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Application Potential of Constructed Wetlands on Different Operation Mode for Biologically Pre-Treatment of Rural Domestic Wastewater

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Abstract: In order to satisfy the requirements of rural domestic sewage, a bio-ecological combination system was proposed, including a biological treatment section (anaerobic hydrolysis tank and aerobic tank) and an ecological post-treatment section. This study observed the application potential of constructed wetlands (CW) on different operation modes for biologically pre-treated rural domestic wastewater. The organics and nutrient removal efficiency of the tidal flow constructed wetland (TFCW) and the horizontal subsurface flow constructed wetland (HFCW) were compared at a temperature range of 20–40 °C. During the stable phase, the higher chemical oxygen demand (COD), ammonia nitrogen (NH_4^+-N), and total phosphorus (TP) removal efficiencies existed in TFCW than HFCW, corresponding to the efficiency of COD 69.46%, NH_4^+-N 96.47%, and TP 57.38%, but lower performance on COD (61.43%), NH_4^+-N (84.99%), and TP (46.75%) removal in HFCW, which should be attributed to the increasement of aerobic heterotrophic bacteria (*Arthrobact* and *Sphingomonas*), nitrifiers (*Nitrospira*), and phosphate accumulating organisms (PAOs) (*Pseudomonas*). The microbial biomass was also increased from 2.13 ± 0.14 mg/g (HFCW) to 4.64 ± 0.18 mg/g (TFCW), which proved to strengthen the formation and growth of biofilm under a better oxygen supplement. Based on the relative abundance of functional genera in the microbial community, it showed that TFCW was more favorable for promoting the growth of heterotrophic bacteria, nitrifiers, and phosphate-accumulating organisms (PAOs). When temperature changed from −4 °C to 15 °C, the two-stage constructed wetlands (TFCW-HFCW and HFCW-TFCW) were used for improving the performance of pollutants removal. The results demonstrated that the effluent concentrations of TFCW-HFCW and HFCW-TFCW met the Class 1A discharge standard of DB32/3462-2020 in JiangSu Province, China. Therefore, this study will provide a useful and easy-to-implement technology for the operation as an ecological post-treatment section.

Keywords: tidal flow constructed wetlands; horizontal subsurface flow constructed wetland; two-stage constructed wetland; pollutant removal; microbial community and diversity

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1. Introduction

In recent years, the treatment of decentralized domestic wastewater in rural areas has attracted much attention [1]. Unlike urban domestic wastewater, rural domestic wastewater has lower concentrations of pollutants [2]. As much as 90% of untreated rural domestic sewage is discharged directly into lakes, rivers, and nearby surface water bodies, making it widely distributed and scattered. [3]. Moreover, water resource sustainability and fresh water supplies are major threats to public sanitation and rural residents' health status. It was not feasible to build a "scaled-down" sewage treatment plant in rural China due to extensive pipe networks and high operation costs. In addition, there is often a change in the quality, quantity, and spatial distribution of rural wastewater; therefore, centralized treatment technology used in municipalities is unsuitable for the treatment of rural wastewater [4]. Rural wastewater is generally characterized by a small amount, a

large pollution range, and intermittent discharge [5]. As a result, it is imperative to develop a simpler, environmentally sustainable, economically affordable, and socially acceptable alternative process to treat rural domestic wastewater.

A great deal of attention has been paid to the study of technologies for rural domestic wastewater treatment. It was reported that subsurface wastewater infiltration systems [6], vertical-flow multi-soil-layering systems [7], septic tanks [8], the activated sludge process [9], modified membrane bioreactors [10], and a three-stage A/O plug-flow step-feed bioreactor [11] had been used for the treatment of rural sewage. These processes can be roughly divided into three categories: ecological treatment processes, activated sludge processes, and integrated bioreactors. However, some shortcomings exist, including a large space occupation in the ecological treatment process, a complex process and professional management in the activated sludge process, and high energy consumption in the integrated bioreactor [12]. To better solve these problems, previous studies have shown that bio-ecological combination systems are seen to be the effective ways for rural domestic wastewater treatment [13,14]. Bio-ecological combination systems possess lower energy consumption, little land utilization, easy management, and flexible operation compared with traditional technologies [14,15]. The biological treatment section would mainly remove organics and ammonia nitrogen, and the subsequent ecological treatment section would remove residual nitrogen and phosphorus [15].

In the development of bio-ecological combination systems, some issues are still not clear. For rural wastewater treatment, previous studies usually focused on the biological treatment optimization parameters in pollutants removal. However, the ecological treatment system's performance to adapt biologically treated rural domestic wastewater is still unclear. As reported, microbial activity, substrate utilization rates, and adsorption rates in biological processes were significantly limited by the low temperatures [16]. Due to the continental monsoonal climate in China, temperatures in domestic sewage have been measured to be as low as 8–15 °C in cold climates, and even below 5 °C has been recorded [17]. It is known that low temperatures can significantly decrease wastewater removal efficiency in biological wastewater treatment facilities [18]. To meet the emission requirements, especially at low temperatures, the pollutants removal performance of different types of ecological treatment technologies remains to be explored in the whole bio-ecological combination system. Note that there is a challenge in selecting suitable technology for ecological treatment, which demands efficient performance, low operating cost, easy maintenance, and convenient operation.

Constructed wetland (CW) is one of the most commonly used ecological technologies owing to its high pollutant removal efficiency, low operating costs, stable operation, and excellent ecological performance [19]. Over the past 50 years, horizontal subsurface flow constructed wetlands (HFCW) have been widely used for sewage treatment [20]. In the HFCW system, the wetland bed has a poor oxygen environment and a low ability to reoxygenate due to the long-term submergence of the substrate [21]. An innovative enhanced-oxygen operation is employed in tidal flow constructed wetland (TFCW), which has been shown to be an effective technology for improving the performance of CWs [22–24]. The TFCW acts as a passive pump, repelling and drawing oxygen from the environment into the matrices in a periodic cycle of "flooding" and "drying" periods [25]. TFCW can greatly improve reoxygenation effect via the tidal operation strategy, and the reported oxygen transfer rate would reach up to 450 g/(m^2·d) under operation with the optimal duration of "wet" and "dry" phases, significantly higher than in conventional CWs [26]. However, the different operation mode on constructed wetlands of ecological system with long-term operation is still unclear. Additionally, the ecological system to adapt temperature change remains to be explored. Therefore, TFCW and HFCW were constructed as the ecological post-treatment sections for the biological pre-treatment of rural domestic wastewater at medium-high temperatures (20–40 °C). The performance using single-stage constructed wetlands is not satisfactory due to the fact that they cannot provide good nitrification and are not favorable for denitrification in cold climates [27]. In regions with

cold climates, multi-stage hybrid CWs have been shown to be effective at dealing with high-strength sewage [28]. The adoption of a hybrid-CW system involving HFCW-VFCW or VFCW-HFCW in a simultaneous combination under cold climates is usually the most effective way to overcome the performance limitation [29]. Thus, two-stage constructed wetlands provide better performance in contaminant removal at low temperatures than single-stage constructed wetlands. To sum up, it is necessary to explore the application potential of different operation modes on constructed wetlands for biologically treated rural domestic wastewater.

