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Performance and Bacteria Communities of a Full-Scale Constructed Wetland Treating the Secondary Effluent after Multi-Years' Operation

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Abstract: Constructed wetlands (CWs) had been widely used to treat the tailwater from sewage treatment plants. However, the enduring effectiveness of CWs was still unclear. Therefore, this study aimed to investigate the chemical oxygen demand (COD), ammonia nitrogen (NH_4^+-N), and total phosphorus (TP) removal efficiencies (RE) of the Hongze CW (HZ-CW) after multi-years' operation. The average COD RE were 7.6% and 15.14% in the 4th and 5th year, respectively. The NH_4^+-N RE was 78.33% and 46.04% in the 4th and 5th year, respectively, while the TP RE remained high at 66.86% and 64.68%. The high-throughput sequencing analysis revealed that the bacterial community of HZ-CW at the end of the 5th year exhibited a substantial abundance and diversity, and Proteobacteria and Bacteroidota were the dominant phyla with a relative abundance of 33.75–71.8% and 11.28–24.53% in different zones of HZ-CW. Ammonia oxidizing organisms (AOMs) presented much higher relative abundance (0.43–0.79%) in aerated pond (AP) and four free water surface flow CWs (FWS1–FWS4) than those of anammox bacteria, indicating the dominant role of nitrification in NH_4^+-N removal.

Keywords: full-scale constructed wetland; secondary effluent; bacterial community; multi-years' of operation

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1. Introduction

Constructed wetlands (CWs) are a well-known and widely recognized solution, due to their good operation performance [1,2], cost-effectiveness [3], and ability to withstand impact loads [4–7]. This technology is capable of treating various types of wastewater, including agricultural wastewater, industrial effluent, polluted river water, and domestic sewage [8–11]. Currently, there is an increase in the application of CWs for the treatment of effluent from sewage treatment plants (STPs) driven by the increasingly stringent environmental protection measures in China [12,13]. In fact, in 2018, Jiangsu Province of China even proposed that "tail-water from STPs need to undergo ecological purification by CWs before being discharged into the surface water system".

The analysis of the operational effectiveness of tail-water CWs had observed a steady increase in conjunction with the rise in their application cases. It has been found that CWs exhibited high removal efficiencies for ammonia nitrogen (NH_4^+-N), chemical oxygen demand (COD) and total phosphorus (TP) [4,14]. Wu et al. conducted an extensive follow-up study for two and a half years on the tail-water from a wastewater treatment plant that was treated by a large-scale vertical-flow CW [4]. It was discovered that the average removal efficiencies for COD and NH_4^+-N by the CW were 53% and 72%, respectively. Additionally, the study highlighted that there was a significant positive correlation between inflow loads and treatment performance. Song et al. findings indicated that CWs exhibited effective removal efficiencies for NH_4^+-N, COD, and TP, with initial operation yielding percentages of 56.33%, 55.64% and 88.44%, respectively [3]. Zhu et al. have carried out a detailed 14-month follow-up investigation into the performance of a large-scale vertical-flow CWs treating wastewater treatment plant tail-water, taking into account seasonal variations [15]. The results of their study revealed that the comprehensive removal rates of COD, NH_4^+-N, and TP were 40.05%, 45.47%, and 62.55%, respectively. Notably, there was no significant seasonal difference in TP removal. An issue prevalent amongst these studies is the limited scope of their research phase, which largely focuses on the initial period of CWs establishment, and the lack of analysis on the long-term effectiveness of CWs after years of operation. However, it should be noted that the performance of CWs changes over time due to substrate blockage, saturation of adsorption capacity, decomposition and release of accumulated organic matter, and an increase in microbial diversity and abundance [16,17]. The tracking studies on the CWs' performance are few, which limits people's comprehensive understanding of them and is not conducive to their optimization and upgrading. Numerous studies have demonstrated that microbial processes serve as the primary pollutant removal pathway, prompting researchers to investigate microorganisms within CWs [15,18]. Zhu et al. discovered a considerable variety of microbial communities within the integrated vertical flow CW, with Proteobacteria, Planctomycetes, Bacteroidetes, and Acidobacteria emerging as the primary strains. Among these, the ratios of nitrifying bacteria, denitrifying bacteria, and anammox bacteria were calculated to fall within the range of 0.28–0.31%, 1.83–1.92%, and 0.34–0.40%, respectively; denitrification and anammox were identified as the core mechanisms for nitrogen removal [15]. Furthermore, Zhang et al. determined that anammox bacteria played a critical role in vertical flow CWs' nitrogen removal. Consequently, the functional properties of microbial communities in surface-flow CWs, including their specific microbial populations, require explicit exploration [18].

