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Simultaneous nitrification and denitrification in a PCL supported constructed wetland with limited aeration

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Research Article

Keywords: constructed wetland, nitrogen, solid organic substrates, nitrification and denitrification, aeration

Posted Date: July 19th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1678498/v1

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Abstract

Considerable advances have been made in constructed wetlands (CWs) substrate design and operation strategies to promote nitrogen transformation and elimination. However, few studies focused on the complex interplay of solid organic substrates and limited aeration on nitrogen removal. Vertical flow constructed wetlands (VFCWs) in gradient distribution of inorganic (volcanic and ceramsite) (C-VFCW) or solid organic substrates (polycaprolactone/PCL) (P-VFCW) with aeration were developed for tertiary removal from municipal wastewater. Results showed that ammonia was almost converted to nitrate while total nitrogen removal efficiency (TNRE) was negligible in C-VFCW. However, an optimal TNRE exceeded 95% with an aeration rate of 0.06 mL·min^{-1} and hydraulic retention time (HRT) of 24 h in P-VFCW, and simultaneous nitrification and denitrification process (SND) in aerobic conditions was verified. As for microbial communities' spatial characteristics, *Comamonas*, closely relating to heterotrophic nitrification and anoxic/aerobic denitrification, was enriched vertically along with the P-VFCW. Autotrophic nitrifier (*Nitrospira)*, anoxic denitrifier (*Methyloversatilis*), and aerobic denitrifier (*Bradyrhizobium* and *Azospira*) were observed and dominated in different depths of P-VFCW. Besides, the biomass growth of *Canna indica* in P-VFCW was much greater than that in C-VFCW. The P-VFCW developed in this study is a promising technology for advanced nitrogen removal from municipal secondary wastewater.

1. Introduction

Constructed wetland (CW) is widely applied for nitrogen removal due to the nature of low cost, easy maintenance, and environmentally-friendliness (Gunes et al. 2021, Parde et al. 2020, Vymazal et al. 2021, Wallace &Waltham 2021). The nitrogen removal pathways in CWs include comprehensive interactions of plants' uptake, substrates' adsorption, and biotransformation, with the microbial nitrification and denitrification processes being the most dominant (Tang et al. 2020, Zhou et al. 2020). In general, oxygen deficiency is always present in the CWs (Nguyen et al. 2019), and the scarcity of electron donors further restricts the denitrification process. Thus, it is a regular occurrence that CWs always exhibit a suboptimal total nitrogen removal efficiency (TNRE) when treating wastewater with a low C/N ratio due to the oxygen and electron donor availability.

Considerable advances have been made in operation strategies and unique designs of CWs to facilitate high nitrogen transformation and elimination. Aeration was frequently employed to improve the overall treatment efficacy of CWs (Boog et al. 2019, Wang et al. 2020). Numerous studies have verified that aeration can largely enhance the total nitrogen removal in CWs (Jehawi et al. 2020, John et al. 2020, Li et al. 2021). For example, compared to the CW without aeration, the aerated CW presented superior nitrogen removal performance with NH_4^+-N removal efficiency of 94.3% and TNRE of 91.5% (Li et al. 2021). Meanwhile, the nitrate originating from the nitrification process cannot be entirely removed with the insufficient electron donor for denitrification, resulting in nitrate accumulation accompanied by a low TNRE would occur in CWs. From this, another critical point is how to simultaneously eliminate the nitrate produced by the aerated nitrification process. Recently, several researchers attempted to tackle the

insufficient electron supply for denitrification in CWs by utilizing solid organic substrates that served as both substrates and the carbon source (Si et al. 2018). Biodegradable polymers with stable and strong electron supply capacity have been shown to significantly enhance the TNRE of CWs (Jia et al. 2021, Shen et al. 2014). These biodegradable polymers, mainly including polycaprolactone (PCL) and poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), have been investigated (Li et al. 2016, Xu et al. 2019, Yang et al. 2020) and demonstrated a great potential for largely increasing nitrate removal efficiency. However, it was discovered that the estimated cost of PHBV (21.0-37.2 €/kg NO_3^--N) is much greater than that of PCL (5.4–8.9 €/kg NO_3^--N) (Zhang et al. 2016). In light of this, PCL is economically more viable.

As such, newly, some research has tried to combine the benifits of aeration and biodegradable polymers in order to promote the TNRE of CWs. For example, Yang (Yang et al. 2017) found that compared with non-aeration, higher ammonia removal efficiency (91.00%) and total nitrogen removal efficiency (97.03%) in a PHBV/PLA and ceramsite supported CW were achieved under limited aeration. Similarly, it was discovered that compared with non-aerated PHBV supported CW, the NH_4^+-N and TN removal efficiencies in an aerated PHBV-supported CW were much higher, reaching 90.4% and 92.1%, respectively (Sun et al. 2018). However, in the experiments stated previously, the biodegradable polymers were placed at the bottom of CWs, which brought some problems. Substrates inextricably link with the other key operation parameters and significantly influence the performance of CWs (Ji et al. 2021). It is well known that the competition of aerobic heterotrophic microorganisms for oxygen would bring detrimental effects on the nitrifiers in the presence of excessive organic matter (Tran et al. 2018, Zhu &Chen 2001). Besides, aeration would accelerate the degradation and consumption of biopolymers by aerobic heterotrophic microorganisms, which would result in clogging of CWs, secondary effluent pollution and a decreased polymers' effectiveness. It can be concluded that the distribution pattern of biopolymers imposed significant effects on nitrogen removal in aerated CWs. Thus, in the case of aeration, it was critical to develop a proper distribution of biopolymer and other inorganic substrates in CWs. Additionally, previous studies verified the feasibility of a combination of aeration and organic substrates, while made little attempt to explore the mechanism underlying simultaneous ammonia and nitrate removal. The complex interplay of biodegradable polymers and aeration in the CWs on the biotransformation of N in various forms was also unclear.

In this study, two vertical flow constructed wetlands (VFCWs) were developed, normal VFCW with micro aeration (C-VFCW) and PCL supported VFCW with micro aeration (P-VFCW), respectively. This study aims to: (i) investigate the difference on TNRE between both systems under different aeration rate and HRT through a long-term continuous operation; (ii) explore the spatial distribution characteristics of microbial community structure, and thereby elucidating the mechanism about simultaneous removal of ammonia and nitrate.

2. Materials And Methods

2.1 Materials

In the present study, two VFCWs were built with multiple inorganic/organic media: volcanic rock, ceramsite and PCL polymer. Volcanic rock and ceramsite granules were purchased from GongYi Songxin filter material industrial company, and the brand of PCL granules was American Solvay Advanced polymers 6800. Volcanic rock and ceramsite granules had particle sizes ranging from 4 mm to 6 mm, from 2 mm to 3 mm, respectively. The physical characteristics of PCL granules were as follows: shape (cylindrical), diameter (3.0 mm), and height (1.00 mm).