In the current study, a bio-ecological combination treatment system, consisting of a biological treatment section (anaerobic hydrolysis tank and aerobic tank) and an ecological post-treatment section, was developed for treating wastewater. The anaerobic hydrolysis tank as a pre-treatment section was supposed to complete the anaerobic hydrolysis process and digest macromolecular organic matter under an appropriate hydraulic retention time (HRT) and organic load rate (OLR). This treatment was followed by an aerobic tank for organics and ammonia nitrogen removal. The ecological post-treatment treatment was designed to further remove the organic matter and nutrients. This article will focus on the ecological post-treatment section, considering the difference and application significance of pollutant removal for biologically pre-treated rural domestic wastewater. Experiments in traditional operation (HFCW) and tidal flow operation (TFCW) in this study were carried out at a temperature range of 20–40 °C. The TFCW-HFCW (TFCW as the first stage and HFCW as the second stage) and HFCW-TFCW (HFCW as the first stage and TFCW as the second stage) systems were set up to investigate pollutant removal at low temperatures ((−4)–15 °C). The objectives of this study were to: (1) explore the removal of organics and nutrients of the two CWs during the long-term operation; (2) assess and analyze the differences in bacterial community structure and microbial diversity between the two CWs; (3) investigate the performance of pollutants removal in TFCW-HFCW and HFCW-TFCW and explore main contributions of pollutants removal at low temperature.

2. Materials and Methods

2.1. Schematic of the Experimental Devices

The whole bio-ecological system was established at the Wuxi campus of Southeast University in Jiangsu province, China (31°28′55.02″ N, 120°22′33.71″ E). Figure 1 shows a schematic illustration of the whole bio-ecological system. The raw sewage was first pumped up to the elevated tank and then entered the anaerobic hydrolysis tank (material: PVC; L × W × H = 1.5 m × 1.5 m × 1.5 m). Combined vertical packing was used in the anaerobic hydrolysis tank to maintain sludge concentration and provide a growing environment for microorganisms. The effluent from the anaerobic hydrolysis tank was introduced to the aerobic tank. Vertical combined packings were also packed in the aerobic tank (material: PVC; length: L × W × H = 1.3 m × 1.3 m × 1.0 m). Moreover, five microporous aerators were installed at the bottom of the aerobic tank. The sewage from the aerobic tank was stored in a storage tank to conduct the experimental study.

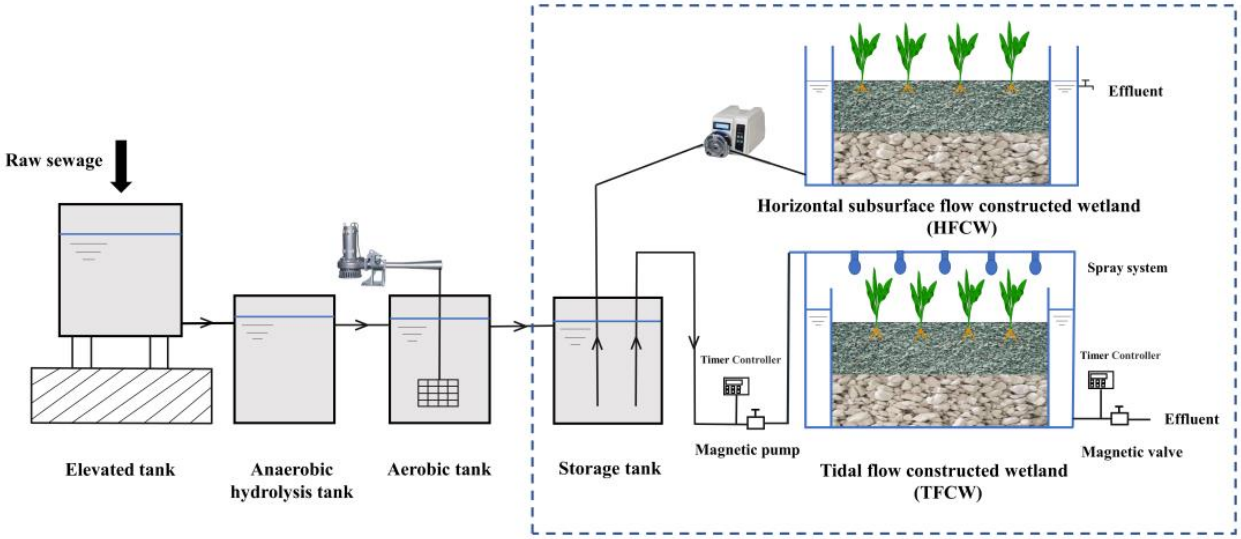

Figure 1. Schematic illustration of the whole bio-ecological system.

The experiments focused on the ecological post-treatment section, including TFCW and HFCW. Two parallel laboratory-scale CWs (TFCW and HFCW) were operated under the same operating conditions. The downflow TFCW and HFCW systems mainly consisted of an inlet area, treatment area, and outlet area. In order to distribute the flowing water evenly, the adjacent area of HFCW was separated by clapboard with uniformly small holes. Continuous operation in HFCW was controlled by a peristaltic pump. In the TFCW system, water distributors consisting of perforated PVC pipes were installed for uniform water distribution. A tidal operation was generated by a spray system and an automatic drain valve controlled by a timer. The two CWs were made of polypropylene (PP) material, and each device was cuboid (L × W × H = 1.22 m × 0.32 m × 0.67 m) with a volume of 260 L. The total thickness of the substrate layers of each system was 40 cm from the top to bottom layers, filled with 20 cm of green zeolite (particle size: 6–12 mm) and 20 cm of limestone (particle size: 10–20 mm), respectively. The porosity was approximately 46%, and water spinach (*Ipomoea aquatica* Forsk) was planted with an initial density of 100 plants/m^2, both in the TFCW and HFCW.

The two-stage CW systems of TFCW-HFCW and HFCW-TFCW are constructed and have been operated in cold climates (Figure S1). Both the TFCW-HFCW and HFCW-TFCW were planted by water dropwort (*Oenanthe javanica* DC) on the surface of the substrate with 100 plants/m^2 density at low temperatures. During the whole experimental phase, the single-stage CW system was performed at a temperature range of 20–40 °C, while the two-stage CW system was conducted between −4 °C and 15 °C.

2.2. Sewage Characterization and Experimental Setup

The wastewater from the ecological post-treatment system was the effluent of the aerobic tank that treated domestic wastewater from a collecting tank; it came from the teaching building, dormitories, and canteens at the Wuxi campus of Southeast University located in Wuxi, China. The primary pollutant concentrations in the influent of the ecological post-treatment system are shown in Table 1.

Table 1. Primary pollutant concentrations of the influent of the ecological post-treatment system.

Type	T (°C)	COD (mg/L)	NH_4^+-N (mg/L)	NO_3^--N (mg/L)	TN (mg/L)	TP (mg/L)	DO (mg/L)
single-stage CW	20–40	59.93–127.67	7.16–14.25	10.04–17.25	21.51–30.36	1.64–2.44	2.48–4.35
two-stage CW	(−4)–15	98.98–132.38	10.23–15.88	13.52–20.46	25.32–34.69	1.93–2.38	3.32–6.56

Operation was controlled by an automatic control system (Timer controller) using the "tidal flow" mode, consisting of the four phases (fill-contact-drain-rest). During the fill phase, sewage from the storage tank was pumped rapidly (15 min) onto the substrate bed via the perforated PVC pipes, and after filling, the duration of contact time was 36 h. After this all wastewater was drained out rapidly (15 min), and the bed was kept to rest for 6 h. The whole tidal cycle was 42 h with the hydraulic load of 0.32 $m^3/(m^2 \cdot d)$ for TFCW. To minimize variability in the experiment, the hydraulic retention time (HRT) was set to 42 h, with the same hydraulic load of 0.32 $m^3/(m^2 \cdot d)$ in HFCW. During the start-up phase, microbial accumulation and plant growth lasted for a month. After that, the duration of the long-term experiment with repeated cycles in the TFCW was 180 days, and continuous operation of the HFCW was also conducted under a 180-day operation.

