2 without assimilation [5]. Actually, the consumed C/N ratio was only 2.0 in phase 3, which was lower than that in phase 1 (4.6) and phase 2 (2.9) (Fig. S2). These results suggested that alternative nitrogen removal pathways, such as autotrophic denitrification and/or AMX, might have occurred in the T-IVCW.

Previous studies have demonstrated that nitrogen removal in CWs is often restricted by insufficient oxygen supply and/or a lack of organic carbon [12]. Vymazal [37] reported that the TN removal efficiencies in conventional CWs vary between 40% and 55% with total nitrogen removal rate (NRR) ranging from $0.68 \text{ g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ to $1.73 \text{ g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ depending on the CW type and inflow loading. As summarized in Table 2, some intensification methods have been used to enhance nitrogen removal, such as intermittent aeration, step-feeding, recirculation, and substrate optimization [10,13,20,27,38–41]. Intermittent aeration employed in vertical flow CWs is more effective at the removal of NH_4^+ -N (> 95%) than traditional CWs [27,38]. In addition, with an influent C/N ratio of 9.39, up to 93.91% of TN has been removed with a corresponding NRR of $3.64 \text{ g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ [38]. Moreover, a relatively short HRT and high HLR were helpful to further achieve a much higher NRR ($8.6 \text{ g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$) in a vertical flow CW with intermittent aeration and recirculation [39]. Step-feeding is another common method to improve TN removal in CWs by regulating the C/N ratio [34]. Wang et al. [15] reported an additional TN removal of up to 75% with the

application of step-feeding of 50% in a TFCW.

In this study, the T-IVCW was developed by converting the upper part of the IVCW into a tidal flow pattern and operated in short cycles (eight cycles per day). As shown in Table 2, the T-IVCW achieved much higher HLR and NRR, and shorter HRT than the IVCWs [17–19]. In comparison, solid sustained-release carbon sources (rice husk and woodchips) employed in the multi-stage IVCWs played a major role in the removal of nitrate [20,40]. Furthermore, the planted multi-stage IVCW yielded a much high NH_4^+ -N removal efficiency (99%) compared to the unplanted unit (49%), which could be ascribed to the radial oxygen loss (ROL) during the long HRT [20]. However, the ROL of aquatic plants in most CWs is $< 5.0 \text{ g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ [42]. Theoretically, one-liter air (containing about 280 mg oxygen at 20°C) will be drawn into the T-IVCW once one-liter sewage was drained due to the tidal operation strategy. Therefore, the oxygen supply of T-IVCW showed a positive correlation with HLR and was $92.4 \text{ g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ at the HLR of $33 \text{ cm}\cdot\text{d}^{-1}$, which was much higher than that of the IVCWs and multi-stage IVCWs. Similarly, because of the high oxygen supply and HLR, the TFCWs achieved much higher CRR (105.7 – $580.1 \text{ g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$) and NRR (8.12 – $53.46 \text{ g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$) than other types of CW (Table 2). By contrast, the T-IVCW achieved comparative TN removal efficiency and NRR under a much lower influent C/N ratio, demonstrating its effectiveness in treating low C/N ratio wastewater. Moreover, the T-IVCW had great potential to achieve higher HLR and NRR by increasing the thickness of the upper tidal zone and substrate optimization.

3.3. Removal performance of the down-flow chamber and up-flow chamber

The removal efficiencies and effluent concentrations of the DF and UF are presented in Fig. 3. The mean concentrations of COD, TN, and NH_4^+ -N of the DF effluent were 34.38 , 13.85 , and $10.81 \text{ mg}\cdot\text{L}^{-1}$, respectively. The low content of NO_3^- -N (0.95 – $3.62 \text{ mg}\cdot\text{L}^{-1}$) in the DF effluent suggested that the influent NO_3^- -N and nitrified NH_3 were mostly removed within this chamber. In addition, over 55% of COD, TN, and NH_4^+ -N were removed in the DF, while only 14.01%, 21.42%, and 29.61% of the corresponding pollutants were removed in the UF. This was in accordance with a previous study that > 80% of NH_4^+ -N was removed in the first half of a planted baffled subsurface-flow CW [20].

The surface area ratio of the DF to UF was 2 in the T-IVCW, which meant that 66.67% of the sewage entering the system in every cycle remained in the tidal zone of the DF, and the rest remained in the saturated zone (Fig. S3). In contrast, approximately 66.67% of the effluent remained in the saturated zone of the UF before draining. As a result, the NH_4^+ -N removal quantity of the UF was lower than that of the DF, but the UF also contributed to the effluent meeting the NH_4^+ -N discharge standard ($< 5 \text{ mg}\cdot\text{L}^{-1}$) (Fig. 3). The coexistence of aerobic and anoxic/anaerobic conditions within partially saturated vertical flow CWs can enhance TN removal efficiencies [21]. Within the T-IVCW, the influent would partly undergo alternating environments in both the DF and UF, including anoxic–oxic conditions in tidal zones and permanently anoxic conditions in saturated zones, thereby contributing to a high nitrogen removal efficiency.

3.4. Nitrogen removal pathways

Many processes could be involved in nitrogen removal within CWs, such as nitrification–denitrification, AMX, NH_3 volatilization, chemical adsorption, and plant uptake [37]. Plant uptake is often considered minor for nitrogen removal in CWs [43], especially under a high load. For example, plant uptake only contributed to approximately 0.10% and 0.77% of TN removal for an IVCW and TFCW [44,45]. In addition to uptake, the plants can also promote nitrogen removal by enhancing microbial activity in CWs [46]. In this study, plants were not involved in order to eliminate the influence of plants in different growth stages on nitrogen removal. Ammonia volatilization was negligible owing to

the lower concentration of NH_3 and neutral pH in the influent of the T-IVCW. Du et al. [44] discovered that the average proportion of nitrogen removed by substrate adsorption decreased from 36.17% to 6.53% with the operation time. Substrate adsorption hardly contributes to nitrogen removal when it is saturated, unless the adsorption capacity is regenerated via microbial oxidization [14]. The contribution of substrate adsorption to nitrogen removal in the T-IVCW was also negligible because it was already saturated during the 10 months pre-experiment. Thus, biological nitrogen removal (nitrification–denitrification, AMX, etc.) should play a pivotal role in nitrogen removal in the T-IVCW, and high-throughput sequencing and qPCR analysis were carried out to gain insight in the bacteria and functional genes responsible for nitrogen removal.