In this study, we focused on the performance and microbial communities of a full-scale surface-flow CW treating the secondary effluent after multi-years' operation. The objectives of this study were: (1) to assess the long-term removal efficiency of a large-scale surface-flow CW in removing pollutants such as COD, NH_4^+-N, and TP, as well as identifying seasonal variations; (2) to analyze the distribution of the bacterial communities in the CWs, with a particular focus on identifying functional microorganisms.

2. Materials and Methods

2.1. Site Description

The HZ-CW is located in the Hongze District of Jiangsu Province, East China, covering an expansive 55.58×10^4 m^2. This system was thoughtfully designed and comprises an aerated pond (AP), a facultative pond (FP), four free-water surface flow CW (FWS), and an ecological pond (EP) (Figure 1). The hydraulic retention times (HRT) of the system elements are 6.7 d, 4.1 d, 3.4 d, and 2.5 d for AP, FP, FWSs, and EP, respectively. The influent water for this system is taken from a sewage treatment plant and has a daily flow rate of 4×10^4 m^3. Emergent aquatic plants, specifically *Phragmites australis* (Cav.) Trin. Ex Steud., *Typha Orientalis* Presl, and *Thalia dealbata* Fraser, were deliberately selected for their extensive root systems and high biomass yields. For a more comprehensive understanding of the system's setup and management approach, reference Song and Liu's work [3].

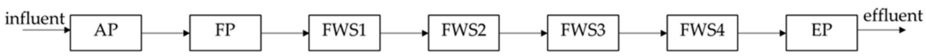

Figure 1. Schematic of HZ-CW system. AP, aerated pond; FP, facultative pond; FWS1–FWS4, four free water surface flow constructed wetlands follow the water flow; EP, ecological pond.

2.2. Water Quality Monitoring

The HZ-CW's inlet and outlet water quality were continuously monitored using an online detector from Nanjing Gangneng, China. The monitored parameters encompassed dissolved oxygen (DO), NH_4^+-N, COD, and TP. Hourly water samples were collected and analyzed while the average data for monitoring points in the HZ-CW were computed every 10 days.

2.3. Microorganism Sampling and Analysis

The bacterial communities in AP, FP, FWSs, and EP were analyzed at the end of the 5th year. Substrate samples of each processing zone were collected, and stored in the icebox during delivery to the laboratory. The substrate samples were collected from the surface layer (0~5 cm) at the bottom of each zone. Three sites were selected in each zone, and three samples were collected from each site and then the samples from the same site were combined for testing. The genomic DNA was extracted and further investigated according to Li et al. [19].

2.4. Data Analysis

The removal efficiency (RE) of pollutants was calculated using the percentage removal rate for each parameter as follows:

$$\text{RE } (\%) = \frac{(\text{InC} - \text{EffC})}{\text{InC}} \times 100 \tag{1}$$

where InC and EffC are the influent and effluent concentrations (mg L^{-1}), respectively. Mean (±s.d.) values were also calculated. Seasonal (spring: March–May; summer: June–August; autumn: September–November; winter: December–February) effects for contaminants removal were investigated using one-way analysis of variance (ANOVA). *t*-tests were used to compare parameters between the influent and the effluent. Nonparametric tests were used for non-normal distribution data. In addition, Tukey's *b*-test was used to determine the significance of differences among seasons, with two-sided $p < 0.05$ taken to indicate significance. All analyses were conducted using SPSS 22.0 (IBM Corp., Armonk, NY, USA).

3. Results and Discussion

3.1. The Performance of HZ-CW after Years Operation

3.1.1. The DO Variations

DO can influence the pollutant removal efficiencies of CWs [20–22]. Therefore, the influent and effluent DO concentrations of HZ-CW in the 4th, 5th, and 1st year (n = 36 for each year) were investigated and presented in Figure 2. As shown in Figure 2, the average influent concentration (InC) of DO in the 4th and 5th years were 3.08 and 1.82 mg L^{-1}, respectively (n = 36 for each year), both higher than that in the 1st year (the average DO was 1.4 mg L^{-1}). Conversely, the average DO EffC of 5.62 and 3.34 mg L^{-1} in the 4th and 5th years were lower than the average of 6.23 mg L^{-1} observed in the 1st year. The low DO EffC in the 5th year might be caused by the aging of solar aerators. Furthermore, the average DO EffC in summer and autumn of the 4th and 5th years was lower than those in spring and winter, which was partly associated with the low solubility and rapid consumption of DO in the high temperature.