The effluent processed from the storage tank became the influent of the two-stage CW system through the pump, going through the first-stage CW (TFCW or HFCW), then being collected in the regulating tank and sequentially pumped into the second-stage CW (HFCW or TFCW). During the operation of the single-stage CW system, the whole experiment was carried out for nearly 180 days, including a start-up phase (from 0 to 30 days with 10 samples) and a stable phase (from 31 to 180 days with 26 samples) under two kinds of operational mode. Furthermore, the duration of the steady-state operation of two-stage CW systems at a low temperature lasted for 60 days, and water samples were collected from the storage tank and outlet of TFCW-HFCW and HFCW-TFCW systems every 6 days.

2.3. Analytical Methods

Water samples from the inflow and outflow were collected in triplicate and immediately taken to the laboratory for evaluation and the removal of organic matter, nitrogen, and phosphorus. Chemical oxygen demand (COD), ammonia nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), total nitrogen (TN), and total phosphorus (TP) concentrations were determined based on the standard methods for the examination of water and wastewater [30]. The dissolved oxygen (DO) was measured using a DO JPSJ-605F meter (REX Company, Shanghai, China). Detailed methods on substrate analysis of microbial biomass and enzyme activity, as well as microbial nitrification and denitrification intensity, were provided in the supplemental material.

2.4. Microbial Community Analysis

After the 180-day operation experiment, the biological film samples of each wetland system were collected, and then the microbial communities were identified by high-throughput sequencing. An E.Z.N.ATM Mag-Bind Soil DNA Kit (OMEGA Bio Tec, Shanghai, China) was used to extract microbial DNA from the samples following the manufacturer's instructions. The DNA extraction and PCR amplification were described in detail in the former study [31]. The bacterial 16S rRNA gene V3–V4 region was PCR amplified from DNA samples with 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) primers [32]. Sequencing was performed on the Illumina MiSeq sequencing platform using the Mothur program (v. 1.43.0) to determine the microbial community structure. The high-quality sequences were organized into operational taxonomic units (OTUs) at a 97% identity threshold. For community structure analysis, the final OTUs were taxonomically classified using Blast against a curated database based on the Ribosomal Database Project (RDA) and the National Center for Biotechnology Information (NCBI).

2.5. Data Analysis

The statistical analyses were processed with Origin Pro 2018b software (Origin Lab Corporation, Northampton, MA, USA) and SPSS statistics software (version 25.0; IBM, New York, NY, USA). The one-way analysis of variance (ANOVA) was used to test for significant differences among different samples. Results were identified as significant when $p < 0.05$.

3. Results and Discussion

3.1. Performance of Pollutant Removal during the Long-Term Operation

The removal performance of COD, NH_4^+-N, NO_3^--N, TN, and TP in TFCW and HFCW during the long-term performance is shown in Figure 2. The experiment consisted of two phases: a start-up phase (phase I) and a stable phase (phase II). During Phase II, the average concentrations of influent COD, NH_4^+-N, NO_3^--N, TN, and TP were 85.59 ± 16.36 mg/L, 11.08 ± 1.51 mg/L, 14.05 ± 1.69 mg/L, 25.97 ± 2.08 mg/L and 1.98 ± 0.20 mg/L. The variations of COD in influent and effluent in HFCW and TFCW during the 180-day running are presented in Figure 2a. During the Phase I, the removal efficiency of COD increased from 39.39% to 62.73% and 33.17% to 54.53% with effluent concentration changing from 50.56 mg/L to 29.24 mg/L and 55.76 mg/L to 35.67 mg/L in TFCW and HFCW, respectively. During the phase II, the TFCW system obtained an average COD effluent concentration and a removal efficiency of 25.94 mg/L and 69.46%, compared with that of 32.81 mg/L and 61.43% in HFCW, respectively. The COD effluent concentration of TFCW and HFCW met the DB32/3462-2020 Class 1A discharge standard (below 50 mg/L). Nevertheless, the statistically significant difference was found between the two CWs ($p < 0.05$). These results suggested that the TFCW system could obtain a better performance of organic matters compared with the HFCW system.