3.4.1. Microbial diversity and structure

High-quality sequences of 789049 were obtained for the 18 substrate samples collected from the three phases. As shown in Table S4,

the number of OTUs ranged from 1335 to 2236, and the Good's coverage value ranged from 97.32% to 99.17%, indicating that all samples were sequenced at high depth. The higher bacterial richness and diversity (ACE, Chao1, and Shannon) in phase 3 suggests that an increment of raw sewage proportion in influent could provide more nutrients and thus stimulate microbial activity under appropriate conditions. Peng et al. [47] demonstrated that both the novel sequences and the bacterial diversity decreased along the rotating biological contactor (RBC) flowpaths as the pollutant concentrations gradually decreased. However, the bacterial richness and diversity showed no downward trend along the flowpaths in T-IVCW, which was similar to the previous study in a partially saturated TFCW [45].

The phyla with a relative abundance > 2% in at least one sample are shown in Fig. S4. The most abundant phylum was Proteobacteria (31.15%), followed by Chloroflexi (16.40%), Planctomycetes (6.56%), Acidobacteria (6.31%), Actinobacteria (6.13%), Bacteroidetes (5.33%), and Nitrospirae (3.93%). The relative abundance of Proteobacteria

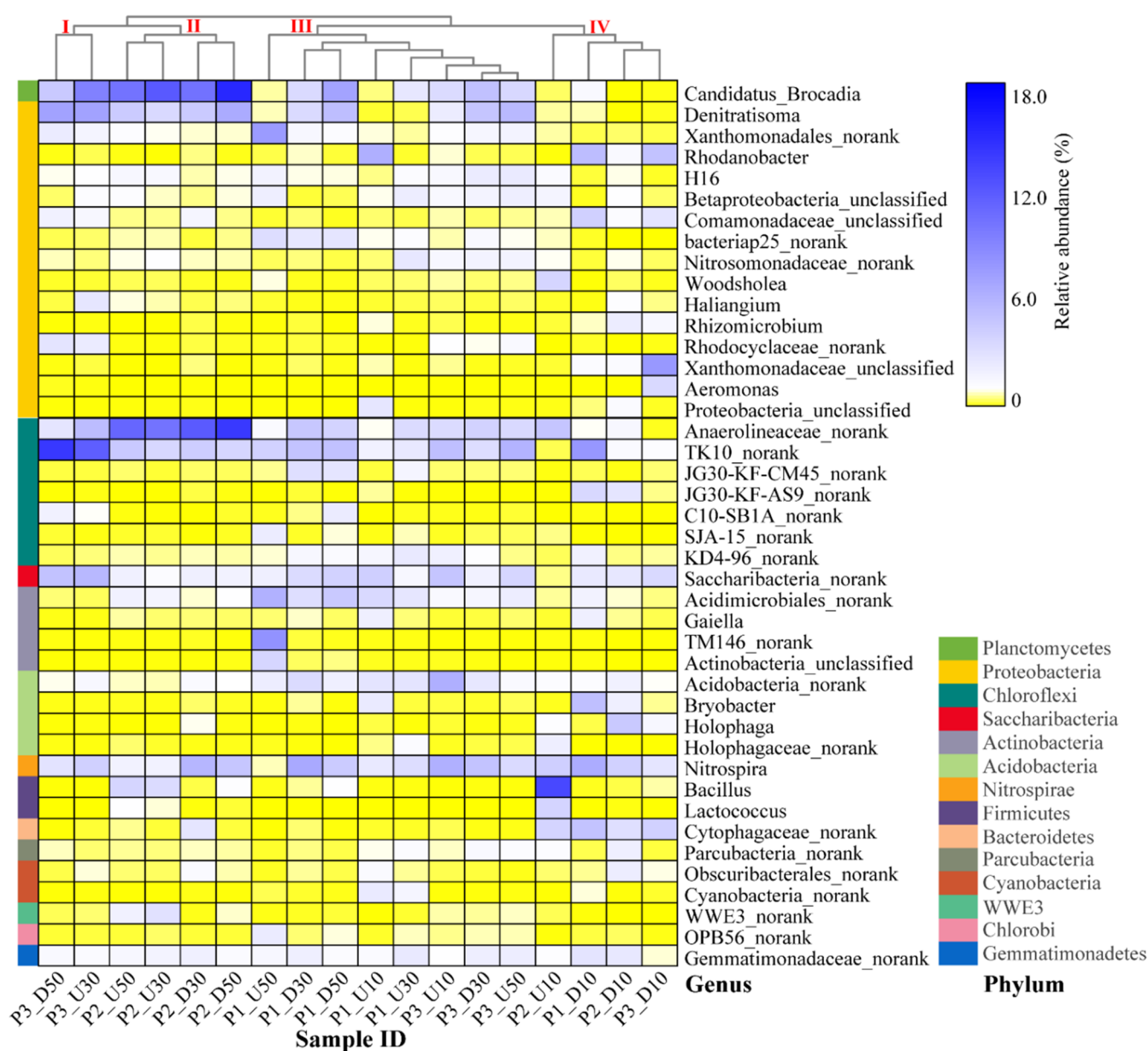


Fig. 4. Heat map depicting the relative abundance of phylotypes at genus level (Genera with relative abundance > 2% at least in one sample). The color bar indicates the range of percentage of a genus in a sample, based on the color key at the top right. Hierarchical cluster analysis grouping samples is shown at the top. The color bar in the left heat map indicates the phylum containing some genera.