Two VFCWs were intended for advanced treatment of the wastewater treatment with a low C/N ratio. According to the “municipal wastewater treatment plant pollutants discharge standard” class A (GB18918-2002), synthetic wastewater with 8 mg/L NH_4^+-N and 7 mg/L NO_3^--N was prepared by adding $NaNO_3$, NH_4Cl and KH_2PO_4 into a 50 L container, and the characteristics of influent during various phases were shown in Table 1. Due to a lower operating temperature and higher changing water frequency in phase I, the influent averaged DO concentrations were higher than that in other phases, while the influent nitrite concentrations were lower. To determine whether PCL biopolymer posed a risk of secondary pollution due to the presence of excessive organics in the effluent, there was no chemical oxygen demand (COD) in the influent.

2.2 Experimental apparatus and procedures

The structure of each VFCW was shown in Fig. 1. Two VFCWs were both performed using a Plexiglas cylinder with 20 cm diameter and 80 cm long. A Plexiglas mesh disc with a 2 mm pore size was to support the substrates. A titanium aeration disc was at the end of the experimental devices. Six sampling ports were arranged on the reactors’ side at 10 cm intervals from 0 cm to 60 cm. Multi-dimensional gradation of substrates was adopted in this study: 10 cm top layer of ceramsite was added for facilitating the growth of plants; the following media PCL polymer (750.00 g)/ceramsite mixture or ceramsite were filled in P-VFCW and C-VFCW with a depth of 30 cm, respectively; 20 cm bottom layer of inorganic substrate-volcanic rock served as the supporting layer to facilitate the nitrification process. Meanwhile, an air pump was installed beneath VFCWs. *Canna indica* (average height of 48.36 ± 1.42 cm, average weight of 262.62 ± 6.02 g) were chosen and 3 plants were planted in each VFCW.

Figure 1. Scheme of the simulated VFCWs

Activated sludge taken from the secondary sediment tank of the Quyang municipal wastewater treatment plant in Shanghai, China was used as an inoculation source with a final biomass concentration of 800 mg MLSS /L for each VFCW. After the inoculation, the synthetic wastewater was introduced into these systems by a peristaltic pump at a flow rate of 13.08 $mL \cdot min^{-1}$ from the bottom of VFCW. The aeration rate of the air pump (Hailea, ACO-5504) was controlled between 0.06 $mL \cdot min^{-1}$ and 0.15 $mL \cdot min^{-1}$ by a rotameter. The whole experiment was divided into three phases to explore the effect of aeration rate and HRT on TN removal performance. Two VFCWs were both exposed to the natural environment (such as air, temperature, light and so on). The whole experiment operated from June 1, 2021 to August 30, 2021 and lasted for three months. The influent characteristics and operation parameters were listed in Table 1.

Table 1
Operation parameters and influent characteristics at different phases

	Phase I	Phase II	Phase III
Operation parameters			
Times (d)	1–25	26–63	64–87
Q ($L \cdot d^{-1}$)	18.80	18.80	30.48
$Q_{aeration}$ ($mL \cdot min^{-1}$)	0.15	0.06	0.06
HRT (h)	24	24	12
Influent characteristics			
NH_4^+-N ($mg \cdot L^{-1}$)	7.09 ± 0.53	6.57 ± 1.22	6.51 ± 1.29
NO_3^--N ($mg \cdot L^{-1}$)	6.14 ± 0.33	6.24 ± 0.62	5.75 ± 0.20
pH	7.40 ± 0.10	7.18 ± 0.19	7.17 ± 0.22
DO ($mg \cdot L^{-1}$)	6.55 ± 0.32	3.26 ± 2.13	3.61 ± 1.79
TN ($mg \cdot L^{-1}$)	13.65 ± 0.61	14.51 ± 0.83	13.84 ± 0.49

2.3 Analytical methods

Influent and effluent samples were collected daily. All the samples were filtrated through a 0.45 μm membrane to remove suspended solids. Total organic carbon (TOC) was determined by a TOC analyzer (TOC V-CPN, Shimadzu, Japan). NO_3^--N, NO_2^--N, NH_4^+-N concentrations were measured according to the standard methods by UV spectrophotometer (UV 1900i, Shimadzu). Dissolved oxygen (DO) and pH were performed by a handled multi-parameter water quality analyzer (Hach, USA). All the tests were conducted in triplicate, and the measurement data were presented as mean values.

The TNRE (%) in this study was defined by the following equation:

$$TNRE = \frac{C_{innitrate} + C_{inammonia} - C_{efammonia} - C_{efnitrate} - C_{efnitrite}}{C_{in}}$$

1

Where $C_{inammonia}$ and $C_{innitrate}$ were influent NH_4^+-N and NO_3^--N concentration ($mg \cdot L^{-1}$); $C_{efammonia}$, $C_{efnitrate}$ and $C_{efnitrite}$ were effluent NH_4^+-N, NO_3^--N and NO_2^--N concentrations ($mg \cdot L^{-1}$), respectively.

2.4 Microbial community analysis

To reveal explore the microbial mechanism for SND performance, five biofilm samples were collected at the vertical intervals of 0–10 cm, 10–20 cm, 20–30 cm, 30–40 cm and 40–50 cm in P-VFCW, marked as 0_10A, 10_20B, 20_30C, 30_40D and 40_50E, respectively. The DNA extraction was eatracted using a microbial DNA extraction kit (Biocolors, Shanghai). The V4-V5 regions of 16SrRNA genes were amplified using primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). To obtain the microbial community, Illumina high-throughput sequencing was performed at the Majorbio Biopharm Biotechnology Company (Shanghai, China). Operational taxonomic units (OTUs) clustering was performed at 97% sequence identity. The phylotype coverage, richness and diversity estimations were calculated by Mothur analysis (http://www.mothur.org) at a 3% distance level.

The Chao and Shannon indices were to evaluate the richness and diversity of microbial structure, respectively. And they were defined as Eqs. (1) and (2), respectively:

$$S_{chao1} = S_{obs} + \left[n_1(n_1 - 1) / 2(n_2 + 1) \right]$$

1

$$Shannon = -\sum_{i=1}^{S} P_i \log_2 P_i$$

2

Where S_{chao1} is the estimated OTUs number, S_{obs} is the observed OTUs number, n_1 was the OTUs number of singletons, and n_2 was the OTUs number of doubletons. S was the number of OTUs and P*i* is the proportion of the community represented by OTU *i*.

3. Results

3.1 Effluent characteristics in two different VFCWs

The difference in treatment performance of the two VFCWs could be disclosed from the effluent quality. Figs.2, 3 and 4 depicted the physical characteristics of pH and DO, nitrogen removal performance and TOC variations for each VFCW during a long-term operation.