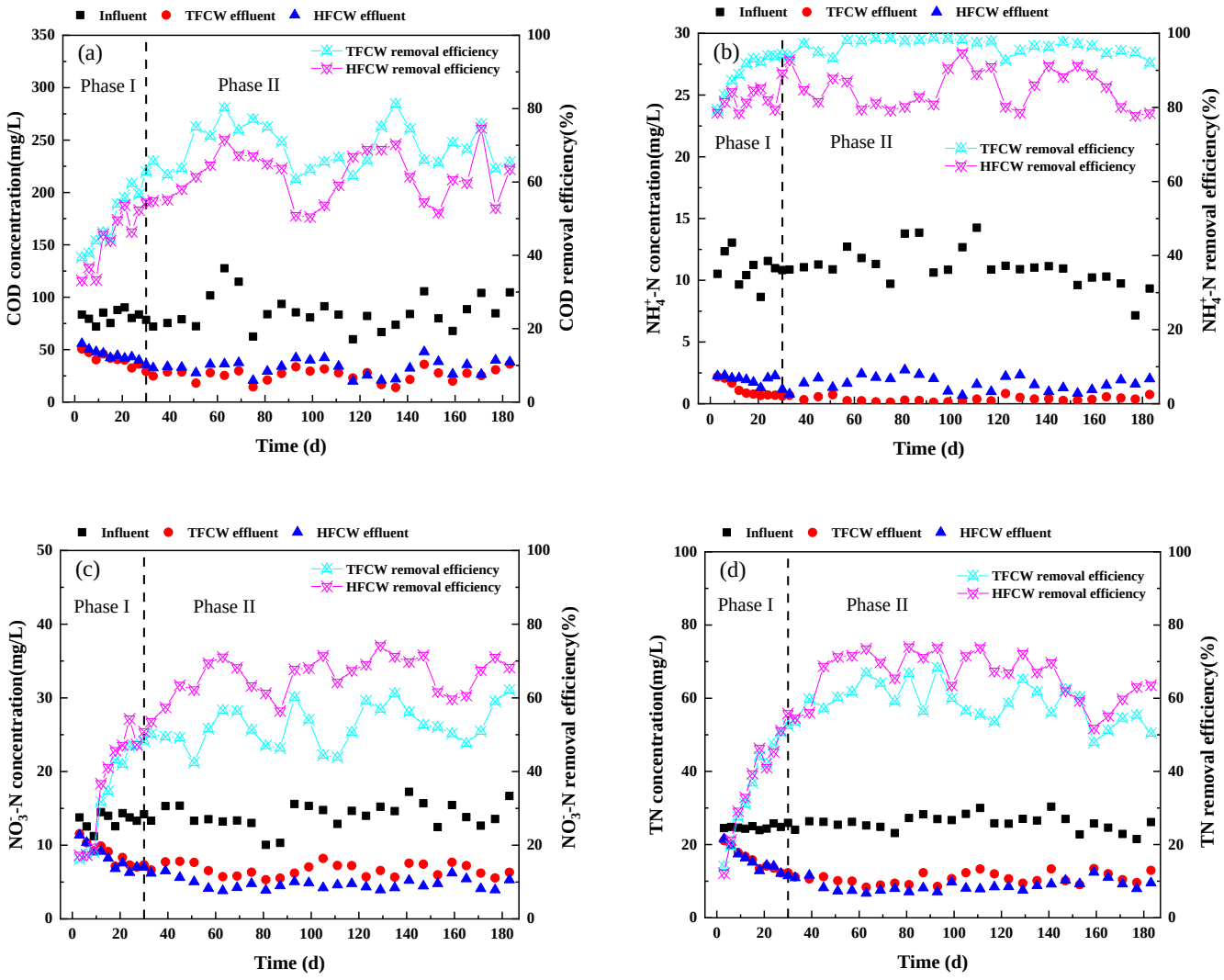

Figure 2. *Cont.*

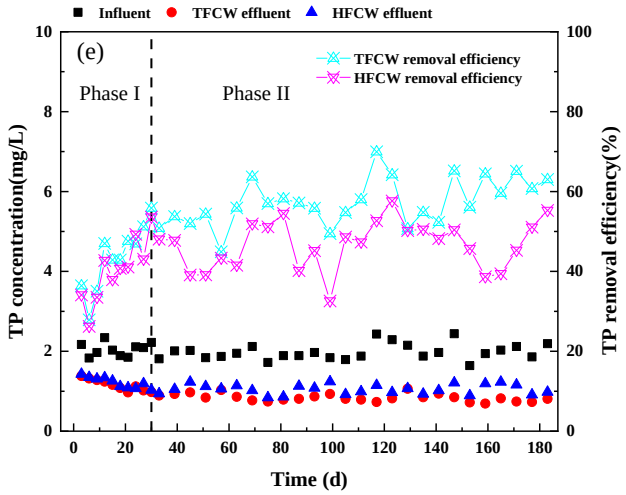

Figure 2. The removal performance of (**a**) COD, (**b**) NH_4^+-N, (**c**) NO_3^--N, (**d**) TN, and (**e**) TP in TFCW and HFCW during the long-term performance.

The tidal operation mode with a regular cycle of a "filled/wet" phase and a "drained/dry" phase greatly improved the reoxygenation capacity of the wetland bed and promoted the removal efficiency of COD in TFCW [33]. Under the regular alternation of the "wetting" and "drying" phases, the relatively aerobic condition in TFCW benefited the nitrification process and organic matter degradation by common nitrifying bacteria and heterotrophic bacteria [34]. In addition, most oxygen is preferentially consumed in organics degradation [35]. Previous studies also indicated that DO usually dropped to a low level within a short distance owing to more oxygen consumption on pollutant degradation than oxygen supply in the HFCW system [36]. Therefore, the tidal operation is beneficial as it enhances the DO capacity, creating an aerobic environment for aerobic microorganisms to utilize organics.

The difference in NH_4^+-N removal in the two CWs during the 180-day operation is presented in Figure 2b. During phase I, the ammonia-nitrogen removal showed an increasing trend, both in TFCW and HFCW. The main mechanism for ammonia-nitrogen removal may be attributed to substrate absorption during the start-up phase. During phase II, the NH_4^+-N effluent concentration was mostly under 2.50 mg/L, with an average value of 1.65 mg/L in HFCW, while the effluent concentration was only 0.38 mg/L on average in TFCW. The removal efficiencies of NH_4^+-N in TFCW (91.96–98.87%) were significantly higher than those in HFCW (77.79–96.44%) during the stable operation ($p < 0.05$). The possible mechanism behind the increased removal performances of TFCW could be explained by the tidal operation strategy which has potential to promote nitrification. The important parameter for nitrification is the presence of oxygen, and the DO concentration was quite good (2.17 mg/L on average) during the experiment. As reported, the DO concentration should be higher than 1.5 mg/L, which is necessary for nitrification [37]. Sufficient oxygen (DO > 1.50 mg/L) promoted nitrifying bacteria growth and enhanced efficiency for nitrification ($2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 2H_2O + 4H^+$ + energy, and $NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$) [38,39]. This illustrated that the repeated "dry phase" and "flood phase" would improve overall NH_4^+-N removal efficiency. The presence of microbial genera representing bacterial nitrificans and microbial nitrification intensity are discussed in the following Section 3.2. Thus, TFCW performed better on ammonia nitrogen removal during the stable operation, which related to a better oxygen supplement by tidal operation mode, resulting in better microbial nitrification process.

The removal performances of NO_3^--N and TN were explored and found to be stable after 30 days in the two CWs (Figure 2c,d). The TN removal efficiency in HFCW effluent (66.03% on average) was higher than that of TFCW (58.58% on average), corresponding to average concentrations of 8.76 mg/L and 10.75 mg/L, respectively. During phase II, the average NO_3^--N removal efficiency in the HFCW and TFCW was 65.76% and 52.37%,

respectively. The TN removal efficiency exhibited similar differences as with NO_3^--N between the two CWs, which indicated the stability of effluent nitrogen related to NO_3^--N reduction. The higher NO_3^--N and TN removal performances of HFCW could be explained by the following factors: first, this aerobic condition (2.17 mg/L of DO on average) and low COD/TN ratio (influent COD/TN = 3.31 on average) in TFCW were not beneficial for the denitrification process and further limited the TN and NO_3^--N removal efficiencies. Due to the better performance on NH_4^+-N removal in TFCW, the majority of the ammonia nitrogen was converted to nitrate nitrogen during Phase II. A former study also showed that substantial NO_x^--N accumulation would likely occur with intermittent aeration in a CW system when the influent COD/TN ratio was below 5 [40]. Despite the effective reoxygenation of TFCW in the performance improvement of NH_4^+-N and COD removal, the anaerobic environment in TFCW and the activity of denitrifiers were quite limited, resulting in an accumulation of effluent nitrate and low TN removal [26,37,41]. Therefore, the growth of denitrifying bacteria to consume nitrate is limited in TFCW. Secondly, the substrate layers of HFCW were in low-oxygen conditions (1.33 mg/L on average), where denitrifying bacteria were effectively enriched. It has been proved that high NO_3^--N removal efficiency can be achieved through denitrification process under low DO condition changing from 1.06 mg/L to 1.66 mg/L in the HFCW ecosystem [42].

The results obtained during the experimental phase for TP removal were promising in TFCW, whereas lower removal efficiency was observed in HFCW (Figure 2e). During phase II, an average TP effluent concentration of 0.84 mg/L and a removal efficiency of 57.38% were reached in TFCW, while in HFCW these were 1.05 mg/L and 46.75%, respectively. The discharge rate of TP to meet the Class 1A discharge standard (below 1.0 mg/L) of DB32/3462-2020 in TFCW and HFCW under the stable phase was 92.31% (24 of 26 samples) and 38.46% (10 of 26 samples). The results suggested that TP was removed more effectively in TFCW than HFCW. This phenomenon might be explained by operational strategies such as flooding, aeration (tidal, intermittent, etc.) [43]. Phosphorus removal in CWs is thought to be mostly related to plant absorption, normal microorganism assimilation, excessive phosphorus uptake by polyphosphate accumulating bacteria (PAOs), and substrate adsorption [44]. It has been reported that TP removal was mainly accomplished by the chemical absorbance of substrate in CWs [45,46]. However, the results showed effective improvement on the TP removal in TFCW than that in HFCW though they filled with the same substrates and cultivated the same plants ($p < 0.05$). It can be inferred that tidal operation mode is associated with the periodically changing aerobic-anaerobic condition, which may enhance the phosphorus absorbance by PAOs. Apart from the chemical adsorption of the substrate and plant absorption, that is another mechanism for phosphorus removal occurred in TFCW. The related functional bacterial genera distribution confirmed this deduction, which would be discussed in the following section.