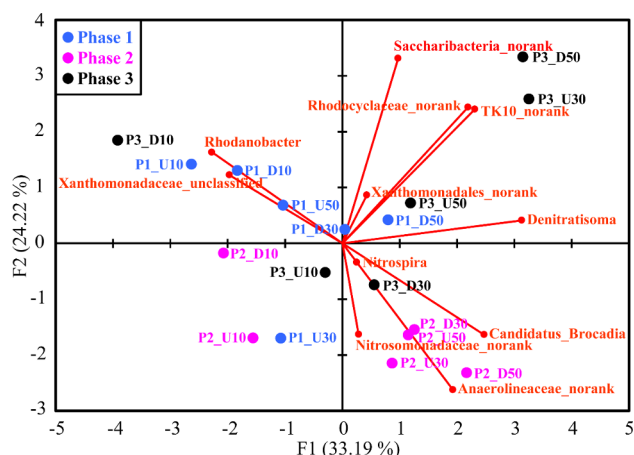


Fig. 5. Principal component analysis of the main genera related nitrogen removal with the sample locations in the DF and UF in three phases.

accounted for 27.19%, 28.30%, and 37.95% in phases 1, 2, and 3, respectively, indicating an increasing trend with the increase in the proportion of raw sewage in the influent. However, the abundances of Planctomycetes (10.13%) in phase 2 and Actinobacteria (11.49%) in phase 1 were much higher than those in the other phases. Proteobacteria is usually the most abundant phylum in CWs [36,45], and has different metabolic activities involved in nitrogen and carbon cycling [48]. The abundance of Planctomycetes was higher in the saturated zone (averaging 8.54%) than that in the tidal zone (averaging 2.60%); this might be partly explained by the aerobic and anoxic conditions in the two zones. Planctomycetes are known for containing unique AMX bacteria, which are fastidious chemoautotrophic bacteria that are specialized in the oxidation of NH_4^+ to N_2 using NO_2^- as the electron acceptor under anoxic conditions [49].

To further elucidate the functional populations involved in nitrogen removal, a heat map generated with major genera is shown in Fig. 4. A total of 42 genera belonging to 14 phyla were selected, and high average abundances of *Candidatus Brocadia* (5.01%), *Anaerolineaceae norank* (4.91%), TK10 norank (4.78%), *Nitrospira* (3.93%), and *Denitratisoma* (3.19%) were found. The abundance of common ammonia-oxidizing bacteria (AOB) of *Nitrosomonadaceae norank* (0.91%) was noticeably lower than the abundance of nitrite-oxidizing bacteria (NOB) of *Nitrospira*, thereby implying that PN could hardly be achieved in the T-IVCW. Furthermore, as a potential AOB, *unclassified Xanthomonadaceae* had a higher relative abundance in the tidal zone (1.24%, 1.27%, and 7.85% in P1_D10, P2_D10, and P3_D10, respectively), and might have participated in NH_4^+ oxidation either as a heterotrophic nitrifier or via autotrophic nitrification through uncharacterized pathways [50].

As the most abundant genus and sole AMX bacteria that was detected in the T-IVCW, *Candidatus Brocadia* was mainly enriched in the saturated zone, and accounted for 3.44%, 12.01%, and 5.65% in phases 1, 2, and 3, respectively, while it was less favored (average value below 1.00%) in the tidal zone (Fig. 4 and Table S5). These results suggested that a TFCW with short F/D cycles might create a more aerobic environment, thereby suppressing the metabolism of AMX bacteria, which are obligate anaerobes whose metabolism is reversibly inhibited above 2 μM of oxygen [51]. Obviously, the relative abundance of AMX bacteria in the T-IVCW was significantly higher than that in partially saturated VFCWs [21,45], TFCWs [36], and PDN/AMX reactors [52].

For the denitrifying groups, *Denitratisoma* accounted for 1.78%, 3.27%, and 4.53% in phases 1, 2, and 3, respectively, and was mainly detected in the saturated zone. Meanwhile, *Rhodanobacter* was identified in greater abundance with proportions of 5.36%, 1.37%, and 5.01% in P1_D10, P2_D10, and P3_D10, respectively. *Rhodanobacter* is a facultative anaerobe that is capable of complete denitrification with an

optimal pH of 6.5 [53]. A high abundance of *Rhodanobacter* was also detected in a rotating biological contactor [47] and TFCW [45], which were not permanently anoxic systems. Moreover, the microbial diversity of the aerobic unit (SWIS) substrates in this study showed that the abundance of *Rhodanobacter* increased along the SWIS flowpaths and reached the highest abundance of 16.88% in the bottom substrate (Table S6), which had a much lower pH (approximately 4) than that of the upper substrate [3]. This observation was consistent with a previous study that the abundance of *Rhodanobacter* is positively correlated with lower pH conditions [54]. Therefore, permanently anoxic conditions might not be suitable for the growth of *Rhodanobacter*, while an anoxic and aerobic alternating environment with low pH would be conducive to its bloom.

3.4.2. Multivariate statistical analysis

Cluster analysis was applied to the 18 substrate samples to measure consistencies among various bacterial communities and to group similar substrate samples at the genus level. As shown in Fig. 4, the 18 substrate samples could be clustered into four groups based on bacterial communities. Clusters 1 and 2 had a higher relative abundance of anaerobic bacteria (e.g., *Candidatus Brocadia*, *Anaerolineaceae norank*, and *Denitratisoma*) and contained substrate samples mainly collected from the saturated zones in phases 2 and 3, while cluster 4 was mainly composed of *Rhodanobacter*, *Cytophagaceae norank*, and *unclassified Xanthomonadaceae*, containing substrate samples collected from the tidal zones. The other substrate samples all belonged to cluster 3.

Principal component analysis based on the relative abundance of the 11 genera also clearly showed variations among the sampling positions (Fig. 5). The first component (F1) demonstrated that the saturated zone biofilm samples were clustered distantly from the tidal zone biofilm samples owing to the high predominance of *Candidatus Brocadia*, *Anaerolineaceae norank*, and *Denitratisoma*. However, in the case of the second component (F2), the separation was not clear. Taken together, these results indicated that mixing ratios and vertical variation of local redox conditions are important in shaping the community composition and abundance of bacteria.

3.4.3. Functional genes associated with nitrogen transformation

The abundances of AMX 16S rRNA and functional genes (*amoA*, *nrxA*, and *nirS*), which were involved in nitrogen metabolism, were quantified by qPCR in phase 3. As shown in Fig. 6, *amoA* and *nrxA* were enriched in the tidal zones (D10 and U10) of the T-IVCW. Furthermore, similar to the results of high-quality sequences, the absolute abundance of *nrxA* was nearly 5 times higher than that of *amoA*, which also indicated that PN could hardly be achieved in the T-IVCW. In contrast, *nirS* and AMX 16S rRNA were predominantly enriched in the saturated zones (D30, D50, and U30) with average abundances of 2.87×10^7 copies- g^{-1} and 2.61×10^7 copies- g^{-1} , respectively.

