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Impacts of Melatonin on Functionalities of Constructed Wetlands for Wastewater Treatment

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Abstract: Constructed wetlands (CWs) are effective wastewater treatment systems, relying on plant and substrate uptake and microbial depletion to remove pollutants. It has been reported that melatonin can promote plant growth and change the structure of microbial communities. The effects of melatonin on stress tolerance of plants have been extensively studied, while the effects of melatonin on the efficiency of wastewater treatment in constructed wetlands are rarely known. In the current study, 1 mM melatonin was added to the constructed wetland systems to determine physiological characteristics of Phragmites australis, microbial enzyme activity, and microbial community structure of CWs. Under melatonin treatment, the An and g_s of Phragmites australis plants were significantly improved compared with the control. In addition, the contents of phosphate and total anion in the xylem sap of Phragmites australis significantly increased. However, the concentration of total phosphorus in the effluent did change significantly. Melatonin treatment improved the dehydrogenase activity and significantly improved the removal efficiency of NH_4^+-N in CWs. Furthermore, melatonin reduced the richness of the microbial community in CWs, while it increased the diversity of bacterial community and altered microbial composition. FARPROTAX analysis showed that melatonin increased the abundance of bacteria involved in nitrogen fixation and ureolysis, which may be related to the improvement of plant photosynthetic performance and improved rhizosphere oxygen environment. These results suggested that melatonin may affect plant performance and microbial composition and functions to improve the purification effect of constructed wetland.

Keywords: constructed wetlands; melatonin; microbial community; *Phragmites australis*

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1. Introduction

Constructed wetlands are widely utilized as wastewater treatment systems to improve water quality, which is effective in the removal of contaminants including inorganic mater, organic matter, and excess nutrients [1]. The pollutant-removal capacity of wetlands is determined by an interactive process that includes plant uptake, microorganism metabolism, and substrate uptake [2]. Wetland plants not only directly absorb nutrients and elements from wastewater, but also transport oxygen down into the root zone, thus providing a dissolved oxygen environment and improving the microbial community structure and purification efficiency in constructed wetlands [3–5]. Common reed (*Phragmites australis*), the plant frequently used to construct wetlands for wastewater treatment, has a high root oxygen secretion capacity and is effective in removing nitrogen from wastewater [6].

Microorganisms also play important roles in the pollutant-removal processes; the removal of organic matter and nitrogen can be attributed to the microbial degradation in CWs [7]. The composition and function of a microbial community directly affect the purification effect of constructed wetlands [8]. Meanwhile, the degradation of organic pollutants is mainly carried out by the bacterial community in wetlands. In addition, some symbiotic fungi, such as mycorrhizal fungi, can also improve pollutant-removal efficiency by promoting the nutrient-uptake capacity of plants in CWs [9].

Melatonin (N-acetyl-5-methoxytryptamine) is a polymorphic molecule found in animals, plants, and microorganisms that has many physiological functions and plays an important role in plant development and stress tolerance [10]. Melatonin improves plant photosynthesis and growth. Furthermore, the changes in photosynthetic capacity can affect the ability of plants to provide oxygen, which in turn determines removal efficiency in constructed wetlands [11,12]. Melatonin also promotes cellular metabolism and enhances plant uptake of nutrients such as nitrogen, phosphorus, and sulfur. Previous studies have shown that exogenous melatonin aids in phytoremediation, a process that removes pollutants from the environment [13,14]. It has been found that exogenous melatonin can promote plant root development and improve the ability of plants to remediate heavy metal ions in the soils [15,16]. Some studies found that exogenous melatonin affects the composition and function of a microbial community in soils [17,18]. Exogenous melatonin has been widely used as a biostimulator for contaminant treatment in soils due to its safety, but few studies have used it in constructed wetland systems. Wetland microbial communities have complex structures and are susceptible to external environmental factors [2]. The physiological activity of plants and external environmental stimulation have impacts on the microbial communities of CWs [4]. *Phragmites australis*, as one of the wetland model-plants, has growth and development that are particularly important to the wetland environment, especially under CWs. Previous studies reported that melatonin could increase the vitality of antioxidase and accumulation of osmoregulation substances, increasing the resistance of *Phragmites* to abiotic stress [19]. Thus, we hypothesized that melatonin could affect the *Phragmites* development and microbial composition in constructed wetlands, which in turn improve the water purification effect of constructed wetlands.