3.2. Microbial Community Structure and Bacterial Diversity in the TFCW and HFCW

The tidal operation modes ("dry phase" and "flood phase") would have better oxygen supplement efficiency by exposing the wetland bed to the atmosphere for a period of time after the drainage period. By this way, the presence of a better oxygen supplement efficiency strengthened the formation and growth of biofilm on the substrate surface for ammonia and COD oxidation. Thus, the microbial biomass and activity and the microbial nitrification and denitrification intensity would be influenced due to the different reoxygenation environment. A total of 161,622 raw sequence reads were obtained by Illumina MiSeq™/Hiseq™ sequencing of 16S hypervariable regions V3 and V4, and then they could be used for analysis of microbial diversity and community under the same sequencing depth after normalization. Table 2 showed the OUTs, diversity, and richness estimators of microbial samples collected after 180 days of running when the two CW systems were stable. The coverage of the samples in the two CWs was 0.996, indicating that the results of the high-throughput sequence were representative and reliable. The richness indexes of the microbial community (Chao and Ace) were higher in TFCW, which reflected

the acceleration of microbial growth in a better oxygen environment (Figure 3a). A higher Shannon index, chao index, and ACE index demonstrated that TFCW contained a more diverse and abundant biological diversity [47].

Table 2. Microbial richness and bacterial diversity in two CWs.

Samples	OTUs	Shannon Index	Chao	ACE Index	Coverage
TFCW	1377	5.42	1658.04	1615.98	0.996
HFCW	1253	4.66	1497.45	1478.52	0.996

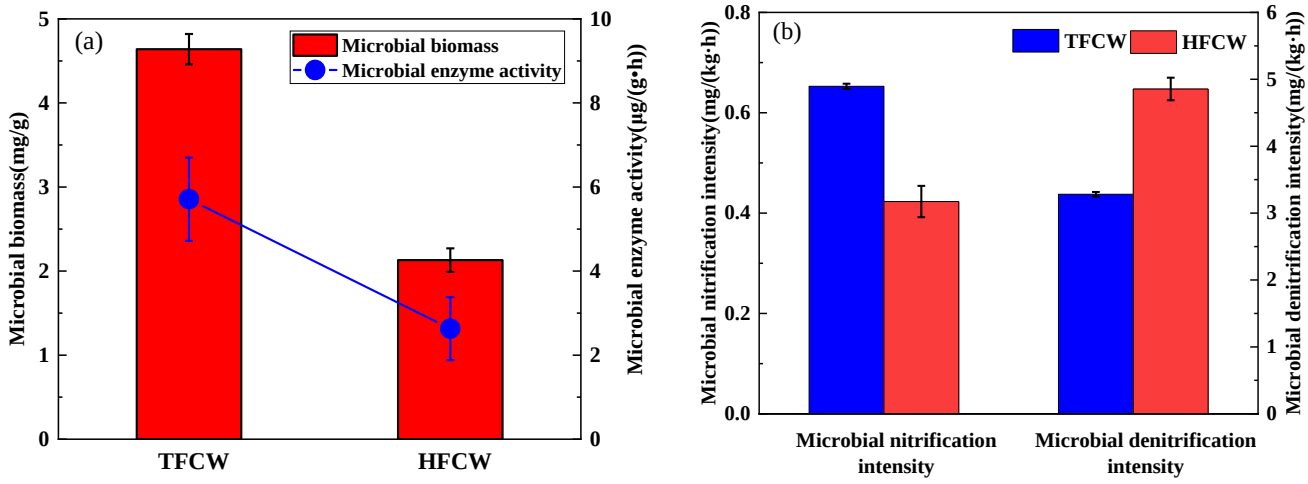

Figure 3. Evaluation the difference of microbial activity between in TFCW and HFCW: (**a**) microbial biomass and enzyme activity; (**b**) microbial nitrification and denitrification intensity.

The difference in microbial activity between TFCW and HFCW is shown in Figure 3. As shown in Figure 3a, the microbial biomass and microbial enzyme activity in TFCW were 4.64 ± 0.18 mg/g and 5.71 ± 0.99 μg/(g·h), which were higher than those in HFCW (2.13 ± 0.14 mg/g and 2.63 ± 0.75 μg/(g·h)). As reported, the greater number of microorganisms, species, and ecological positions were associated with stronger enzymatic degradation activity and efficiency during the biodegradation process [48]. Therefore, these results indicated that better oxygen was beneficial to the growth of organisms. Generally, the abundance of nitrifying bacteria and denitrificans positively correlated with nitrification and denitrification rates, respectively [49]. As presented in Figure 3b, microbial nitrification intensity in TFCW (0.65 mg/(kg·h) on average) was higher than HFCW (0.44 mg/(kg·h) on average), but microbial denitrification in TFCW (3.17 ± 0.23 mg/(kg·h)) was lower than HFCW (4.86 ± 0.17 mg/(kg·h)). These results explained that NH_4^+-N removal of TFCW was better than that in HFCW, but TN removal efficiency was opposite between the TFCW and HFCW.

Figure 4a shows the relative abundance at phyla level from microbial samples in the two CWs. In general, *Proteobacteria*, *Bacteroidetes*, and *Chloroplast* have been found to be widespread in CWs [50]. In TFCW, *Proteobacteria* accounted for the highest percentage of phyla (43.63%), followed by *Bacteroidetes*, *Chloroplast* and *Planctomycetes*, with proportions of 16.84%, 9.92%, and 5.23%, respectively. Nonetheless, *Bacteroidetes* were the dominant phyla in HFCW (32.32%), which contained a relatively large proportion of *Proteobacteria* (27.74%) and a considerable fraction of *Saccharibacteria* (13.15%) but least *Nitrospirae* (0.19%).