3.1.2. The Performance for COD Removal

As shown in Figure 3a, the EffC of COD in the 4th and 5th years were 35.99 ± 8.65 mg L^{-1} and 24.09 ± 6.90 mg L^{-1}, both meeting the effluent requirement for COD (≤40 mg L^{-1}). *t*-tests revealed that InC and EffC of COD in both the 4th and 5th year presented a significant difference ($p < 0.01$). Compared to the 1st year (55.64%), the average COD RE declined in the 4th (7.60%) (n = 36) and 5th year (15.14%) (n = 36) (Figure 3c) and the average RE per season in the 4th and 5th year were lower than that in the 1st year (Figure 3b). One-way ANOVA showed that there was a significant difference in RE between the 4th~5th year and the 1st year ($p < 0.05$). Zhu et al. [15] and Zhang et al. [18] have shown that both full-scale integrated vertical-flow CWs and surface-flow CWs were able to achieve COD RE of 40.05% and 53%, respectively, when treating secondary effluent, which was comparable to the COD removal rate in 1st year of the study. These results demonstrated the effectiveness of CWs in COD removal, irrespective of the types of CWs, especially in the initial years of operation. However, the COD RE of the HZ-CW decreased significantly in the 4th and 5th years of operation. According to previous studies, the COD removal efficiency of CWs showed a significant positive correlation with the influent. In this study, the average COD InC in the 4th and 5th years were 43.93 ± 16.20 mg L^{-1} and 32.79 ± 17.05 mg L^{-1}, markedly lower than that in the 1st year (64.59 ± 27.57 mg L^{-1}), which was likely to be the dominant factor contributing to the COD RE decline in the 4th and 5th years. As shown in Figure 3a, the COD EffC reached lower than 40 mg L^{-1}, despite exceeding an influent COD concentration of 100 mg L^{-1} in the 5th year of operation, indicating HZ-CW still boasted strong COD removal capabilities. These results further support the notion that the decline of influent COD load was the primary driver for the decrease in COD RE.

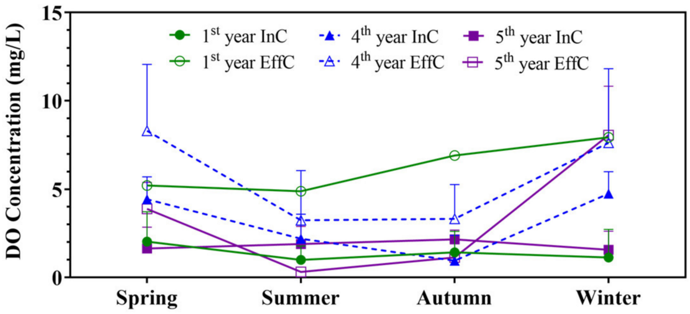

Figure 2. The influent and effluent DO concentrations of HZ-CW in the 4th, 5th and 1st year (*n* = 36 for each year), and the DO concentrations in different seasons (*n* = 9 for each season). Data are given as the mean ± s.d. InC, influent concentration; EffC, effluent concentration.

The One-way ANOVA also presented that the COD RE among seasons in the 1st year, 4th year, and 5th year showed no significant differences ($p > 0.05$) (Figure 3b). However, these findings differed from those studies by Zhu et al., who found that COD RE in winter was lower than in summer due to decreased microbial degradation capacity [15]. In fact, the removal of COD in CWs involves various mechanisms including precipitation, filtration, adsorption, and microbial degradation [23–25]. Temperature is believed to have a greater impact on microbial degradation, and a lesser effect on physico-chemical processes. Therefore, the small seasonal variation in COD removal in HZ-CW might be the result of the combined effects of various mechanisms.

3.1.3. The Performance for NH_4^+-N Removal

Nitrogen (N) is recognized as one of the key contributors to eutrophication in water; therefore, the removal performance of NH_4^+-N serves as a significant indicator for assessing the water purification effectiveness of the full-scale CWs [4,26,27]. As shown in Figure 4a, the average NH_4^+-N EffC in the 4th and 5th year were 0.84 ± 1.35 mg L^{-1} and 0.18 ± 0.23 mg L^{-1}, respectively, with almost all of the EffC of NH_4^+-N were lower than the effluent standard (Figure 4a). *t*-tests confirmed the significant difference between InC and EffC of NH_4^+-N ($p < 0.01$) during each year. However, the mean NH_4^+-N EffC in the 1st year was 4.40 ± 3.60 mg L^{-1}, failing to meet the effluent requirement, which might be due to the unstable establishment of the bacterial community and the relatively high InC of NH_4^+-N [15,28]. The average NH_4^+-N RE were 56.33%, 78.33%, and 46.04% in the 1st, 4th, and 5th year, respectively (Figure 4c), presenting a significant difference in RE between the 1st year and the 4th year ($p < 0.05$). According to the former studies, the NH_4^+-N RE in CWs had a positive correlation with the inflow load [4,15] in the 5th year, the average NH_4^+-N InC (1.63 ± 2.20 mg L^{-1}) was significantly lower than that in the 4th year (4.58 ± 4.31 mg L^{-1}), so the NH_4^+-N RE in the 5th year was significantly lower than that in the 4th year.