The current study investigated the effects of melatonin treatment on the physiological characteristics of wetland plants (*Phragmites australis*) and the structure and function of microbial communities in constructed wetland systems based on 16S/ITS rRNA gene sequencing, aiming to assess the prospects of application of melatonin to improve pollutant removal efficiency in constructed wetlands.

2. Materials and Methods

2.1. Experimental Setup and Synthetic Wastewater

Six subsurface flow-constructed wetlands (CWs, 45 cm in height and 35 cm in diameter) were set up in a climate chamber in the Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Changchun, China. The CWs were filled with coal cinder (3–10 mm in diameter) to the same depth of 35 cm. The water surface was 5 cm below the grave bed. Six *Phragmites australis* seedlings with uniform size (approximately 55 cm in plant height) and vigor were planted in each CW with fresh water on 10 August 2020. Regarding the environmental factors setting in the climate chamber, the air temperature and relative humidity during the whole experiment period were 25/22 °C (day/night) and 65%, respectively, and the concentration of CO_2 was 400 ppm. Meanwhile, the photoperiod was 14 h with above 450 μmol m^{-2} s^{-1} PAR (photosynthetic active radiation). *Phragmites australis* plants were fed with 1/10 Hoagland solution for 30 days before the formal experiment; during this period, the plants had developed new leaves and roots. When the formal experiment began, melatonin (1 mmol L^{-1}) was added to the wastewater of three CWs, while the other three CWs added the same volume of water to the wastewater as a control group.

Synthetic wastewater was prepared following Wiessner et al. [20], containing 10 mg L^{-1} Total Phosphorus (TP), 45 mg L^{-1} Total Nitrogen (TN), and a Chemical Oxygen Demand (COD) of 200 mg L^{-1}. During the experiment, 40 L of synthetic wastewater was added to each CW. Furthermore, 7 days after the formal experiment, the wastewater, plant, and microbiome samples of CWs were collected for subsequent determination and analysis.

2.2. Water Samplings and Analysis

From the first day of the formal experiment, a 250 mL water sample was collected daily through a drain tap at 1 cm from the bottom of the CWs on the 1st, 3rd, and 7th day. The concentration of TP in the effluent was determined by spectrophotometer method after digesting with HNO_3-HCl at 200 °C. The TN content was determined by the Kjeldahl nitrogen method, and the contents of NH_4^+-N, NO_3^--N, as well as COD_{Mn} in the effluent were analyzed following by the Water and Wastewater Monitoring and Analysis Methods [21]. Three replicates were included in each treatment.

2.3. Physiological Performance of Phragmites australis

On the 7th day of the experiment, the gas exchange parameters, including photosynthetic rate (An) and stomatal conductance (g_s) of the last fully expanded upper canopy leaf of *Phragmites australis*, were determined. Additionally, the An and g_s of leaves were measured using an infrared gas analyzer-based portable photosynthesis system (Li-6400XT, LI-COR, Lincoln, NE, USA).

The xylem sap of *Phragmites australis* was collected by a Scholander-type pressure chamber to determine the concentrations of anions, cations, nitrogen, ammonium, and phosphorus. The xylem sap samples (1 mL) were stored in an Eppendorf vial wrapped with aluminum foil and stored at −80°C for subsequent analysis. The anions (including F^-, Cl^-, SO_4^{2-}, NO_3^-, NO_2^-, PO_4^{3-}, and Br^-) were measured on a Metrosep-A-Supp 4 analytical column and cations (including Li^+, Na^+, K^+, Ca^{2+}, Mg^{2+}, and NH_4^+) were measured by a Metrosep C4-100 analytical column on ion chromatography (Metrohm, Herisau, Switzerland).