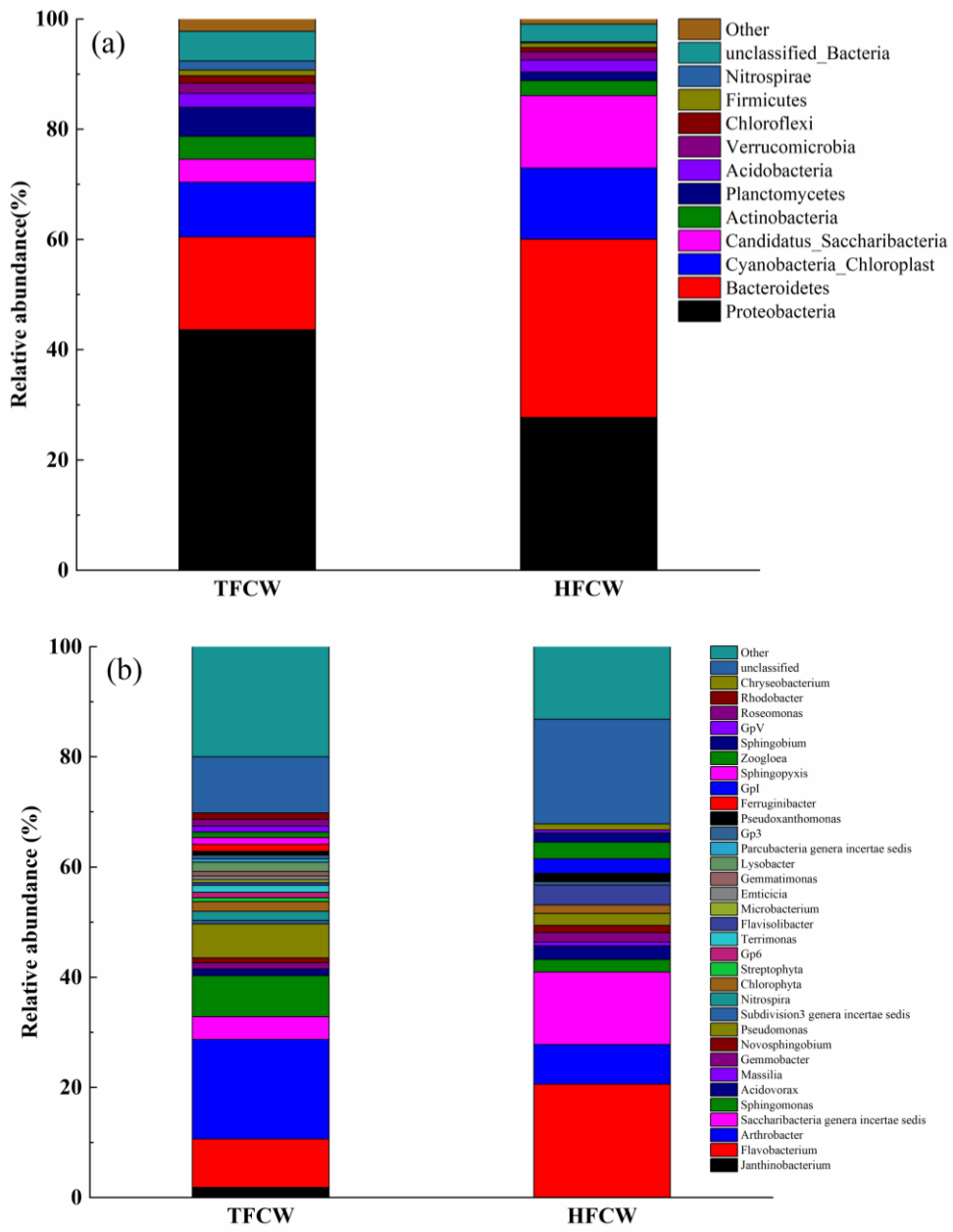

Figure 4. Relative abundance at the (**a**) phyla and the (**b**) genera levels (<0.5% defined as Others) within the different CWs.

As presented in Figure 4a, the percentages of *Proteobacteria* and *Nitrospirae* in TFCW were 15.89% and 1.65% higher than those in HFCW, respectively, which might be related to the better removal of organics, nitrogen, and phosphorus in TFCW. As reported, *Proteobacteria* contained many species involved in carbon and nitrogen cycling, which played a key role in the removal of conventional pollutants [51,52]. The proportion of *Bacteroidetes*, which was a typically related phylum to heterotrophic denitrification in CWs [53], was 15.48% in HFCW, and higher than that in TFCW. A limited dissolved oxygen environment existed in HFCW, creating an anaerobic environment for the growth of denitrificans. It was reported that *Proteobacteria* also was supposed to be recognized as the dominant PAOs and nitrifying strain in denitrifying phosphorus removal system [54], which explained the significant difference on phosphorus removal in the TFCW and HFCW.

Figure 4b shows the relative abundance (>0.5%) of different bacteria at genera level within the different CWs. A total of 29 genera were identified, and more genera were detected by high throughput sequencing in TFCW, which was in accordance with the

higher Shannon index of the microbial sample. *Arthrobact* was the dominant genus in TFCW with an abundance of 18.06%, followed by *Flavobacterium* (8.76%), *Sphingomonas* (7.42%), *Pseudomonas* (6.18%), *Saccharibacteria genera incertae sedis* (4.16%). The relative abundance of *Arthrobact* and *Sphingomonas* in TFCW was higher than that in HFCW (7.20% and 2.27%). *Arthrobact* has been proven to be related to the degradation of various organic pollutants [55]. *Sphingomonas,* a common aerobic heterotrophic bacteria, was also reported to be involved in the degradation of organics [56]. While in HFCW, the top five genera were *Flavobacterium* (20.59%), *Saccharibacteria genera incertae sedis* (13.15%), *Flavisolibacter* (3.62%), *Zoogloea* (3.02%) and *Acidovorax* (2.48%), which were supposed to contain common denitrifying bacteria; together, these accounted for 42.86% of the detected genera [49,57–60]. In contrast, *Flavobacterium*, *Saccharibacteria genera incertae sedis*, *Acidovorax* and *Zoogloea* were the main detrifiers, accounting for 15.16% in TFCW. Except for denitrifying bacteria, *Nitrospira,* which were detected in two CWs, had a higher relative abundance in TFCW (1.66%), leading to a better performance of nitrification (96.49% in TFCW vs. 84.83% in HFCW) (Figure 2b). The results indicated that *Nitrospira* played pivotal roles in nitrification process [61]. *Pseudomonas* has been proven to be capable of the degradation of various polymers and TP removal [62]. As a whole, compared with HFCW, TFCW was more beneficial as a supporter for the growth of heterotrophic bacteria, nitrifiers, and PAOs.

The microbial community structure and microbial activity help explain the difference in contaminant removal. According to the above discussion, a schematic model of pollutant removal in TFCW and HFCW is proposed and demonstrated in Figure 5. During the contact period, organic matter, NH_4^+-N, NO_3^--N and TP enter into biological film to convert, and residual nitrogen and phosphorus are adsorbed onto the wetland substrates and roots. Denitrificans were generally considered facultative bacteria, and anoxic condition was conducive to denitrification. Although the removal of NO_3^--N in TFCW was lower than that in HFCW, it still reached 52.37%. The former study indicated that DO >1.7 mg/L is beneficial for denitrification by aerobic denitrifying bacteria under oxygen-rich environments [63]. The average effluent DO was 2.17 mg/L in TFCW (Figure S2). Thus, there was likely aerobic denitrification occurring in TFCW. Phosphorus removal is commonly dependent on the hydrolysis of intracellular polyphosphate when organics are stored as polyhydroxyalkanoates (PHAs) by PAOs, and then PHAs are oxidized by denitrifying phosphate-accumulating organisms (DPAOs) in the aerobic zone to accomplish phosphate removal [64]. After the bulk wastewater is drained out, the bed is exposed directly to the atmosphere, and then aerobic processes (organics oxidization, ammonia nitrogen nitrification, and phosphorus conversion) occur during the bed resting phase. In addition, the denitrification process could also take place during this period.

Figure 5b demonstrates the pathways of pollutant removal in HFCW. The deficiencies of inappropriate oxygen distribution and insufficient oxygen supply exist in HFCW [35]. TN is commonly removed by the combination of NH_4^+-N adsorption, NH_4^+-N oxidation, and NO_3^--N reduction (nitrification/denitrification processes) in HFCW. TP removal is mainly achieved by the chemical absorbance of substrate particles and plant uptake. The thicker biofilm and developed root system can intercept suspended matter, particulate organics, and particulate phosphorus, which contributes to keeping the removal efficiency of inorganic and organic matter at low temperatures in HFCW. Sedimentation and adsorption are also important pathways to remove particulate matter and other forms of pollution. In a cold climate, a fraction of the influent COD may become colloidal and be susceptible to removal by filtration.