3.1.1 Effluent pH and DO characteristics

Fig.2 depicted the variations of pH and DO in the influent and effluent. During the whole operation process, the influent and effluent pH were 6.73-7.56 and 6.63-7.70, respectively. The difference in effluent pH between P-VFCW and C-VFCW was subtle. On the whole, the difference in pH between influent and effluent in each VFCW was negligible. It was reported that the effluent pH in the biodegradable polymer supported denitrification system always decreased due to the residual acidic intermediate products from the hydrolysis of polymers (Xu et al. 2018a). It is known that H^+ and OH^- are produced during the

nitrification and denitrification processes, respectively, thus SND could alleviate pH fluctuations (Hu et al. 2021). Meanwhile, the influent DO in phase I kept relatively stable, ranging from 6.09 to 7.16 mg·L^{-1}. Due to the volatility of influent supply, the influent DO in phases II and III considerably fluctuated. The DO concentrations in the influent tank were 0.17-5.97 mg·L^{-1} and 0.32-5.92 mg·L^{-1}, respectively. Whereas, the effluent DO in three stages slightly fluctuated. The effluent DO in C-VFCW ranged from 5.00 mg/L to 8.46 mg/L with an average of 7.61±0.55 mg/L. The effluent DO in P-VFCW were 7.48-8.41 mg·L^{-1}, 4.35-7.32 mg·L^{-1} and 3.72-7.55 mg·L^{-1} with an average of 8.07±0.28, 6.03±0.93 and 5.44±0.90 mg·L^{-1}, corresponding to phases I, II and III, respectively. In comparison to the C-VFCW, the decrease of effluent DO in P-VFCW might be ascribed to the DO consumption of aerobic heterotrophic bacteria. Additionally, no substantial suppression of the nitrification occurred when DO exceeded 1.7-2.0 mg·L^{-1}; nevertheless, concerning the denitrification process, the DO should be kept below 0.3-0.5 mg·L^{-1} to accomplish NO_3^--N reduction (Lin et al. 2020). Thus, excessive DO concentrations in this CW might lead to a suppression of denitrification, thereby hindering the effective removal of TN.

Fig.2. Variations of pH and DO in C-VFCW and P-VFCW

3.1.2 Effluent nitrogen characteristics

Fig.3 depicted the variations of nitrogen in different forms in each VFCW. The influent primarily consisted of ammonia and nitrate, with a TN of approximately 14.07±0.78 mg·L^{-1}. During the whole operation, the influent NH_4^+-N varied from 3.82 to 8.38 mg·L^{-1} with an average of 6.71±1.10 mg/L, while the effluent NH_4^+-N concentrations was negligible in each VFCW (Fig.3a). It was evident that the NH_4^+-N removal efficiencies in all phases exceeded 95%, indicating that an aeration rate of 0.06-0.15 ml/min all achieved a good nitrification performance. It has been demonstrated that when compared to the non-aerated CW, the intermittent-aerated CW had a better NH_4^+-N removal efficiency of 94.3% (Li et al. 2021), indicating the DO supply was beneficial for nitrogen removal in CW.

Fig.3. Nitrogen removal performance in C-VFCW and P-VFCW

However, in phase I, the effluent nitrogen was mainly nitrate in each VFCW (Figs.3b and 3d). Due to the residual organic carbon source or nonviable microorganisms in the inoculated activated sludge, the TN slightly decreased in each VFCW in the first few days. However, effluent NO_3^--N concentrations averaged at 12.54±1.84 mg·L^{-1} and 7.91±0.56 mg·L^{-1} with low TNREs of 8% and 40% in C-VFCW and P-VFCW, respectively. It was previously verified that the biodegradable polymer-PCL served as the electron donor for the denitrification process, resulting in a higher TNRE of P-VFCW. Meanwhile, it also indicated that an excessive amount of DO would create a high redox potential and further seriously hindered the denitrification process. When the aeration rate decreased from 0.15 ml/min to 0.06 ml min^{-1} (phase II), effluent nitrogen primarily in form of NO_3^--N remained high in C-VFCW, but sharply declined in P-VFCW. After a 16-day adaption period, the effluent NO_3^--N was extremely low, and the effluent TN averaged at 1.50±1.34 mg·L^{-1} with a remarkable TNRE of 90% in P-VFCW. Similarly, in an intermittent-aeration CW,

there was a better TNRE at a lower DO of 3 mg l^{-1} and shorter HRT of 2 days than a DO of 4 mg·L^{-1} and HRT of 3 days (Li et al. 2021), indicating that a lower DO was closely related to a higher TNRE. It was reported that the expression of nitrate reduction enzyme-Nap located at periplasm is not sensitive to the inhibition of DO, thus, Nap still can drive the reduction of NO_3^--N (Chen et al. 2018). Nevertheless, DO remained a crucial factor influencing the denitrification performance under aerobic conditions. In general, the optimal DO concentration for aerobic denitrification was generally found in the range of 3.0-5.0 mg·L^{-1} (Hu et al. 2021). According to the above-mentioned effluent quality, it was deduced that a considerable TNRE in P-VFCW attributed to the SND process and aerobic denitrification occurred.

When the HRT decreased from 24 h (phase II) to 12 h (phase III), there was no noticeable difference in the effluent of C-VFCW. However, the effluent NO_3^--N and the TN concentrations both increased and averaged at 2.63±1.01 mg·L^{-1} and 3.28±1.08 mg·L^{-1} in P-VFCW, respectively, thus the TNRE decreased from 90% to 76%. It indicated that TNRE decreased with a decline of HRT in P-VFCW. Despite this, the TNRE was unexpectedly high under a shorter HRT and aerobic condition (DO>4 mg·L^{-1}). As an intermediate product of nitrification and denitrification processes, effluent nitrite accumulation was both negligible in each VFCW (Fig.3c), revealing that the nitrification and denitrification processes were relatively complete. Additionally, the cost of using PCL polymers was approximately 5.4-8.9 €/kg NO_3^--N, and it was estimated that the cost of using PCL was about 2-7 times that of using methanol and ethanol, but the same as that of using acetic acid (Wang &Chu 2016). There are no safety concerns during transport and operation in terms of toxicity and inflammability for PCL at all. If the price of PCL can be reduced further, it is reasonable to assume that an aerated P-VFCW will become a viable technology for advanced nitrogen treatment.