The activities of 13 key carbohydrate metabolism enzymes in *Phragmites australis* leaves were measured following the methods of Jammer et al. [22]. The enzyme activities of phosphofructokinase (PFK), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), fructokinase (FK), hexokinase (HXK), SuSy, UDP-glucose pyrophorylase (UGPase), glucose-6-phosphated dehydrogenase (G6PDH), ADP-glucose pyrophorylase (AGPase), and aldolase (Ald) were determined in kinetic enzyme assays. The enzyme activities of invertases including cytoplasmic invertase (cytInv), vacuolar invertase (vacInv), and cell wall invertase (cwInv) were analyzed in a miniaturized end-point assay. All the enzymes were expressed in nkat mg^{-1}, FW. Additionally, the measurement of enzyme activities was run three times with blanks using an Epoch Take3 spectrophotometer (BioTek Instruments, Inc., Winosky, VT, USA) with a 96-well microtiter format.

2.4. Microbial Enzyme Activities

The dehydrogenase (DHA) activity was determined according to the method reported by Hu et al. [23]. The urease (URE) activity was determined by the method of Klose and Tabatabai [24]. The ammonia monooxygenase (AMO) activity was measured according to the method of Zheng et al. [25]. Phosphatase (PHO) activity was analyzed following the method described by Hu et al. [23]. Three replicates were included in each treatment. Additionally, all these methods have been used in our previous study [26].

2.5. DNA Extraction and 16S/ITS rRNA Gene Sequencing

After the water samples were filtered by membrane filter (0.45 μm pore size), microbial DNA of CWs was extracted by OMEGA Water DNA Kit (D5525) (Omega Bio−Tek, Norcross, GA, USA) using the primers of bacterial 16S rRNA forward 338F (5′−ACTCCTACGGGAGGCAGCAG−3′) and 806R (5′−GGACTACHVGGGTWTCTAAT−3′), and the

primers of ITS rRNA forward ITS1F (5′−AAGTCGTAACAAGGTTTCCGTAG−3′) and ITS2R (5′−CCGTCAATTCCTTTGAGTTT−3′) for PCR amplification of extracted microbial genomic DNA. PCR amplification conditions: 98 °C (3 min) initial denaturation, followed by 25 cycles of denaturation (98 °C for 30s), annealing at 50 °C for 30 s, extension at 72 °C for 30 s, and extension at 72 °C for the last 5 min. The reads based on representative 16S or ITS sequences were clustered into operational units (OTUs) (similarity threshold of 97%). High-throughput sequencing services were provided by Shanghai Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

2.6. Statistical Analysis

All the physiological data were subjected to the one-way ANOVA using SPSS 22.0 (SPSS Inc., Chicago, IL, USA). The microbial data were analyzed on the online tool of Majorbio Cloud Platform "https://cloud.majorbio.com/page/tools/" (accessed on 10 August 2020) " and all data were tested for significant differences at the $p < 0.05$ level.

3. Results

3.1. Treatment Performance of CWs as Affected by Melatonin

Figure 1 depicts the dynamics of pollutant concentrations in the two wastewater treatment systems. On the 1st day, the levels of TP, TN, NH_4^+-N, NO_3^--N, and COD_{Mn} were significantly lower in both CK and MT treatments, and under the influence of melatonin, the NH_4^+-N concentration in the effluent was significantly lower than in CK, but the level of COD_{Mn} was significantly higher than in CK. There was no significant difference in the changes of COD_{Mn} in the effluent between the MT and CK treatments except on days 1 and 5. Throughout the experiment, the NH_4^+-N concentration in the effluent under melatonin was much lower than that under CK, and exogenous melatonin would favorably affect the NH_4^+-N treatment efficiency of the CWs system, while there was no significant effect on the treatment effects of TN, NO_3^--N, and TP. The activities of the four main microbial enzymes in CWs under CK and MT treatments are shown in Figure 2, where the activities of URE, AMO, and PHO under melatonin treatment were increased but not significant compared with those of the control. However, DHA activity was significantly enhanced in response to exogenous melatonin.