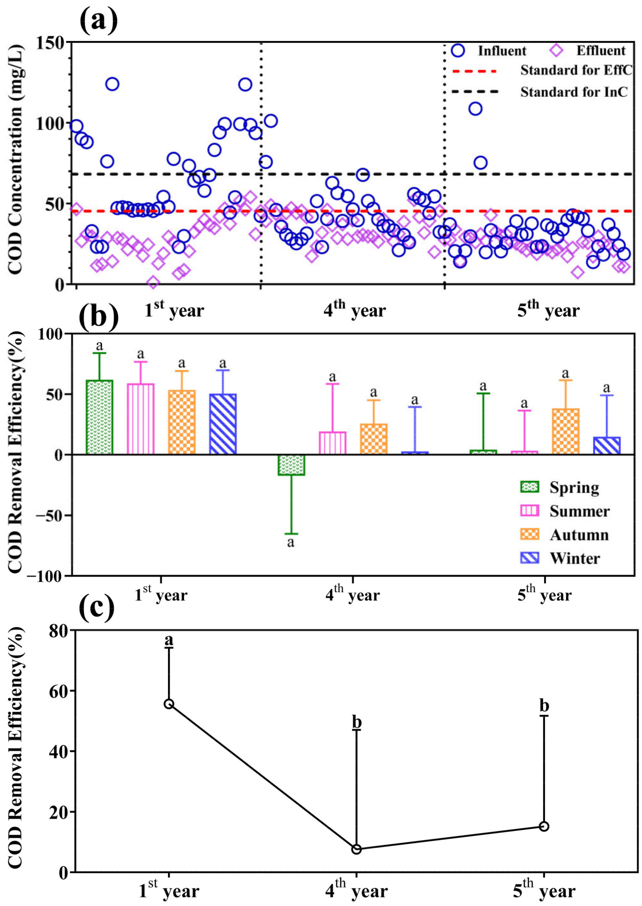

Figure 3. The performance of HZ-CW for COD removal during the study period. (**a**) The influent and effluent concentrations, (**b**) COD removal efficiencies (%) in different seasons (n = 9 for each season), (**c**) the mean COD removal efficiencies (%) in each year (the 1st year, 4th year, and 5th year, n = 36 for each year). Different letters indicate significant differences ($p < 0.05$) among different years. Data are given as the mean ± s.d. InC: influent concentration; EffC: effluent concentration.

This study also revealed a significant difference in NH_4^+-N RE between autumn and spring–winter in both the 4th and 5th years ($p < 0.05$) (Figure 4b), potentially due to the higher InC NH_4^+-N in the autumn of the 4th than that in the spring and winter. Similarly, the InC NH_4^+-N in the autumn of the 5th year was lower than that in the spring and winter of the same year. This result was inconsistent with former studies, which showed that a low temperature limited microbial activity and plant growth, thereby leading to a decrease in NH_4^+-N RE in CWs in winter and spring. Therefore, it is necessary to consider both temperature and NH_4^+-N InC when evaluating the removal effect of CWs on NH_4^+-N.

3.1.4. The Performance for TP Removal

As shown in Figure 5a, the average EffC of TP were 0.08, 0.67, and 0.08 mg L^{-1} in the 1st, 4th, and 5th year, respectively. Specifically, the mean EffC of TP in the 4th year failed to meet the effluent TP requirement of ≤0.4 mg L^{-1}, which was attributed to the elevated TP InC of 2.97 mg L^{-1}, approximately three times the design influent value, and higher than those recorded in the 1st (0.73 mg L^{-1}) and 5th year (0.30 mg L^{-1}). In the 1st and 5th years, there was no significant seasonal difference in TP RE ($p > 0.05$). Nevertheless, between summer and autumn–winter in the 4th year, there were seasonal variations ($p < 0.05$) (Figure 5b), potentially because of the high TP InC. Compared to the RE of NH_4^+-N and COD, the TP RE was relatively stable ($p > 0.05$), totalling 88.44%, 66.86%, and 64.68% in the 1st year, 4th year, and 5th year, respectively, which was higher than 60% (Figure 4c), as reported by Saggai, Ainouche [29].