As an important step in denitrification, *nirS* is involved in the reduction of NO_2^- to NO (nitric oxide). Similarly, once the inorganic nitrogen compounds are transported into the AMX cell, nitrite is assumed to be converted into NO by *nirS* [55], then NO is involved in the production of hydrazine as an intermediate of AMX metabolism [51,56]. Furthermore, the AMX bacterium *Candidatus Kuenenia stuttgartiensis* is able to grow by coupling NO reduction to NH_4^+ oxidation in the absence of NO_2^- [57]. However, the apparent lack of a *nirS* gene in the *Candidatus Brocadia* assemblies suggested that *Candidatus Brocadia* might employ an unidentified nitrite reductase for the production of NO in the AMX process [55]. The above researches are carried out in the activated sludge systems, e.g. sequencing batch reactors and membrane bioreactors, while no relative study to date has been performed in CWs. Therefore, whether the high abundance of *nirS* in the saturated zone provide NO for AMX process should be further investigated in the future.

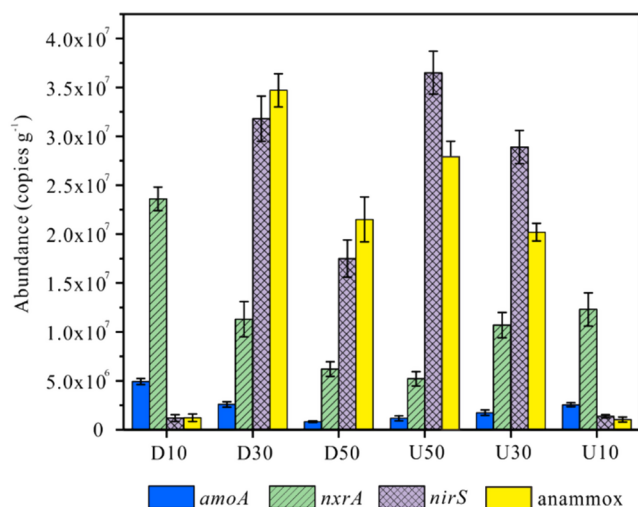


Fig. 6. Absolute abundance of nitrogen transformation functional genes in the substrate samples collected from the T-IVCW in phase 3.

3.4.4. Proposed microbial nitrogen removal pathways

Achieving a stable and high rate AMX process in CWs is still a challenge owing to the difficulty of selectively inhibiting NOB [58]. DO is the key parameter for PN control in domestic sewage, and the values should be controlled at a low level of $< 0.5 \text{ mg}\cdot\text{L}^{-1}$ [26]. However, it is difficult to regulate DO for most CWs (e.g., VFCW and TFCW) because aeration is usually not employed. In sequencing batch reactors, PDN can facilitate stable NO_2^- -N accumulation in appropriate COD/ NO_3^- -N ratios without a complex controlling strategy [52], and a high nitrogen removal efficiency via PDN/AMX has been achieved in different activated sludge reactors [25,26]. Although NO_2^- -N accumulation was not observed in the T-IVCW with low C/N ratios, it is likely that the production of NO_2^- -N via PDN and the consumption of NO_2^- -N via AMX occurred simultaneously in the anoxic condition. Similar result was also observed in denitrifying bioreactors with different media [59]. Moreover, in the presence of NH_4^+ and acetate, AMX bacteria had a higher affinity toward NO_2^- than the denitrifiers, thereby resulting in a strong selective advantage [51], which further reinforced the synergy between PDN and AMX.

The unique structure of the T-IVCW was crucial for the appropriate substrates (NH_4^+ -N, COD, and NO_3^- -N) to enter the saturated zone where PDN/AMX occurred. The tidal zone at the upper layer developed an aerobic-dominated condition, which stimulated the nitrification process, while the saturated zone at the lower layer created an anoxic/anaerobic condition for denitrification and the AMX process. More importantly, sewage just entering the T-IVCW could be divided into two

parts, namely one part retained in the tidal zone and the other in the saturated zone, which could be regulated by the surface area ratio of the DF to UF (Fig. S3). As a result, NO_2^- that produced by PDN and the introduced NH_4^+ in the saturated zone provides substrates for AMX bacteria, thereby promoting the PDN/AMX process.

On the other hand, the influent mixing ratios also played an important role in the successful implementation of PDN/AMX. For example, the influent NH_4^+ -N concentration was only $8.93 \text{ mg}\cdot\text{L}^{-1}$ in phase 1, which resulted in low NH_4^+ -N in the saturated zone and was not conducive to the flourishing of AMX bacteria. It should be noted that the influent NH_4^+ and COD, rather than NO_3^- , are crucial to PDN/AMX in T-IVCW because NO_3^- can be produced in the tidal zone. Consequently, T-IVCW is not only an alternative strategy for treating secondary effluent, but also has the potential to treat domestic sewage directly. In contrast, as an anoxic unit, PDN/AMX reactors based on activated sludge cannot perform nitrification, and thus are not suitable for domestic sewage treatment alone [26].

Besides, the real domestic sewage employed in the present study might have contributed to biofilm formation of AMX bacteria owing to the continuous inoculation. According to Vymazal [60], the development of bacterial assemblages in systems treating real sewage is entirely different from that treating artificial wastewater. Furthermore, our recent investigations also showed that rapid start-up (within 50 d) of the AMX process in a saturated VFCW was achieved using the same real domestic sewage by adding an appropriate amount of NO_2^- -N but without external seeding sludge (unpublished data). Therefore, as long as the key substrate NO_2^- -N can be supplied stably, either via PDN or by direct addition, AMX bacteria can be easily cultured without inoculation and play a vital role in nitrogen removal in CWs.

Based on the results of microbial community and functional genes, and the aforementioned discussion, the possible nitrogen transformation and removal pathways in the T-IVCW were proposed. As shown in Fig. 7, aerobic ammonia oxidation mainly occurred in the tidal zone, while PDN/AMX and denitrification, which were the major nitrogen removal pathways, mainly occurred in the saturated zone. In addition, denitrification via *Rhodanobacter* in the tidal zone also contributed to nitrogen removal.