3.2. Physiological Response of Phragmites australis Aerial Portion as Affected by Melatonin

The physiological characteristics of *Phragmites australis* were detected here in order to investigate whether exogenous melatonin affects the plant physiological activity of the CWs. As shown in Figure 3, phosphorus concentrations in the xylem sap of *Phragmites australis* increased significantly under melatonin treatment compared to the control. In contrast, the levels of nitrate and ammonium in xylem sap had no significant difference between the two treatments. In addition, melatonin treatment resulted in a significant increase in anion and total ground anion content, but not in cation concentration. Exogenous melatonin treatment also altered the photosynthetic parameters of common reed leaves (Figure 4), with photosynthetic capacity (An) and stomatal conductance (g_s) of melatonin-treated leaves being much higher than those of normal controlled leaves.

Enzymatic profiling analysis further revealed changes in carbohydrate-metabolizing enzymes in fully expanded leaves of *Phragmites australis* (Figure 5). The activities of glucose-6-phosphate dehydrogenase (G6PDH), hexokinase (HXK), cytoplasmic convertase (cytInv), and vacuolar convertase (vacInv) were significantly increased in melatonin-affected leaves compared to the control. Additionally, melatonin application greatly increased the activity of UDP-glucose pyrophosphatase (UGPase) and decreased the activity of sucrose synthase (Susy) compared to the control. In addition, total soluble sugar concentration in the leaves under melatonin treatment was significantly increased compared to the control, while sucrose concentration did not differ between the two groups (Figure 4).

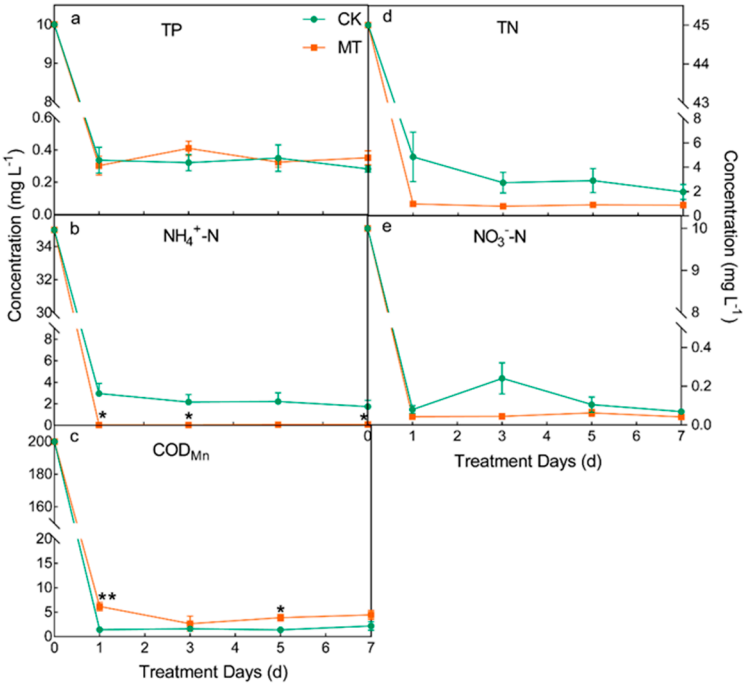

Figure 1. Effluent concentrations of TP (**a**), NH_4^+-N (**b**), COD_{Mn} (**c**), TN (**d**), and NO_3^--N (**e**) in the control (CK) and melatonin (MT). TP, total phosphorus; NH_4^+-N, ammonia nitrogen; COD_{Mn}, chemical oxygen demand; TN, total nitrogen; NO_3^--N, nitrate nitrogen. "ns" indicates not significant, "*" indicates significance at $p < 0.05$, and "**" indicates significance at $p < 0.01$. Data are expressed as means ± SE (n = 3).