3.1.3 Effluent TOC characteristics

Fig.4. Effluent TOC characteristics in P-VFCW

It was found that TOC accumulation occurred in biodegradable polymer supported denitrification systems for nitrate removal in previous studies (Xu et al. 2019, Xu et al. 2018b). The effluent TOC concentrations were depicted to explore whether effluent TOC accumulation occurred in VFCWs (Fig.4). During the whole experiment, the effluent TOC was negligible in C-VFCW due to the absence of organics in the influent. Meanwhile, the effluent TOC concentrations were in the range of 1.11-5.95 mg·L^{-1} with minor fluctuations in P-VFCW. The average effluent TOC concentrations were 2.53±0.66, 2.71±1.22 and 2.13±0.68 mg·L^{-1} corresponding to phases I, II and III, respectively. According to the environmental quality standards for surface water (GB3838-2002), the effluent residual TOC concentrations in the P-VFCW were too low to pose a risk of secondary pollution for the surface water. However, it was discovered that in an aerated PHBV/PLA-supported SND system, effluent TOC rapidly increased up to a maximal concentration of 36.40 mg·L^{-1} (Sun et al. 2020), revealing that the gradient structure of biopolymer and other inorganic substrates had an obvious advantages on decreasing effluent organics. Meanwhile, in a aerated PHBV and ceramsite supported VFCW, the COD concentrations were about 19.44±7.91 mg/L which was still

higher than that in the present study (Sun et al. 2018), indicating that aeration might aggravate the risk of deteriorated effluent when the biodegradable polymers deposited at the bottom of systems without proper gradient structure. Additionally, a low effluent TOC in P-VFCW was attributed to the proper distribution pattern of organic and inorganic substrates under aerobic conditions.

3.2 Canna indica

Plants require copious amounts of nitrogen to sustain growth, and thus, nitrogen acquisition is frequently a limiting factor of plant growth. It was reported that plants in CWs make an important contribution in terms of contaminant uptake (Schierano et al. 2020, Zhang et al. 2021). It has been estimated that approximately 60%-70% of TN is removed via denitrification, while 20%-30% of TN can be consumed by plant uptake in CWs (J.Spieles &J.Mitsch 2000). After the whole operation for 90 days, the average height of *Canna indica* increased from 37.55±5.53 cm to 75.20±4.08 cm and 105.97±15.36 cm in C-VFCW and P-VFCW, respectively; and its wet weight rose from 64±6.10 g to 173.13±2.16 g and 733.96±4.04 g in C-VFCW and P-VFCW, respectively (Fig.1). Additionally, it indicated that some of influent NH_4^+-N and NO_3^--N were removed by *Canna indica* for its growth in each VFCW.

Nitrogen response mainly refers to the plant adaptation responses to diverse nitrogen conditions or fluctuations in the nitrogen supply, which is a critical component of the regulatory network controlling plant growth (Ueda et al. 2017). The primary forms of nitrogen available to plants for assimilation are NO_3^--N and NH_4^+-N. It was reported that the relative growth rate of *Canna indica* exceeded 0.01 $g \cdot d^{-1}$ in the presence of 0 mg/L NH_4^+-N (Wang et al. 2016), indicating that a low effluent NH_4^+-N concentration in both C-VFCW and P-VFCW would not inhibit the growth of *Canna indica*. Meanwhile, it has been verified that when plants were supplied with both NO_3^--N and NH_4^+-N, they preferred NH_4^+-N uptake more than NO_3^--N. For example, it has been demonstrated that when a candidate plant for wetland- *Arundo donax* was fed with NO_3^--N, it had significantly less above-ground biomass than when NH_4^+-N was fed (Tho et al. 2017). Besides, it was also found that high nitrate concentrations (>5 mM) also negatively affected axial root growth in maize (Tian et al. 2008). Thus, a higher NO_3^--N concentration likely inhibited the growth of *Canna indica*, resulting in a lower biomass growth in C-VFCW than in P-VFCW.

3.3 Microbial community structure evolution along with the vertical profile of the constructed wetland

The microbial community structure of the biofilm samples collected along the vertical regions of P-VFCW was revealed by Illumina high-throughput sequencing for 16S rRNA genes. Table 2 summarized the sequencing analysis results and the diversity of microbial communities. The sequences without sampling obtained from five samples were respectively 72251, 60581, 69620, 71239, and 72995 with a length range of 414-420 bp. They were resampled to 54460 for the subsequent analysis. The rarefaction curves indicated that the sequencing depth was adequate for the microbial community analysis. The Chao and Shannon indices can be used to estimate the richness and diversity of the microbial community,

respectively. The richness and diversity among these five samples differed significantly, according to the structures of microbial communities.

Table 2 The characteristics of sequencing results and microbial community richness

	0-10A	10-20B	20-30C	30-40D	40-50E
Number sequences without resamples	72251	60581	69620	71239	72995
Chao index	1182.57	1902.85	1852.24	2064.82	2129.99
Shannon index	4.77	4.58	5.20	4.66	5.69

The microbial communities were further identified at phylum and genus levels. The relative abundances of the major phyla (sequence percentage >1% in at least one sample) were shown in Fig.5a. The main phyla among all samples were divided into 12 phyla, but their relative abundances varied. *Proteobacteria* was the predominant phylum in all samples, with relative abundances of *Proteobacteria* significantly higher in samples 20_30C (60%), 30_40D (60%) and 40_50E (52%) than in samples 0_10A (32%) and 10_20B (40%) (Fig.5a). In terms of the biological nitrogen removal processes, ammonia-oxidizing bacteria and denitrifying bacteria in PCL supported system both lay in *Proteobacteria* (Bucci et al. 2021, Zhang et al. 2016). The difference in the relative abundance of *Proteobacteria* among five samples might be associated with nitrogen biological processes in different zones of P-VFCW.

Fig.5b depicted the top 30 genera with the most read sequences. It was found that the microbial community evolution occurred along with the profile of CW. It has been reported that aeration intensity regulates the substrate distribution and growth of bacteria with depth in CWs, and the distribution of bacteria species depends on aeration intensity (John et al. 2020). *Comamonas* dominated in all samples, with a relative abundance of approximately 30% in samples 20_30C and 30_40D. Some strains of *Comamonas* were identified based on the denitrifying gene coding for *nirS* in a PHBV-supported denitrification system (Shams Tabrez Khan 2007), and some of which were also capable of heterotrophic nitrification and aerobic heterotrophic denitrification (Cao et al. 2021, Liu et al. 2021, Moura et al. 2018). *Comamonas* and *Longilinea*, for example, has been observed in a SND bioreactor under oxygen-rich conditions (Lai et al. 2020).