In order to calculate the relative contribution of PDN/AMX and denitrification, it was assumed that 100% or 80% of the COD removed in the T-IVCW was consumed by denitrification (including PDN). In the former assumption, the contribution of AMX to TN removal was severely underrated because a fraction of COD would always be removed by aerobic decomposition; the latter assumption might have over-estimated or underrated the contribution of AMX. In addition, the average values (Table 1) and the following stoichiometric relationships were used for the calculation [61]: (i) the molar ratio of NO_3^- -N/ NO_2^- -N in PDN is 1:1; (ii) the stoichiometric consumption (molar ratio) of NH_4^+ -N/ NO_2^- -N in the AMX process is 1:1.32 and produces

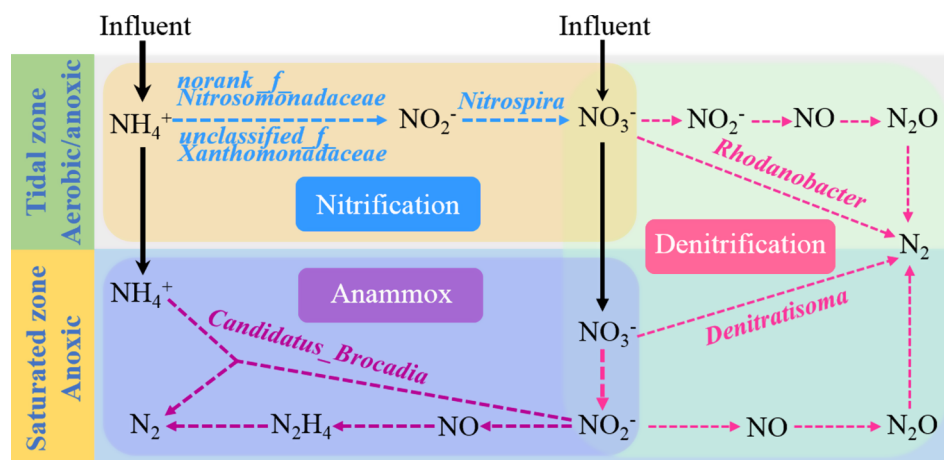


Fig. 7. Possible nitrogen transformation and removal pathways coupling nitrification, PDN/AMX, and denitrification in the T-IVCW.

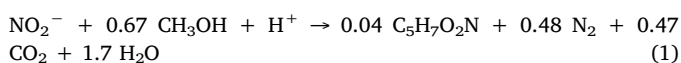
Table 3
The estimated contribution of PDN/AMX and denitrification to TN removal in three phases.

COD consumed by denitrification	Route	Nitrogen transformed (mg·L ⁻¹)			Contribution to TN removal (%)		
		Phase 1	Phase 2	Phase 3	Phase 1	Phase 2	Phase 3
100% ^a	PDN/AMX	0	4.14	14.45	0	27.77	52.91
	Denitrification	8.42	10.77	12.86	100	72.23	47.09
80% ^a	PDN/AMX	1.12	6.95	18.27	13.30	46.61	66.90
	Denitrification	7.30	7.96	9.04	86.70	53.39	33.10

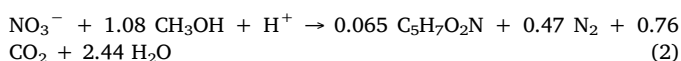
^a The assumed percentage that the removed COD in the T-IVCW is used for denitrification (including PDN).

0.26 mol NO₃⁻-N [62]; since NO₂⁻ is derived from the PDN of NO₃⁻, the molar ratio of NH₄⁺-N/NO₂⁻-N can be reduced to 1:1.06; and (iii) based on Eqs. (1) and (2) [63], 1.00 mg·L⁻¹ of NO₃⁻-N is used for consuming 3.705 mg·L⁻¹ of COD in complete denitrification (1.0 mg of methanol is equivalent to 1.5 mg of COD) and 1.00 mg·L⁻¹ of NO₃⁻-N is used for consuming 1.41 mg·L⁻¹ of COD in PDN.

NO₂⁻ removal:



NO₃⁻ removal:



The simulation results (Table 3) showed that from phase 1 to phase 3, the TN removal by PDN/AMX increased gradually in the two assumptions. Specifically, in phases 2 and 3, the contributions of PDN/AMX to TN removal were at least approximately 28% and 53%, respectively, while in phase 1, denitrification played the leading role in nitrogen removal.

Although simultaneous nitrification, AMX, and denitrification (SNAD) has been achieved in various CWs such as TFCW [12], IVCW [17], recirculating VFCW [41], it is still a big challenge to achieve high rate AMX in CWs when treating low C/N ratio domestic sewage. First, due to insufficient oxygen supply, permanently saturated CWs (e.g., IVCW, VFCW) failed to produce NO₂⁻ efficiently, thereby limiting the AMX process; Second, TFCW created alternating aerobic and anoxic conditions, which might not be conducive to the metabolism of AMX bacteria. For example, even a long flood/drain cycle (24 h/12 h) was adopted, aerobic ammonia oxidation still dominated the NH₄⁺-N removal when the influent C/N ratio was ≤ 6, while AMX was notably enhanced when the C/N ratio was higher than six [12], suggesting that TFCW was not suitable to the treatment of low C/N ratio sewage. In this study, T-IVCW achieved high rate AMX via PDN owing to the unique structure and operation strategy. When treating low C/N ratio domestic sewage in CWs, the PDN/AMX may be a more feasible and promising nitrogen removal pathway than PN/AMX and should be paid more attention to in future investigations.