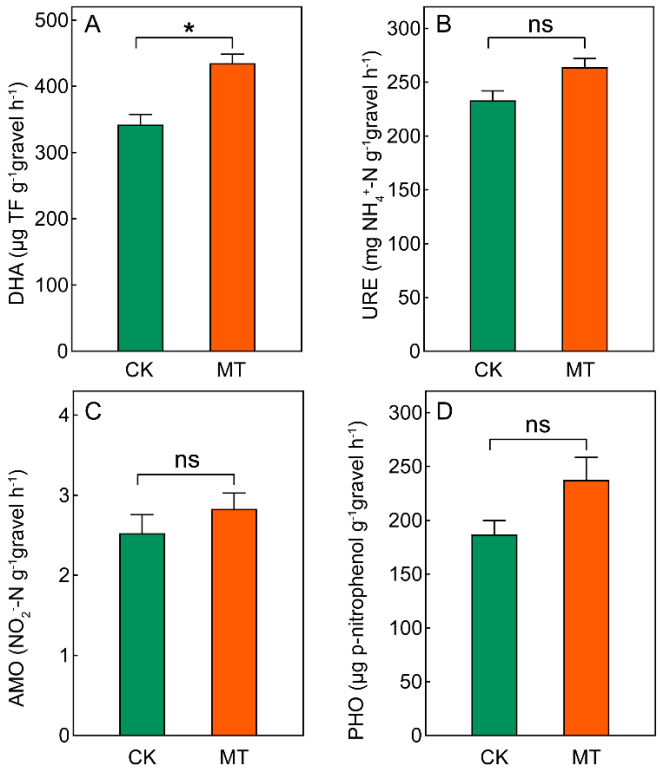

Figure 2. Activities of DHA (**A**), URE (**B**), AMO (**C**), and PHO (**D**) of substrates on the gravel in constructed wetland under control (CK) and melatonin (MT). DHA, dehydrogenase; URE, urease; AMO, ammonia monooxygenase; PHO, phosphatase. "ns" indicates not significant and "*" indicates significance at $p < 0.05$. Data are expressed as means ± SE (n = 3).

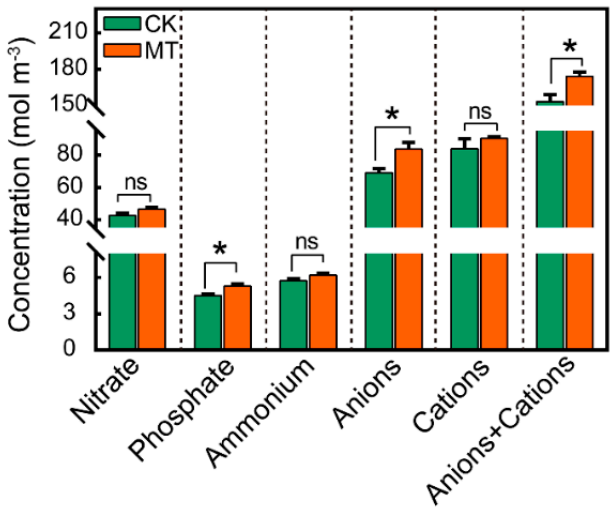

Figure 3. The concentration of nitrate, phosphate, ammonium, anions, cations, and anions and cations in leaves of *Phragmites australis* under control (CK) and melatonin (MT). "ns" indicates not significant and "*" indicates significance at $p < 0.05$. Data are expressed as means ± SE (n = 3).