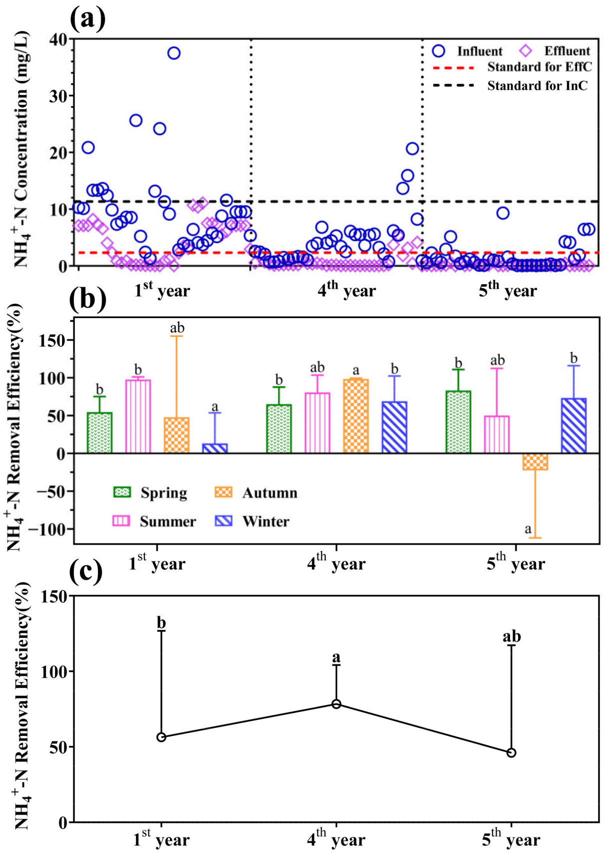

Figure 4. The performance of HZ-WC for NH_4^+-N removal during the study period. (**a**) The influent and effluent concentrations, (**b**) NH_4^+-N removal efficiencies (%) in different seasons during the study period (n = 9 for each season), (**c**) the mean NH_4^+-N removal efficiencies (%) in each year (the 1st year, 4th year, and 5th year, n = 36 for each year). Different letters indicate significant differences ($p < 0.05$) among different years. Data are given as the mean ± s.d. InC: influent concentration; EffC: effluent concentration.

Enhanced biological phosphorus removal (EBPR) processes have proven to be effective in removing TP from STPs through the accumulation of polyphosphate accumulating organisms (PAOs) [30]. However, the tail-water lacked organic matter and did not necessarily have alternating anaerobic/aerobic or anaerobic/anoxic conditions, so the role of EBPR in tail-water CWs was limited [15]. The substrate adsorption and filtration are the primary pathways for TP removal in these processes and are less affected by environmental factors, such as temperature. This explained the absence of seasonal variation in TP removal observed in the HZ-CW in the 1st and 5th years of operation. It is worth noting that clogging was reported as a factor affecting the RE of TP after years of operation [31], while this study found that the HZ-CW still exhibited effective TP removal, even after several years of operation.

3.2. The Bacterial Communities Diversity and Composition in HZ-CW

The bacterial communities in each zone of the HZ-CW at the end of the 5th year of operation were revealed by the high-throughput sequencing.