4. Conclusion

In this study, a novel T-IVCW was developed to treat secondary effluent with low C/N ratio raw sewage as an electron donor at different mixing ratios. High NH₄⁺-N and TN removal rates (6.84 g·m⁻²·d⁻¹ and 9.28 g·m⁻²·d⁻¹) were obtained along with satisfactory effluent qualities (3.66 mg·L⁻¹ and 6.50 mg·L⁻¹) at a mixing ratio of 7:3. The aerobic and anoxic conditions of different zones dominated the microbial community composition. Specifically, *Cytophagaceae norank*, *Rhodanobacter*, and *unclassified_Xanthomonadaceae* were enriched in the tidal zone, whereas *Candidatus Brocadia* and *Denitratisoma* were mainly present in the saturated zone. A similar trend was observed for the corresponding functional genes (*amoA*, *nxrA*, *nirS*, and *anammox*). Both microbial community and molecular biological analysis confirmed that PDN/AMX and

denitrification were the main nitrogen removal pathways in T-IVCW. Moreover, PDN/AMX played an increasingly important role in nitrogen removal with the increase of NH₄⁺-N in the influent. The influent NH₄⁺ and COD, rather than NO₃⁻, are crucial to the successful implementation of PDN/AMX in T-IVCW. Thus, T-IVCW may be an alternative strategy for treating low C/N ratio sewage. Future studies are needed to investigate the influence factors of PDN/AMX and the metabolic pathways of AMX bacteria (*Candidatus Brocadia*) in T-IVCW.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the Fundamental and Applied Fundamental Research Major Program of Guangdong Province, China (2019B030302013), the Science and Technology Planning Project of Guangdong Province, China (2017B020236003 and 2017B030314175), and the One-Three-Five Program of the Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, China (135PY201604). This is contribution No. IS-2853 from GIGCAS.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cej.2020.125165>.

References

- [1] Q.H. Zhang, W.N. Yang, H.H. Ngo, W.S. Guo, P.K. Jin, M. Dzakpasu, S.J. Yang, Q. Wang, X.C. Wang, D. Ao, Current status of urban wastewater treatment plants in China, *Environ. Int.* 92–93 (2016) 11–22.
- [2] U.S. Environmental Protection Agency, Decentralized Systems Technology Fact Sheet-Septic Tank Systems for Large Flow Applications, EPA 832-F-00-079. Office of Water, Washington, D.C. 2000.
- [3] Y.Q. Yang, X. Zhan, S.J. Wu, M.L. Kang, J.N. Guo, F.R. Chen, Effect of hydraulic loading rate on pollutant removal efficiency in subsurface infiltration system under intermittent operation and micro-power aeration, *Bioresour. Technol.* 205 (2016) 174–182.
- [4] L. Gao, W. Zhou, J.C. Huang, S.B. He, Y.J. Yan, W.Y. Zhu, S.Q. Wu, X. Zhang, Nitrogen removal by the enhanced floating treatment wetlands from the secondary effluent, *Bioresour. Technol.* 234 (2017) 243–252.
- [5] T.M. Misiti, M.G. Hajaya, S.G. Pavlostathis, Nitrate reduction in a simulated free-water surface wetland system, *Water Res.* 45 (2011) 5587–5598.
- [6] Y. Fernández-Nava, E. Marañón, J. Soons, L. Castrillón, Denitrification of high nitrate concentration wastewater using alternative carbon sources, *J. Hazard. Mater.* 173 (2010) 682–688.
- [7] G.P. Fu, L.K. HuangShen, Z.P. Guo, Q.H. Zhou, Z.B. Wu, Effect of plant-based carbon sources on denitrifying microorganisms in a vertical flow constructed wetland, *Bioresour. Technol.* 224 (2017) 214–221.
- [8] N.B. Martínez, A. Tejada, A. Del Toro, M.P. Sánchez, F. Zurita, Nitrogen removal in pilot-scale partially saturated vertical wetlands with and without an internal source of carbon, *Sci. Total Environ.* 645 (2018) 524–532.
- [9] F.M. Li, L. Lu, X. Zheng, H.H. Ngo, S. Liang, W.S. Guo, X.W. Zhang, Enhanced nitrogen removal in constructed wetlands: effects of dissolved oxygen and step-feeding, *Bioresour. Technol.* 169 (2014) 395–402.
- [10] V. Torrijos, O. Gonzalo, A. Trueba-Santiso, I. Ruiz, M. Soto, Effect of by-pass and