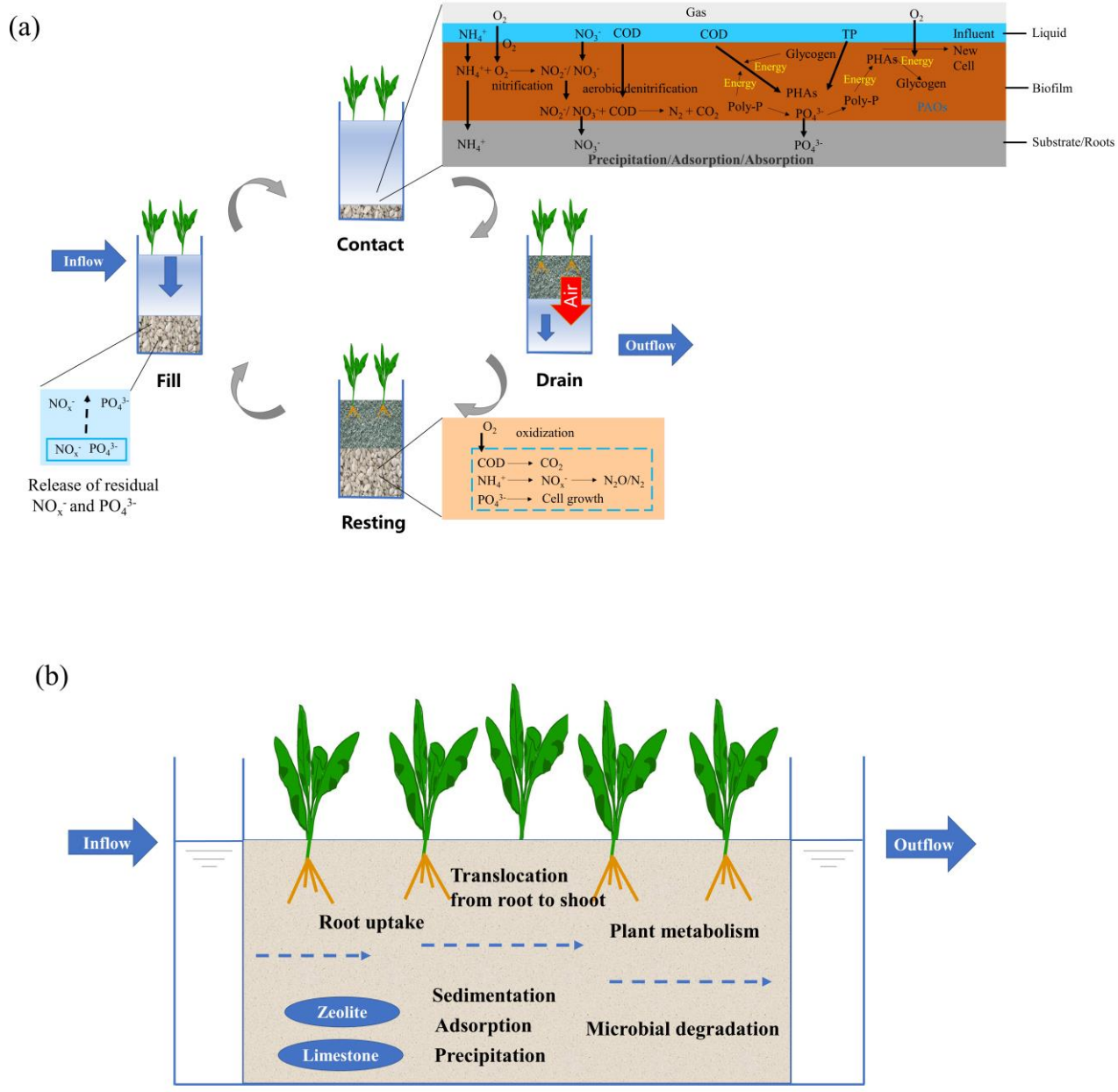

Figure 5. The schematic diagram of pollutants removal in (**a**) TFCW and (**b**) HFCW.

3.3. Performance for Pollutant Removal at Low Temperatures

The variations of COD, NH_4^+-N, NO_3^--N, TN, and TP in influent and effluent concentrations, as well as the corresponding removal efficiencies in TFCW-HFCW and TFCW-HFCW systems, are shown in Figure 6. The influent COD concentration fluctuated between 98.98 mg/L and 132.38 mg/L, with an average content of 115.27 mg/L during the cold-temperature phase (Figure 6a). In addition, the results also showed sustained and stable contaminant removal in both TFCW-HFCW and HFCW-TFCW systems, where the average effluent concentrations of COD were 23.13 mg/L and 26.32 mg/L, respectively. The effluent concentration of COD both in TFCW-HFCW and HFCW-TFCW systems could satisfy the DB32/3462-2020 Class 1A discharge standard at low temperatures. Therefore, the two-stage design of TFCW-HFCW and TFCW-HFCW ensures stable COD removal performance at low temperatures. Furthermore, there was no significant difference in the removal efficiency of COD between TFCW-HFCW and HFCW-TFCW ($p > 0.05$). In the TFCW-HFCW system, the first-stage TFCW and second-stage HFCW accomplished 46.53% and 33.26% of total COD removal, respectively. In the HFCW-TFCW system, the first-stage

HFCW and second-stage TFCW achieved 40.11% and 36.82% of total COD removal, respectively. For COD removal, degradation in aerobic and anaerobic conditions is the main pathway in CWs [65]. The TFCW-HFCW and HFCW-TFCW in this study provided both aerobic and anoxic environments, which were more beneficial for COD reduction. Plants on the CW would also provide surface area and oxygen for the microbial growth in the rhizosphere, improving the degradation of organic pollutants, although their contribution to total organic pollutant removal was lower than that of microorganisms attached to the substrates [66].

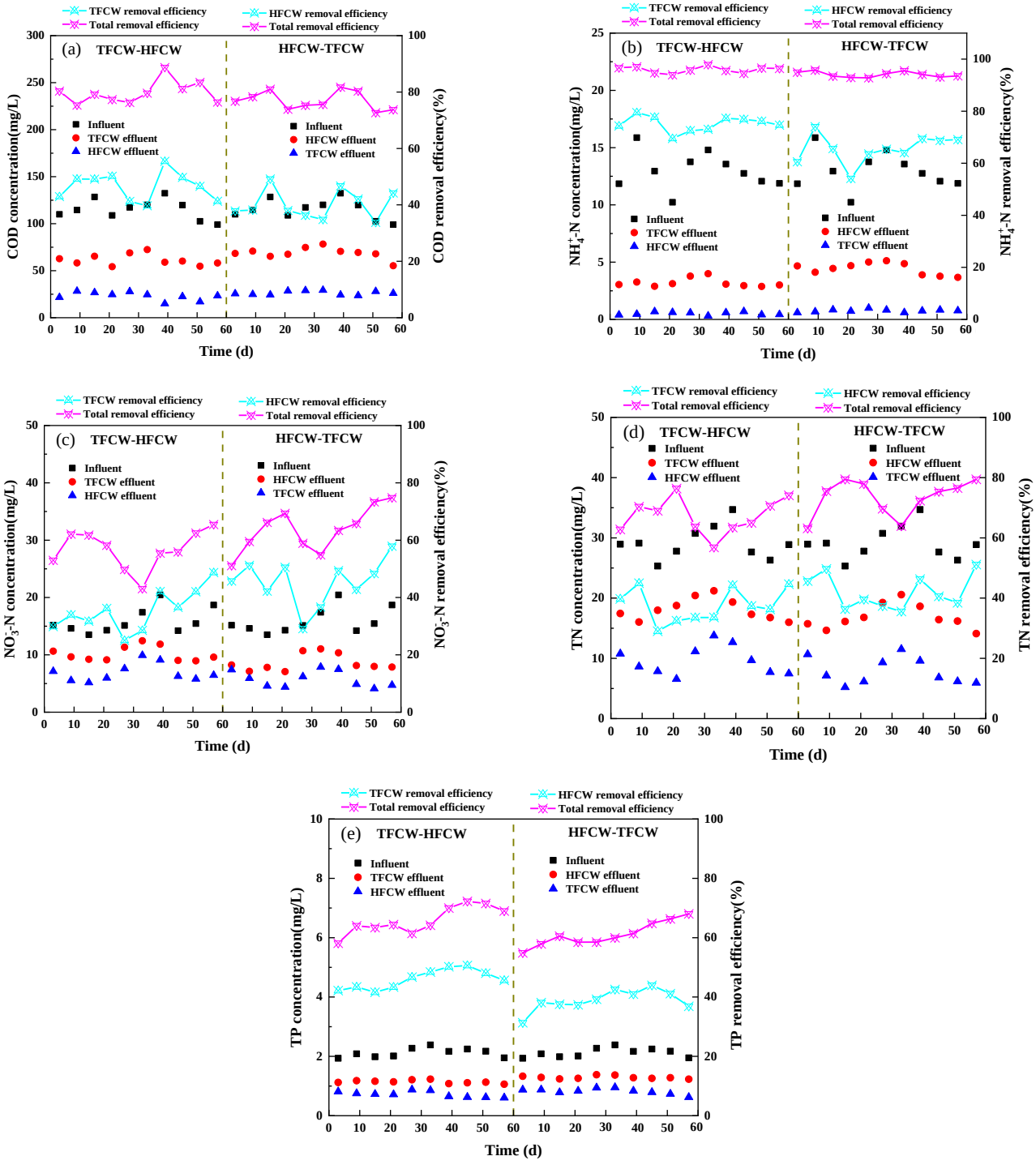

Figure 6. Pollutants removal performance in the TFCW-HFCW and HFCW-TFCW at low temperature: (**a**) COD, (**b**) NH_4^+-N, (**c**) NO_3^--N, (**d**) TN, (**e**) TP.