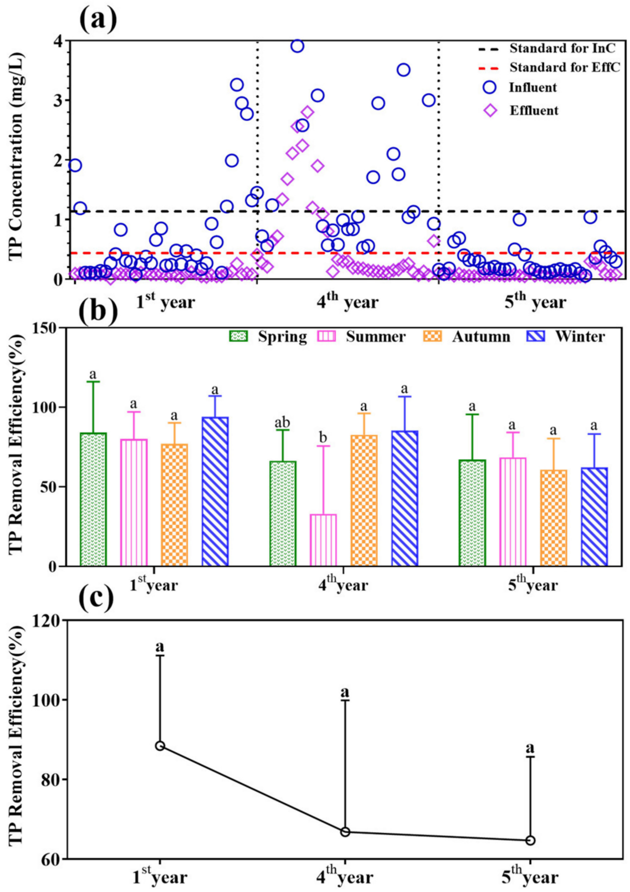

Figure 5. The performance of HZ-CW for TP removal during the study period. (**a**) The influent and effluent concentrations, (**b**) TP removal efficiencies (%) in different seasons (n = 9 for each season), (**c**) the mean TP removal efficiencies (%) in each year (the 1st year, 4th year, and 5th year, n = 36 for each year). Different letters indicate significant differences ($p < 0.05$) among different years. Data are given as the mean ± s.d. InC: influent concentration; EffC: effluent concentration.

3.2.1. The Richness and Diversity Analysis of the Bacterial Communities

Bacterial richness and diversity within each zone of the HZ-CW were listed in Table 1. The Coverage of the sequences were 0.969, 0.990, 0.966, 0.968, 0.967, 0.964, and 0.982 in AP, FP, FWS1, FWS2, FWS3, FWS4, and EP, respectively, which indicated that the majority of the OTUs have been captured [32]. ACE estimator showed that the community richness of FWS1, FWS2, FWS3, and FWS4 was similar to the ACE from 6223 to 6797, indicating their similar function. The community abundance of samples in the AP was slightly lower than that in the FWS1–4 with the ACE estimator of 5861, which might result from the relatively abundant matrix and the aerobic conditions [33]. The ACE estimators in FP and EP were 1997 and 3349, which might be caused by the greater depth of FP and EP (2.0 m and 1.5 m) and the non-aerated environment. Similar phenomena were observed for bacterial diversity, which FP and EP presented the lower diversity according to Shannon indices.

According to Wang et al., the Chao1 and Shannon index of bacterial communities in an STP were 1498 and 5.46 [30], respectively; both were significantly lower than those in the HZ-CW, except for those in FP. This result was attributed to the CWs' proximity to the natural environment and minimal artificial intervention. The high richness and diversity of microbial communities in the CWs indicate the greater adaptability in pollutant removal, and the CWs have greater potential for the elimination of emerging contaminants, such as antibiotics, estrogens, and personal care products [34,35].

Table 1. Comparison of phylotype coverage and diversity estimation of the 16S rRNA gene libraries at 97% similarity from the Miseq high-throughput analysis.

Samples	97% Similarity			
	OTUs	**Coverage**	**Chao1**	**Shannon**
AP	4371 ± 437 [ab]	0.969	5638 ± 137 [ab]	8.608 ± 1.41 [a]
FP	1464 ± 948 [c]	0.990	1883 ± 1014 [c]	5.486 ± 0.28 [b]
FWS1	4691 ± 1633 [ab]	0.966	6116 ± 2031 [ab]	8.894 ± 1.93 [a]
FWS2	4924 ± 1948 [a]	0.968	6099 ± 2384 [ab]	9.317 ± 2.40 [a]
FWS3	4661 ± 1831 [ab]	0.967	5989 ± 2205 [ab]	8.9785 ± 2.55 [a]
FWS4	5154 ± 761 [a]	0.964	3267 ± 2116 [bc]	9.331 ± 1.66 [a]
EP	2481 ± 1459 [bc]	0.982	6572 ± 2220 [a]	5.7825 ± 1.85 [b]

Coverage (Good's coverage), richness indices (chao1), diversity indices (Shannon) were calculated by the Mothur program. Different superscript lower case letters indicate statistically significant differences among HZ-CW elements ($p < 0.05$); AP, aerated pond; FP, facultative pond; FWS1–FWS4, four free water surface flow constructed wetlands follow the water flow; EP, ecological pond.