Nitrospira was found to be more abundant in the samples 0_10A (2.06%) and 10_20B (1.21%) than in other samples, implying that the nitrification process occurred primarily in the 0-20 cm of P-VFCW where ceramsite served as substrate. *Nitrospira* as a kind of autotrophic nitrifying microorganism was discovered, and it was frequently proved to have a close relationship with nitrite oxidation (Xiang et al. 2020), as well as a critical role in the nitrification process in an aerated PHBV supported denitrification system (Sun et al. 2019). Meanwhile, *Bdellovibrio*, heterotrophic aerobic bacteria involving in the degradation of organic matters (Iannacone et al. 2021), was observed with a high relative abundance in samples 20_30C (2.23%) and 30_40D (6.09%), which was related to the organic substrates- PCL polymers placed at the 20-40 cm of P-VFCW.

Besides, denitrification-associated microbes such as *Ignavibacterium, Bradyrhizobium*, *Methyloversatilis*, *Azospira*, *Stenotrophobacter*, *Thiothrix*, and *Simplicispira* (Ali. et al. 2016, Shams Tabrez Khan 2007, Vymazal et al. 2021, Wu et al. 2013) were identified, and their total relative abundance exceeded 6% in each sample. Among these denitrification bacteria, *Methyloversatilis* belongs to an anoxic denitrifier for reducing nitrate or nitrite (Liu et al. 2020). *Methyloversatilis* was found to be more abundant in samples 30-40D (1.32%) and 40-50E (2.79%), revealing that it facilitated anoxic denitrification in the upper zone of P-VFCW as DO decreased longitudinally. Members of *Stenotrophobacter*、*Dokdonella, Simplicispira* and *Terrimonas* have been reported to possess aerobic denitrification ability. In many SND systems, *Stenotrophobacter* dominated and capable of aerobic denitrification (Qiao et al. 2020). A MBBR reactor inoculated with heterotrophic nitrifying and aerobic denitrifying bacterium *Stenotrophomonas maltophilia* DQ01 could achieve a remarkable TN removal efficiency of 94.43% (Jia et al. 2020). *Terrimonas* was frequently detected in the SND system as a kind of aerobic denitrifying bacteria, and some of *Dokdonella* strain were also capable of aerobic denitrification (Liu et al. 2020). In general, the traditional SND process was restricted by the stringent operation conditions and the inhibition of heterotrophic bacteria to ammonia-oxidizing bacteria. It was concluded that a considerable TNRE in P-VFCW was attributed to the heterotrophic nitrification and aerobic denitrification bacterial mentioned previously.

Additionally, a PCA analysis was performed and presented in Fig.5c. The PCA analysis revealed the resemblance of community structures among these five samples. Environmental factors such as DO concentration, nitrogen concentration and form (mainly including NH_4^+-N and NO_3^--N) and substrate (inorganic or organic substances) were believed to be responsible for the microbial community structures. There was no obvious resemblance between 0_10A and 10_20B samples despite the fact that both were volcanic, which could be attributed to the concentrations and form of nitrogen. However, samples 20_30C, 30_40D and 40_50E (the blue oval) were in close proximity, indicating that three samples shared some microbial structure. The similarity among these three samples was contributed to the same substrates (PCL polymer and ceramsite), while the longitudinally decreasing NO_3^--N concentrations added subtle differences among these three samples. Overall, the difference in the microbial structure could be a result of the different nitrogen metabolic capacities along with the profile of P-VFCW.

Fig.5. The taxonomic affiliation and relative abundance of major groups at phylum (a)、genus (b) level and the PCA analysis among five samples along P-VFCW (c)

3.4 Simultaneous nitrification and denitrification mechanism

It was discovered that the aeration and organic substrate-PCL polymers were both critical factors for microbial community structure evolution along the P-VFCW. The association of aeration and PCL polymers led to a spatial microbial distribution involved in nitrification and denitrification processes (Fig.5). It was reported that simultaneous nitrification and denitrification occurred in the first 5 cm and denitrification only in higher column sections in a PHBV/PLA supported biofilter with aeration (Sun et al.

2020). The DO concentration was greatest at the bottom of P-VFCW, and it decreased with the depth of the system owing to the DO consumption of the nitrification process. *Nitrospira* and *Comamonas*, both autotrophic/heterotrophic microbes, were implicated in the nitrification process occurred in the region of 0-20 cm. Denitrification also occurred simultaneously in the zone of 10-20 cm, owing to a considerable relative abundance of aerobic denitrification bacteria (*Comamonas*, *Azospira*, *Stenotrophobacter*, and so on). There is no doubt that PCL granules facilitated the denitrification process in 20-50 cm., PCL granules were hydrolyzed and degraded into some low-molecular-weight substances as a high-molecular-weight biopolymer (Fang et al. 2020). These dissolved intermediate degradation products of PCL granules would diffuse into the adjacent region of 10-20 cm, resulting in an aerobic heterotrophic denitrification process in this region. Despite the presence of aerobic denitrifying microbes, anoxic heterotrophic microbes continued to dominate in the nitrate removal pathways in 20-50 cm of P-VFCW. Besides, the mixture pattern of PCL and ceramsite would increase the effective utilization ratio of PCL polymers for denitrification, as both the biofilm on the PCL polymer and ceramsite played an essential role in nitrate reduction. As a result, the nitrogen transformation and elimination mechanism in a PCL-supported CW with restricted aeration was deduced and illustrated in Fig.6.

Fig.6. Simultaneous nitrification and denitrification mechanism in aerated P-VFCW

4. Conclusion

Results showed that SND occurred in the P-VFCW rather than C-VFCW, and that increasing the aeration rate and shortening the HRT both reduced the TNRE of P-VFCW. However, even with a shorter HRT of 12 h and a higher aeration rate of 0.15 $mL \cdot min^{-1}$, the TNRE of P-VFCW still reached 76%. Illumina sequencing analysis implied that the dominant microbes involved in nitrification and denitrification differed along the P-VFCW, and heterotrophic nitrification and aerobic denitrification probably occurred. After the whole operation, a bigger biomass growth of *Canna indica* (increasing by 58.41 cm and 471.97 g) occurred in P-VFCW than that in C-VFCW. This study developed a novel VFCW for advanced nitrogen removal from municipal secondary wastewater.

Declarations

CRediT authorship contribution statement

AcknowledgementsWe would like to acknowledge the support from Shanghai Sailing Program (20YF1400200), the Fundamental Research Funds for the Center Universities (20D111324) and the National key research and development project (2019YFC0408603).

Funding This work was supported by Shanghai Sailing Program (20YF1400200), the Fundamental Research Funds for the Center Universities (20D111324) and the National key research and development project (2019YFC0408603).

Author contribution Yuhui Wang: Conceptualization, Methodology, Writing-original draft. Panpan Zhou: Data curation and analysis. Xinshan Song: Writing-review & editing. Zhongshuo Xu: Investigation, Supervision, Writing-review & editing.

Data availability The manuscript has not been published elsewhere. The article is not a consideration in any other journal in full or in part.