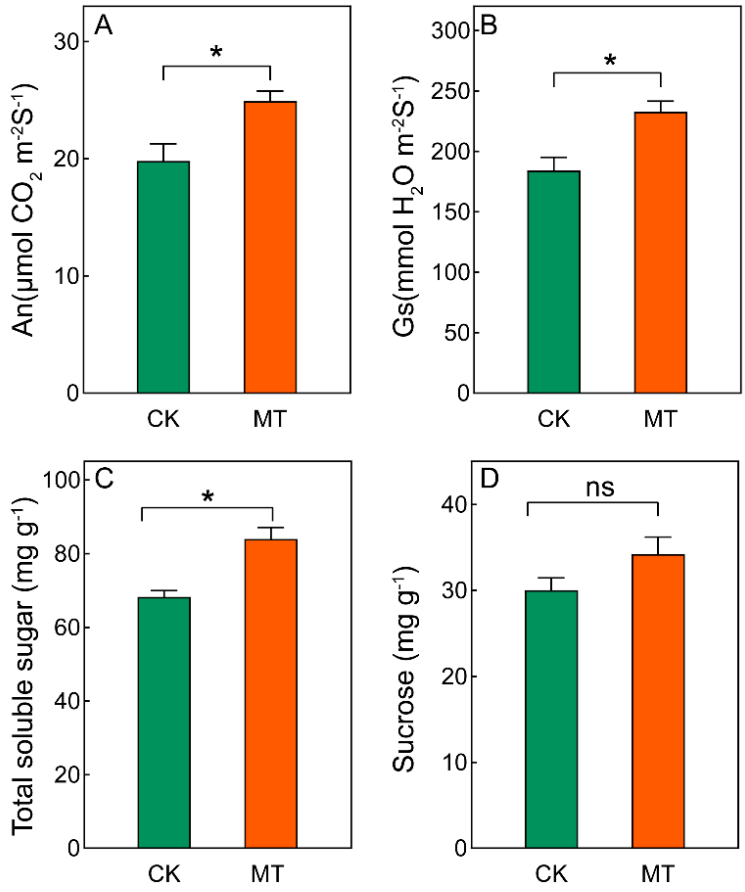

Figure 4. Photosynthetic rate (An) (**A**), stomatal conductance (g_s) (**B**), total soluble sugar concentration (**C**), and sucrose concentration (**D**) in the leaves of *Phragmites australis* under control (CK) and melatonin (MT) conditions. "ns" indicates not significant and "*" indicates significance at $p < 0.05$. Data are expressed as means ± SE (n = 3).