3.2.2. Bacterial Community Composition

The bacterial community structure in different samples was analyzed and shown in Figure 6. On the phylum level, Proteobacteria dominated in all sediment samples, and the relative abundance was 33.75%, 48.62%, 39.07%, 39.99%, 46.07%, 71.80%, and 36.74% in AP, FP, FWS1, FWS2, FWS3, FWS4, and EP, respectively. The result was similar to that reported by Hu et al. (30.38–52.28%) [23] and Li et al. (26.4–52.6%) [36]. The phylum Proteobacteria plays an integral role in carbon, nitrogen, and sulfur cycling within the ecosystem [37–39], with a significant prevalence observed in CWs, STPs, and soils [32,40]. The Bacillota was the subdominant phylum with a relative abundance of 23.36%, 24.53%, 14.04%, 18.83%, 18.79%, 11.28%, and 15.94%, respectively. Bacteroidota, Chloroflexota, and Actinomycetota were phyla with a relative abundance above 1.0% in at least one sample. These taxa exhibited a high level of prevalence in STPs. Actinomycetota was found to represent a significant proportion in FP, which was attributed to the anaerobic conditions situated at the bed of FP and the saprophytic tendency of Actinomycetota [41]. Acidobacteriota was also the top 10 phylum in the system with a relative abundance ranging from 0.4% to 3.1%, which could improve the degradation of plant residues. As shown in Figure 6a, FWS1, FWS2, FWS3, and FWS4 which were sequentially connected presented similar community composition on the phylum level.

On the genus level, 25 primary genera (relative abundance > 1.00% in at least one sample) were observed (Figure 6b). In AP, *Bacillus* was the dominant genus with a relative abundance of 15.30%, followed by *Rokubacteria*, *Hymenobacter*, *Phyllobacterium*, *unidentified-Deltaproteobacteria*, and *Sphingomonas*. In FP, the dominant genus was *Phyllobacterium*, followed by *Lactobacillus Delftia*, *unidentified-Cyanobacteria*, *Rhodococcus*, *Staphylococcus*, and *Megasphaera*. In FWS1, FWS2, FWS3, and FWS4, *Phyllobacterium* was the dominant genus with relative abundance of 9.33%, 6.39%, 8.04%, and 4.66%, respectively. For *Bacillus*, the relative abundances were 3.97%, 3.17%, 2%, and 11.84% in FWS1, FWS2, FWS3, and FWS4. Acinetobacter, Staphylococcus, and Clostridiales also presented high abundance. In EP, the relative abundance of Phyllobacterium was as high as 31.06%, similar to that in FP, which should result from a comparable ecosystem. The relative abundances of *Delftia*, *Acinetobacter*, *Bacillus*, *Staphylococcus*, as well as *Sphingomonas* in EP, were 11.93%, 8.82%, 4.49%, 2.76%, and 2.41%.

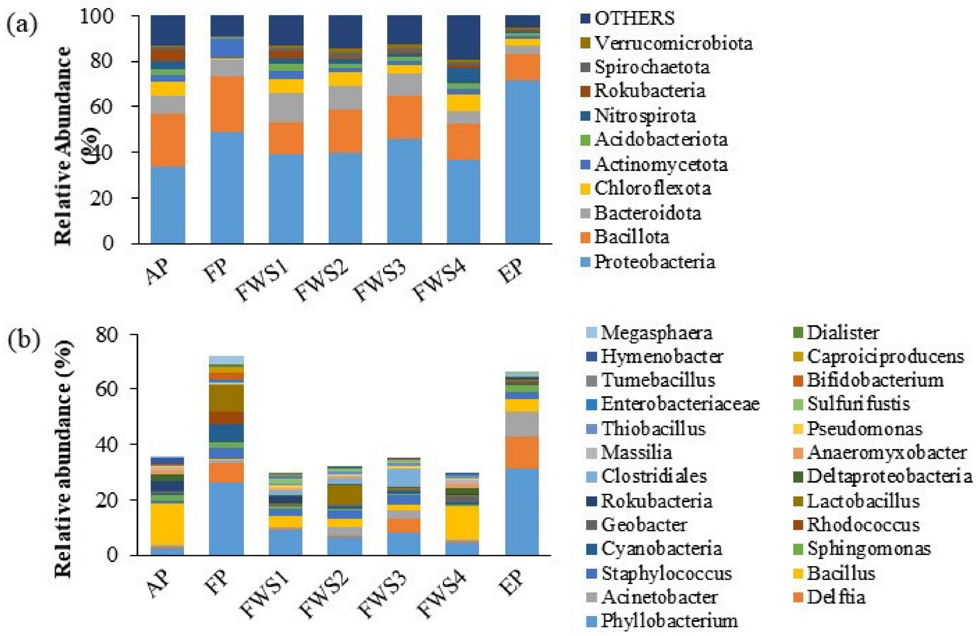

Figure 6. Relative abundance of bacterial community composition of each stage at the (**a**) phylum and (**b**) genus level by 16S rRNA MiSeq sequencing. The sum of the relative abundance of all the other phyla in the figure is grouped into "OTHERS". AP, aerated pond; FP, facultative pond; FWS1–FWS4, four free water surface flow constructed wetlands follow the water flow; EP, ecological pond.