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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Figures

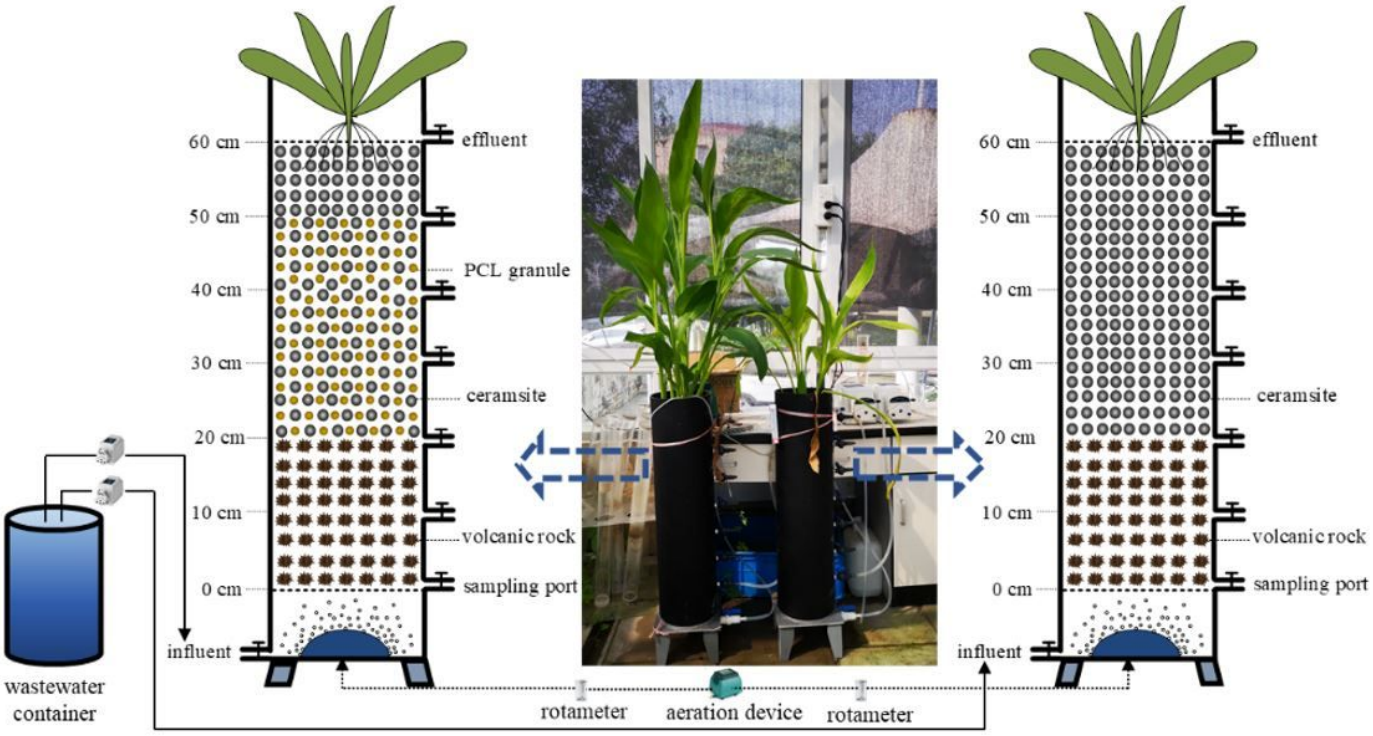


Figure 1

Scheme of the simulated VFCWs

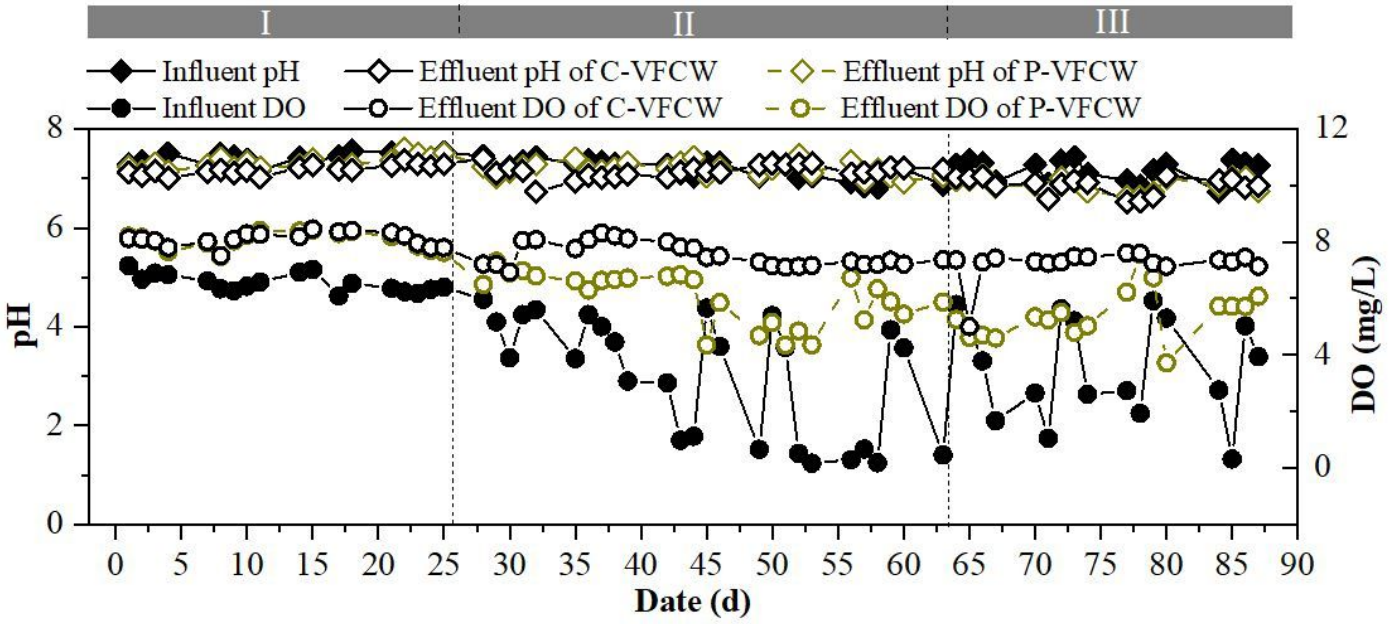


Figure 2

Variations of pH and DO in C-VFCW and P-VFCW