- effluent recirculation on nitrogen removal in hybrid constructed wetlands for domestic and industrial wastewater treatment, *Water Res.* 103 (2016) 92–100.
- [11] S.B. Wu, D.X. Zhang, D. Austin, R.J. Dong, C.L. Pang, Evaluation of a lab-scale tidal flow constructed wetland performance: oxygen transfer capacity, organic matter and ammonium removal, *Ecol. Eng.* 37 (2011) 1789–1795.
- [12] W. Zhi, G.D. Ji, Quantitative response relationships between nitrogen transformation rates and nitrogen functional genes in a tidal flow constructed wetland under C/N ratio constraints, *Water Res.* 64 (2014) 32–41.
- [13] T. Saeed, M.J. Miah, T. Khan, A. Ove, Pollutant removal employing tidal flow constructed wetlands: Media and feeding strategies, *Chem. Eng. J.* 382 (2020) 122874.
- [14] J.J. Chang, J. Mei, W. Jia, J.Q. Chen, X. Li, B.H. Ji, H.M. Wu, Treatment of heavily polluted river water by tidal-operated biofilters with organic/inorganic media: Evaluation of performance and bacterial community, *Bioresour. Technol.* 279 (2019) 34–42.
- [15] Z. Wang, M.L. Huang, R. Qi, S.S. Fan, Y. Wang, T. Fan, Enhanced nitrogen removal and associated microbial characteristics in a modified single-stage tidal flow constructed wetland with step-feeding, *Chem. Eng. J.* 314 (2017) 291–300.
- [16] Y.S. Hu, Y.Q. Zhao, A. Rymaszewicz, Robust biological nitrogen removal by creating multiple tides in a single bed tidal flow constructed wetland, *Sci. Total Environ.* 470 (2014) 1197–1204.
- [17] Y. Hu, F. He, L. Ma, Y. Zhang, Z.B. Wu, Microbial nitrogen removal pathways in integrated vertical-flow constructed wetland systems, *Bioresour. Technol.* 207 (2016) 339–345.
- [18] G.P. Fu, T.Y. Yu, K.L. Ning, Z.P. Guo, M.H. Wong, Effects of nitrogen removal microbes and partial nitrification-denitrification in the integrated vertical-flow constructed wetland, *Ecol. Eng.* 95 (2016) 83–89.
- [19] J.J. Chang, S.Q. Wu, Y.R. Dai, W. Liang, Z.B. Wu, Treatment performance of integrated vertical-flow constructed wetland plots for domestic wastewater, *Ecol. Eng.* 44 (2012) 152–159.
- [20] H.C. Tee, P.E. Lim, C.-E. Seng, M.-A.M. Nawi, Newly developed baffled subsurface flow constructed wetland for the enhancement of nitrogen removal, *Bioresour. Technol.* 104 (2012) 235–242.
- [21] C. Pellissari, M. Guivernau, M. Viñas, J. García, M. Velasco-Galilea, S.S. Souza, P.H. Sezerino, C. Ávila, Effects of partially saturated conditions on the metabolically active microbiome and on nitrogen removal in vertical subsurface flow constructed wetlands, *Water Res.* 141 (2018) 185–195.
- [22] S. Lackner, E.M. Gilbert, S.E. Vlaeminck, A. Joss, H. Horn, M.C. van Loosdrecht, Full-scale partial nitrification/anammox experiences—an application survey, *Water Res.* 55 (2014) 292–303.
- [23] R. Chen, J.Y. Ji, Y.J. Chen, Y. Takemura, Y. Liu, K. Kubota, H.Y. Ma, Y.Y. Li, Successful operation performance and syntrophic micro-granule in partial nitrification and anammox reactor treating low-strength ammonia wastewater, *Water Res.* 155 (2019) 288–299.
- [24] M. Laurenzi, P. Falás, O. Robin, A. Wick, D.G. Weissbrodt, J.L. Nielsen, T.A. Termes, E. Morgenroth, A. Joss, Mainstream partial nitrification and anammox: long-term process stability and effluent quality at low temperatures, *Water Res.* 101 (2016) 628–639.
- [25] R. Du, Y.Z. Peng, J.T. Ji, L.L. Shi, R.T. Gao, X.C. Li, Partial denitrification providing nitrite: Opportunities of extending application for anammox, *Environ. Int.* 131 (2019) 707–716.
- [26] M. Zhang, S.Y. Wang, B. Ji, Y. Liu, Towards mainstream deammonification of municipal wastewater: Partial nitrification-anammox versus partial denitrification-anammox, *Sci. Total Environ.* 692 (2019) 393–401.
- [27] L.X. Jia, R.G. Wang, L.K. Feng, X. Zhou, J.L. Lv, H.M. Wu, Intensified nitrogen removal in intermittently-aerated vertical flow constructed wetlands with agricultural biomass: effect of influent C/N ratios, *Chem. Eng. J.* 345 (2018) 22–30.
- [28] X. Zhai, N. Piwpuan, C.A. Arias, T. Headley, H. Brix, Can root exudates from emergent wetland plants fuel denitrification in subsurface flow constructed wetland systems? *Ecol. Eng.* 61 (2013) 555–563.
- [29] SEPA, Monitoring and analysis methods of water and wastewater, fourthed, State Environmental Protection Administration, Xicheng district, Beijing, China, 2002.
- [30] X. Zhan, J. Chen, Y.Q. Yang, S.J. Wu, F.R. Chen, G.G. Ying, C.J. Zhang, L.L. Zhang, Q. Zhang, X.W. Sun, Removal of antibiotics and antibiotic resistance genes from domestic sewage by a subsurface wastewater infiltration system with long-term operations, *Environ. Eng. Sci.* 36 (2019) 863–872.
- [31] J.J. Liu, Y. Yuan, B.K. Li, Q. Zhang, L. Wu, X.Y. Li, Y.Z. Peng, Enhanced nitrogen and phosphorus removal from municipal wastewater in an anaerobic-aerobic-anoxic sequencing batch reactor with sludge fermentation products as carbon source, *Bioresour. Technol.* 244 (2017) 1158–1165.
- [32] J.L. Hayward, Y. Huang, C.K. Yost, L.T. Hansen, C. Lake, A. Tong, R.C. Jamieson, Lateral flow sand filters are effective for removal of antibiotic resistance genes from domestic wastewater, *Water Res.* 162 (2019) 482–491.
- [33] A. Fumasoli, E. Morgenroth, K.M. Udert, Modeling the low pH limit of *Nitrosomonas europaea* in high-strength nitrogen wastewaters, *Water Res.* 83 (2015) 161–170.
- [34] Y.S. Hu, Y.Q. Zhao, X.H. Zhao, J.L.G. Kumar, Comprehensive analysis of step-feeding strategy to enhance biological nitrogen removal in alum sludge-based tidal flow constructed wetlands, *Bioresour. Technol.* 111 (2012) 27–35.
- [35] J. Vymazal, The use of hybrid constructed wetlands for wastewater treatment with special attention to nitrogen removal: a review of a recent development, *Water Res.* 47 (2013) 4795–4811.
- [36] X. Tan, Y.L. Yang, Y.W. Liu, X. Li, X.Y. Fan, Z.W. Zhou, C.J. Liu, W.C. Yin, Enhanced simultaneous organics and nutrients removal in tidal flow constructed wetland using activated alumina as substrate treating domestic wastewater, *Bioresour. Technol.* 280 (2019) 441–446.