3.2.3. Bacteria Related to Nitrogen and Phosphorus Removal

The bacteria related to nitrogen and phosphorus removal were listed in Table 2. The dominant ammonia oxidizers (AOB) were found to be *Unidentified_Nitrosomonadaceae*, with a relative abundance ranging from 0.171–0.593% in AP and FWS1–4, 0.026% in FP, and 0.036% in EP, respectively. *Nitrosomonas* was another AOB with a relative abundance below 0.013%. All samples obtained three genera of nitrite oxidizers (NOB), and the relative abundance were 0.25%, 0.026%, 0.18%, 0.25%, 0.19%, 0.19%, and 0.047% in AP, FP, FWS1, FWS2, FWS3, FWS4, and EP, respectively. Results showed that the relative abundance of AOB and NOB in AP and FWS1–4 was significantly higher than that in FP and EP, which was mainly caused by the lack of DO at the bottom of FP and EP. The relative abundance of nitrifying microorganisms was comparable to that of integrated subsurface-flow CW in Zhu's paper [15], which ensured the effectiveness of the CWs in removing NH_4^+-N. Three potential anammox bacteria were also found in sediment samples, *Candidatus_Anammoximicrobium*, *unidentified_Brocadiales*, and *Candidatus_Brocadia*, but their relative abundance in each region was less than 0.1%, which was significantly lower than other researches [15,18]. This was mainly because HZ-CW was a surface-flow CW, whereas those were subsurface-flow CWs. Therefore, based on the abundance of nitrifying bacteria and anammox bacteria, it could be inferred that nitrification, rather than anammox, was the main process of ammonia removal in surface-flow CWs.

Three putative genera of PAOs Thiothrix, Microlunatus, and Tessaracoccus were found in at least one sample, but the relative abundance was all below 0.01%. *Accumulibacter* and *Tetrasphaera*, the two typical phosphorus accumulating organisms (PAOs) in STPs, were not observed in all the samples, which might be caused by the absence of volatile fatty acids (VFA) or proteins and polysaccharides in the influent and the alternation of aerobic and anaerobic conditions. [30,42,43] The findings suggested that EBPR did not represent the principal mechanism for TP removal in the HZ-CW; instead, adsorption, filtration, and other processes appeared to be the driving factors. Consequently, TP removal remained unaffected by seasonal variances [44].

Table 2. The genera of nitrifying and polyphosphate accumulating bacteria in each stage of SHZ-CW.

Genus		Relative Abundances (%)						
		AP	FP	FWS1	FWS2	FWS3	FWS4	EP
AOB	*unidentified_Nitrosomonadaceae*	0.178	0.026	0.287	0.171	0.222	0.593	0.036
	Nitrosomonas	0.013	0.011	0.007	0.007	0.008	0.005	0.002
NOB	*unidentified_Nitrospirae*	0.107	0.003	0.114	0.200	0.173	0.135	0.014
	unidentified_Nitrospiraceae	0.104	0.021	0.045	0.035	0.007	0.034	0.030
	Candidatus_Nitrotoga	0.042	0.002	0.022	0.013	0.010	0.019	0.003
Anammox	*Candidatus_Anammoximicrobium*	0.026	0.000	0.020	0.043	0.026	0.026	0.005
	unidentified_Brocadiales	0.000	0.000	0.000	0.001	0.002	0.018	0.000
PAOs	*Candidatus_Brocadia*	0.001	0.001	0.007	0.000	0.002	0.071	0.000
	Thiothrix	0.000	0.001	0.002	0.009	0.002	0.001	0.000
	Microlunatus	0.000	0.000	0.001	0.001	0.003	0.000	0.001

4. Conclusions

After several years of operation, the HZ-CW still exhibited a good contaminant removal efficiency, the average removal efficiencies of COD, NH_4^+-N, and TP were 7.6%, 78.33%, 66.86% and 15.14%, 64.68%, 46.04% in the 4th and 5th year, respectively. Fluctuation of the influent concentration was demonstrated as the main factor contributing to the inter- and intra-annual variation of COD, NH_4^+-N, and TP removal efficiency. The high-throughput sequencing analysis showed the high richness and diversity of the bacteria community in the HZ-CW, indicating the greater adaptability and potential for contaminants elimination. The relative abundance of ammonia-oxidizing organisms (AOMs) and anammox bacteria revealed that nitrification was the dominant NH_4^+-N removal pathway in the HZ-CW, and adsorption and filtration was the primary TP removal mechanism due to the fairly low abundance of PAOs. This study revealed that CWs were a promising choice for advanced treatment of tailwater even after multi-years' operation, which deepened the understanding of CW performance and could promote their wider application.

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