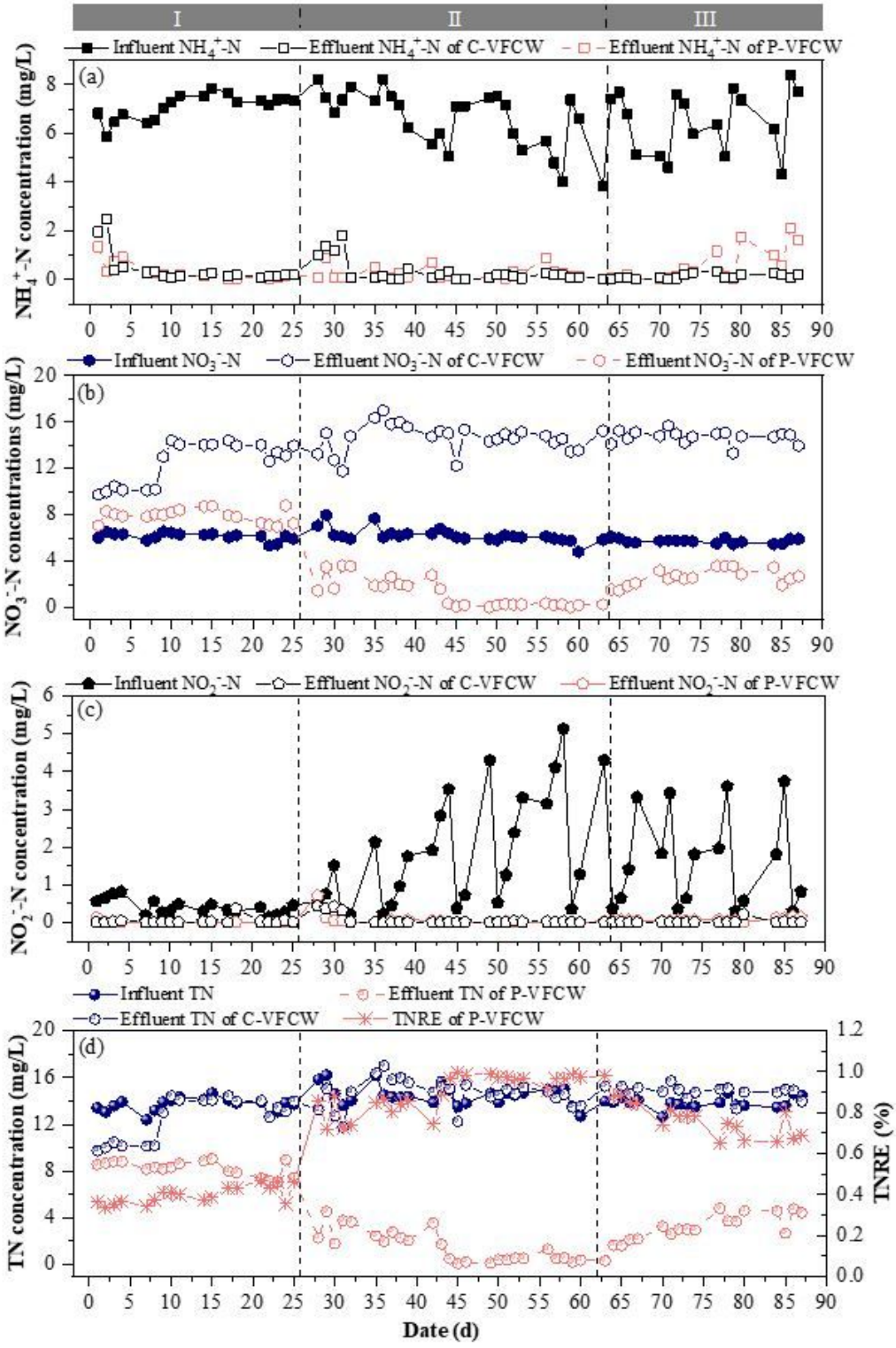


Figure 3

Nitrogen removal performance in C-VFCW and P-VFCW

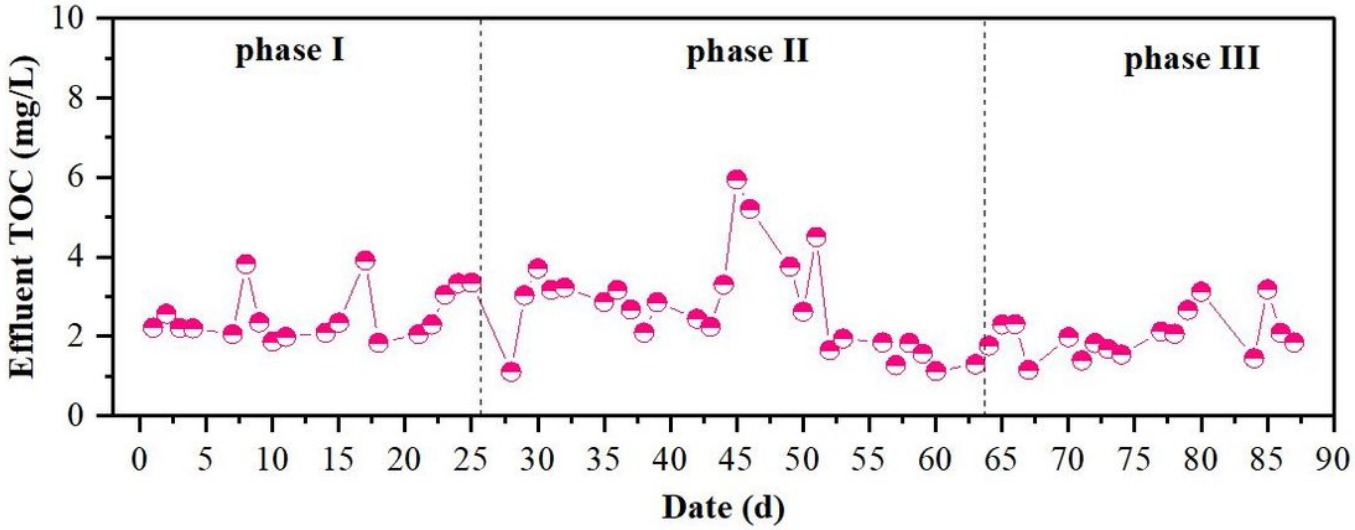


Figure 4

Effluent TOC characteristics in P-VFCW

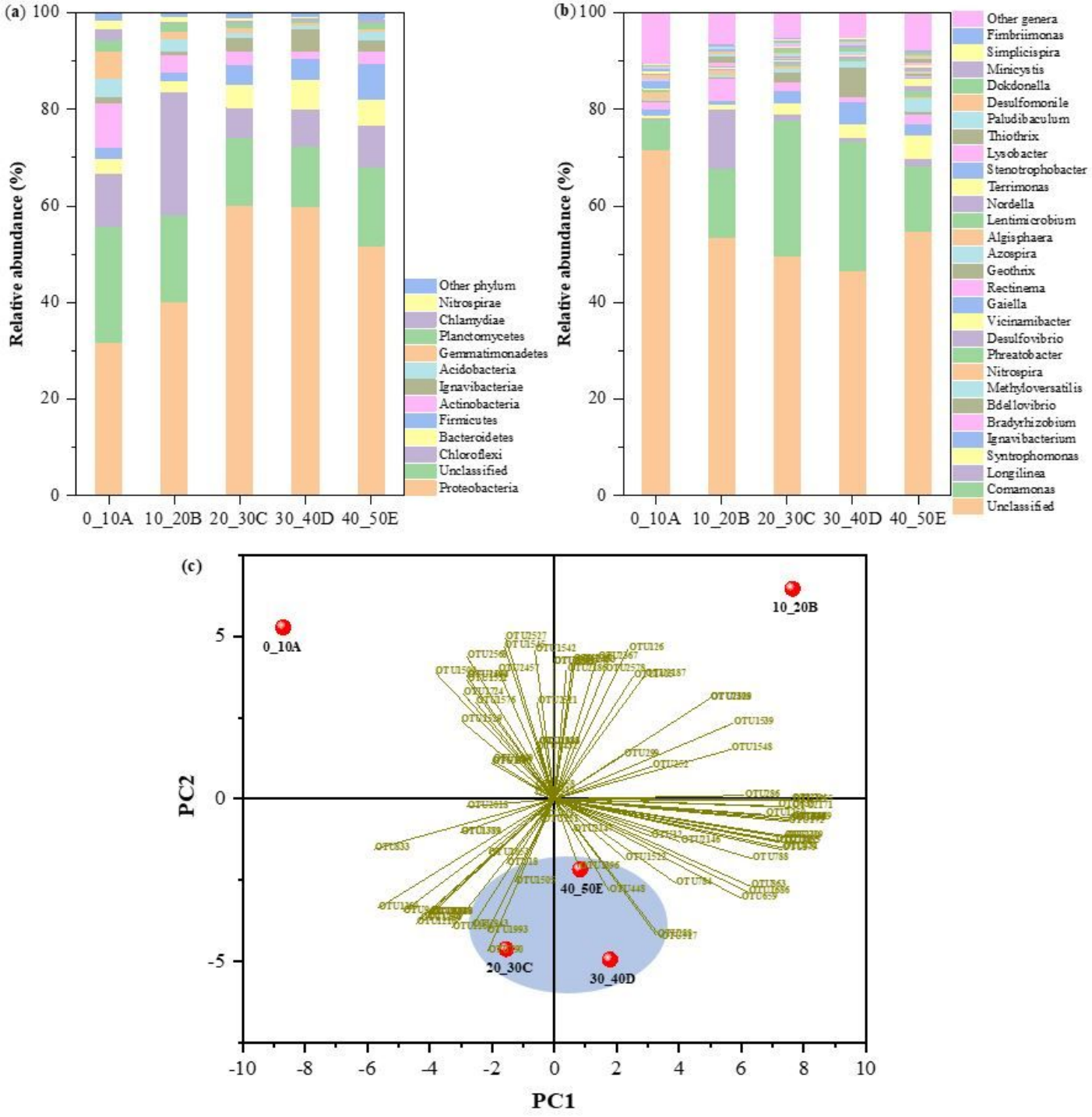


Figure 5