- [37] J. Vymazal, Removal of nutrients in various types of constructed wetlands, *Sci. Total Environ.* 380 (2007) 48–65.
- [38] F.F. Liu, J.L. Fan, J.H. Du, X. Shi, J. Zhang, Y.H. Shen, Intensified nitrogen transformation in intermittently aerated constructed wetlands: Removal pathways and microbial response mechanism, *Sci. Total Environ.* 650 (2019) 2880–2887.
- [39] P. Foladori, J. Ruaben, A.R. Ortigara, Recirculation or artificial aeration in vertical flow constructed wetlands: a comparative study for treating high load wastewater, *Bioresour. Technol.* 149 (2013) 398–405.
- [40] C.B. Yuan, F.C. Zhao, X.H. Zhao, Y.Q. Zhao, Woodchips as sustained-release carbon source to enhance the nitrogen transformation of low C/N wastewater in a baffle subsurface flow constructed wetland, *Chem. Eng. J.* (2020), <https://doi.org/10.1016/j.cej.2020.124840>.
- [41] H.Y. Li, W.D. Tao, Efficient ammonia removal in recirculating vertical flow constructed wetlands: Complementary roles of anammox and denitrification in simultaneous nitrification, anammox and denitrification process, *Chem. Eng. J.* 317 (2017) 972–979.
- [42] H.S. Sun, S.J. Xu, S.H. Wu, R. Wang, X.L. Zhuang, Enhancement of facultative anaerobic denitrifying communities by oxygen release from roots of the macrophyte in constructed wetlands, *J. Environ. Manage.* 246 (2019) 157–163.
- [43] U. Stottmeister, A. Wiefner, P. Kusch, U. Kappelmeyer, M. Kästner, O. Bederski, R. Müller, H. Moormann, Effects of plants and microorganisms in constructed wetlands for wastewater treatment, *Biotechnol. Adv.* 22 (2003) 93–117.
- [44] L. Du, X.T. Trinh, Q.R. Chen, C. Wang, H.H. Wang, X. Xia, Q.H. Zhou, D. Xu, Z.B. Wu, Enhancement of microbial nitrogen removal pathway by vegetation in Integrated Vertical-Flow Constructed Wetlands (IVCWs) for treating reclaimed water, *Bioresour. Technol.* 249 (2018) 644–651.
- [45] Z.F. Han, J. Dong, Z.Q. Shen, R. Mou, Y.X. Zhou, X.M. Chen, X.Y. Fu, C.P. Yang, Nitrogen removal of anaerobically digested swine wastewater by pilot-scale tidal flow constructed wetland based on in-situ biological regeneration of zeolite, *Chemosphere* 217 (2019) 364–373.
- [46] V. Gagnon, F. Chazarenc, Y. Comeau, J. Brisson, Influence of macrophyte species on microbial density and activity in constructed wetlands, *Water Sci. Technol.* 56 (2007) 249–254.
- [47] X.X. Peng, F. Guo, F. Ju, T. Zhang, Shifts in the microbial community, nitrifiers and denitrifiers in the biofilm in a full-scale rotating biological contactor, *Environ. Sci. Technol.* 48 (2014) 8044–8052.
- [48] G. Ansola, P. Arroyo, L.E.S. de Miera, Characterisation of the soil bacterial community structure and composition of natural and constructed wetlands, *Sci. Total Environ.* 473 (2014) 63–71.
- [49] M.C. Van Teeseling, R.J. Mesman, E. Kuru, A. Espaillet, F. Cava, Y.V. Brun, M.S. VanNieuwenhze, B. Kartal, L. Van Niftrik, Anammox Planctomycetes have a peptidoglycan cell wall, *Nat. Commun.* 6 (2015) 6878.
- [50] C.M. Fitzgerald, P. Camejo, J.Z. Oshlag, D.R. Noguera, Ammonia-oxidizing microbial communities in reactors with efficient nitrification at low-dissolved oxygen, *Water Res.* 70 (2015) 38–51.
- [51] M.S. Jetten, L.V. Niftrik, M. Strous, B. Kartal, J.T. Keltjens, H.J. Op den Camp, Biochemistry and molecular biology of anammox bacteria, *Crit. Rev. Biochem. Mol. Biol.* 44 (2009) 65–84.
- [52] R. Du, S.B. Cao, B.K. Li, M. Niu, S.Y. Wang, Y.Z. Peng, Performance and microbial community analysis of a novel DEAMOX based on partial-denitrification and anammox treating ammonia and nitrate wastewaters, *Water Res.* 108 (2017) 46–56.
- [53] O. Prakash, S.J. Green, P. Jasrotia, W.A. Overholt, A. Canion, D.B. Watson, S.C. Brooks, J.E. Kostka, *Rhodanobacter denitrificans* sp. nov., isolated from nitrate-rich zones of a contaminated aquifer, *Int. J. Syst. Evol. Microb.* 62 (2012) 2457–2462.
- [54] S.J. Green, O. Prakash, P. Jasrotia, W.A. Overholt, E. Cardenas, D. Hubbard, J.M. Tiedje, D.B. Watson, C.W. Schadt, S.C. Brooks, Denitrifying bacteria from the genus *Rhodanobacter* dominate bacterial communities in the highly contaminated subsurface of a nuclear legacy waste site, *Appl. Environ. Microbiol.* 78 (2012) 1039–1047.
- [55] F. Gori, S.G. Tringe, B. Kartal, E. Marchiori, M.S. Jetten, The metagenomic basis of anammox metabolism in *Candidatus Brocadia fulgida*, *Biochem. Soc. Trans.* 39 (2011) 1799–1804.
- [56] J.A. Fuerst, E. Sagulenko, Beyond the bacterium: planctomycetes challenge our concepts of microbial structure and function, *Nat. Rev. Microbiol.* 9 (2011) 403–411.
- [57] Z.Y. Hu, H.J. Wessels, T. van Alen, M.S. Jetten, B. Kartal, Nitric oxide-dependent anaerobic ammonium oxidation, *Nat. Commun.* 10 (2019) 1244–1250.
- [58] J. Hou, X. Wang, J. Wang, L. Xia, Y.Q. Zhang, D.P. Li, X.F. Ma, Pathway governing nitrogen removal in artificially aerated constructed wetlands: impact of aeration mode and influent chemical oxygen demand to nitrogen ratios, *Bioresour. Technol.* 257 (2018) 137–146.
- [59] F. Rambags, C.C. Tanner, L.A. Schipper, Denitrification and anammox remove nitrogen in denitrifying bioreactors, *Ecol. Eng.* 138 (2019) 38–45.
- [60] J. Vymazal, Do laboratory scale experiments improve constructed wetland treatment technology? *Environ. Sci. Technol.* 52 (2018) 12956–12957.
- [61] C.J. Lan, M. Kumar, C.C. Wang, J.G. Lin, Development of simultaneous partial nitrification, anammox and denitrification (SNAD) process in a sequential batch reactor, *Bioresour. Technol.* 102 (2011) 5514–5519.
- [62] M. Strous, J. Heijnen, J.G. Kuenen, M. Jetten, The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms, *Appl. Microbiol. Biotechnol.* 50 (1998) 589–596.
- [63] P.L. McCarty, L. Beck, P.S. Amant, Biological denitrification of wastewaters by addition of organic materials, 24th Industrial Waste Conference, Purdue Univ. 1969, pp. 1271–1285.