From Figure 6b, the effluent concentration of NH_4^+-N demonstrated a lower value in the effluent of TFCW-HFCW (0.52 mg/L) and HFCW-TFCW (0.76 mg/L) with little fluctuation during the experimental period. The removal efficiency of NH_4^+-N in TFCW-HFCW and HFCW-TFCW systems demonstrated a similar tendency; the average removal efficiencies were 95.93% in TFCW-HFCW and 94.06% in HFCW-TFCW, which was not a significant difference ($p > 0.05$). First-stage TFCW contributed to 75.15% of total NH_4^+-N removal, and first-stage HFCW contributed to 65.48% of total NH_4^+-N removal, respectively. It has been reported that the level of DO in CW system is the major factor influencing the activity and growth of nitrifiers during the nitrification process [67]. In general, oxygen stemming from rhizosphere exudates of plants is always insufficient in CWs, resulting in low oxygen transfer rates and limiting NH_4^+-N removal [68,69]. The tidal operation mode was adopted to overcome oxygen limitations in TFCW-HFCW and HFCW-TFCW, thus achieving better and more stable NH_4^+-N removal in the two systems, whose result was in accordance with the description of two pilot-scale tidal CWs [70].

Figure 6c,d present the NO_3^--N and TN removal performance of TFCW-HFCW and HFCW-TFCW. The effluent removal efficiencies of NO_3^--N and TN in two CWs demonstrated a similar variation trend, and the average removal efficiencies were 56.72% and 67.23% in TFCW-HFCW and 63.73% and 73.33% in HFCW-TFCW, respectively. The lowest removal efficiency of TN was 56.85% in TFCW-HFCW and 63.92% in HFCW-TFCW after a month of operation, with corresponding effluent concentrations of 13.78 mg/L and 11.52 mg/L, which were still lower than the TN limit of Grade 1A (20 mg/L) in the environmental quality standards for rural domestic sewage treatment facilities in JiangSu Province, China (DB32/3462-2020). Clearly, the NO_3^--N and TN removal performances of the HFCW-TFCW system were significantly higher than those of the TFCW-HFCW system ($p < 0.05$). First-stage TFCW contributed to 35.47% and 3 7.58% of total NO_3^--N and TN removal, respectively. However, the first-stage HFCW contributed to 45.40% and 42.07% of total NO_3^--N and TN removal, respectively. The better NO_3^--N and TN removal performances of HFCW-TFCW could be explained as follows: on the one hand, the first-stage HFCW (HFCW-TFCW) performed well on the denitrification process, whereas the limited denitrification process occurred in first-stage TFCW (TFCW-HFCW); on the other hand, as the degradation of organic matter occurred in first-stage TFCW, the denitrification process in second-stage HFCW was limited because of the lack of carbon source. We observe a large fluctuation in the removal efficiencies of NO_3^--N and TN in TFCW-HFCW and HFCW-TFCW due to changes in temperature. It has been proved that the climatic condition, especially air temperature significantly affected the removal efficiencies of pollutants in CWs [66,71]. In addition, air temperature could have a considerable impact on the growth and establishment of CW plants, which in turn influenced water purification [72,73]. Nevertheless, the TFCW-HFCW and HFCW-TFCW could still ensure efficient and stable nitrogen removal for rural wastewater at low temperatures in this study.

The variation of TP in influent and effluent for TFCW-HFCW and HFCW-TFCW compared here is shown in Figure 6e. During the cold-climate phase, the average TP removal efficiency in TFCW-HFCW and HFCW-TFCW was 65.83% and 61.08%, respectively. The average effluent concentration of TP in TFCW-HFCW was 0.72 mg/L, which was lower than that in HFCW-TFCW (0.83 mg/L). Furthermore, the TP effluent concentration met the DB32/3462-2020 Class 1A discharge standard (below 1.0 mg/L) at cold temperatures. In general, the phosphorus removal could be mainly attributed to substrate adsorption, plant uptake, and biological assimilation in CWs [74]. However, under the low environmental temperatures, the adsorption of phosphorus dominated by chemical adsorption is limited because it is an exothermic reaction. In addition, it was also found that TP was removed more effectively in TFCW-HFCW than HFCW-TFCW. There was a significant difference in TP removal efficiency between TFCW-HFCW and HFCW-TFCW, though both were filled with the same substrate and cultivated the same plants. This phenomenon might be attributed to the operation modes with changeable aerobic and anaerobic conditions in first-stage TFCW (TFCW-HFCW). As previously reported, the substrates adsorption

may be chiefly pathway for phosphorus removal at the initial period of CWs, and after medium adsorption becomes saturated, TP removal would be dominated by PAOs [75]. As for the biological removal process, the quantity and quality of carbon sources may have a crucial effect on phosphorus removal [76]. In general, an influent BOD_5/TP ratio higher than 20 was conducive to biological phosphorus removal [77]. Moreover, phosphorus adsorption dominated by chemisorption is less intensive because it is an exothermic reaction in cold climate conditions [29]. Based on a high COD/TP ratio in influent wastewater and cold condition, TFCW acted as the first-stage treatment unit and performed better on TP absorbance by PAOs. From another perspective, the phosphorus removal was limited by the lack of carbon sources in second-stage TFCW (HFCW-TFCW) because of the consumption of organic matter in first-stage HFCW.

4. Conclusions

In this study, TFCW and HFCW were carried out to explore the purification performance and demonstrate the removal mechanism for biologically pre-treated rural domestic sewage in eastern China. Results showed an effective removal performance of COD (69.46%), NH_4^+-N (96.47%) and TP (57.38%), which could be obtained in TFCW and was better than in HFCW with COD (61.43%), NH_4^+-N (84.99%) and TP (46.75%). TFCW system contained the more abundant biological diversity and microbial community structure, related to the heterotrophic bacteria, nitrifiers and PAOs. This study highlights that the TFCW system has a strong potential for enhancing COD, NH_4^+-N and TP removal, making it an attractive technique for pollutants treatment as ecological post-treatment system. Furthermore, the TFCW-HFCW and HFCW-TFCW performed well on pollutants removal at low temperatures, while the effluent concentration of the two systems satisfied Class 1A discharge standard of DB32/3462-2020. With respect to both the pollutants removal and low costs, the two-stage constructed wetlands as part of a bio-ecological system have a broad application prospect for domestic wastewater treatment solution under cold climate in China.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su15031799/s1, Figure S1. The schematic diagram of the whole bio-ecological system at low temperatures (<15 °C). Figure S2. The variations of DO in the influent and effluent of the TFCW and HFCW. Figure S3. Changes of DO, COD, NH_4^+-N, NO_3^--N, TN, TP for the tidal cycle in TFCW.

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