The taxonomic affiliation and relative abundance of major groups at phylum (a)　genus (b) level and the PCA analysis among five samples along P-VFCW (c)

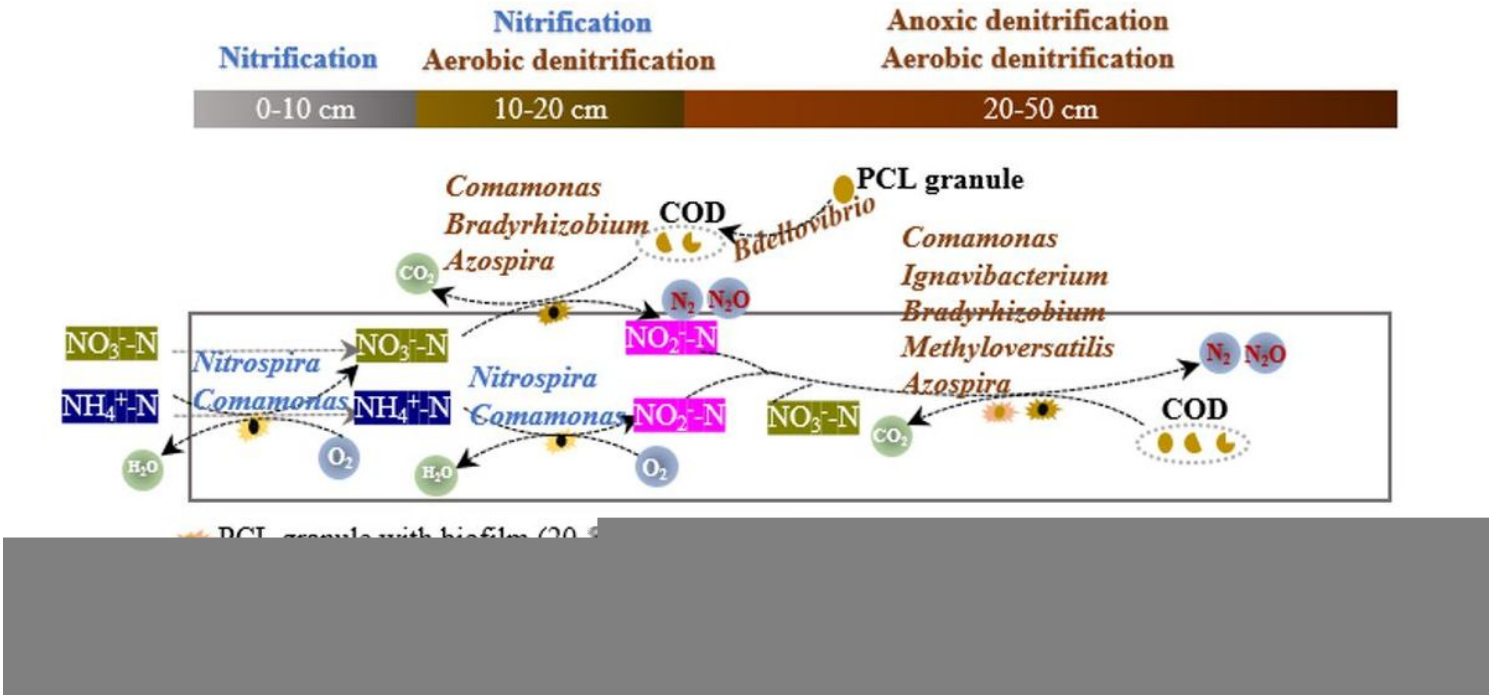


Figure 6

Simultaneous nitrification and denitrification mechanism in aerated P-VFCW