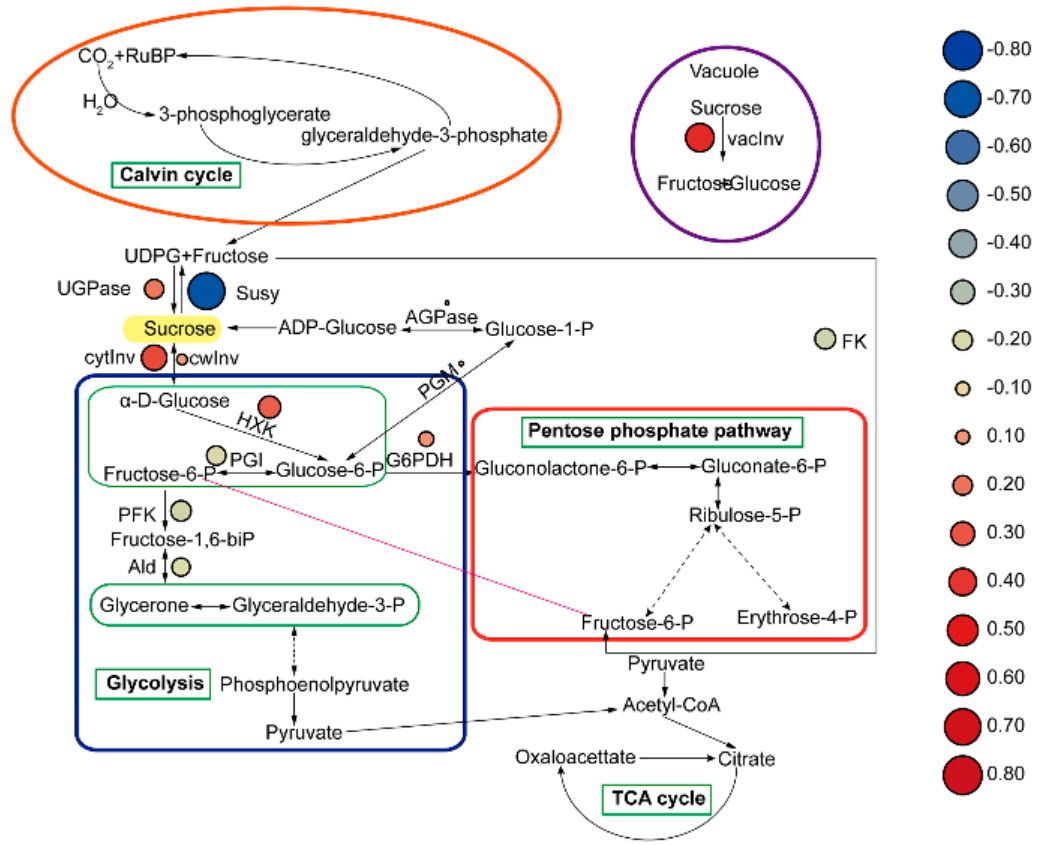

Figure 5. Heat map of activities of key carbohydrate metabolism enzymes in the leaves of *Phragmites australis* under control (CK) and melatonin (MT) conditions. Ald, aldolase; UGPase, UDP-glucose pyrophosphorylase; Susy, sucrose synthase; AGPase, ADP-glucose pyrophosphorylase; HXK, hexokinase; PGI, phosphoglucomutase; G6PDH, glucose-6-phosphate dehydrogenase; PFK, phosphofructokinase; PGM, phosphoglucomutase; FK, fructokinase; vacInv, vacuolar invertase; cytInv, cytoplasmic invertase; cwInv, cell wall invertase. The difference of activity for a given enzyme among these treatments is deviation standardization and converted to a circle. Circles indicates log2 fold change (MT/CK).

3.3. Microbiota of CWs as Affected by Melatonin

3.3.1. Microbial Community Structure

To explore the effect of melatonin on the microbial processes in constructed wetlands, we further investigated the microbial composition using 16S/ITS rRNA gene sequencing. As shown in Table 1, the Good's coverage of bacterial and fungal communities both exceeded 0.98. Alpha diversity of the microbiome in CWs, including Ace, Chao 1, Shannon, and Simpson indexes, showed significant changes, with melatonin significantly altering the richness and diversity of bacterial and fungal communities in CWs. Compared to the control, exogenous melatonin treatment significantly reduced the richness of bacterial and fungal communities and led to the decrease of fungal community diversity, but the bacterial community increased by melatonin treatment in CWs.

As shown in Figure 6, the dominant bacteria in CWs were distributed among the five main phyla, including *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Patescibacteria*, and *Desulfobacterota*, while the dominant fungi were mainly concentrated in the three main phyla, *Ascomycota*, *Rozellomycota*, and *Basidiomycota* (Figure 6A,B). Melatonin application altered the composition of microbial communities in CWs, with melatonin treatment resulting in changes in the relative abundance of bacteria and fungi at the phylum level compared to the control. Melatonin led to an increase in the relative abundance of the phylum *Proteobacteria* and the phylum *Bacteroidota*, and a decrease in the relative abundance of the phylum *Firmicutes* and *Patescibacteria*. In addition, in the fungal community, melatonin led

to an increase in the relative abundance of the phylum *Ascomycota*. The relative abundance of *Basidiomycota* also increased compared to the control, while the relative abundance of *Rozellomycota* decreased. The main dominant bacterial genera in the CWs included *Azospirillum*, *Neisseriaceae*, *Rikenellaceae*, *Kaiserbact*, *Hydrogenophaga*, *Paludibacter*, *Legionella*, *Desulfovibrio*, and *Erysipelothrix*, and the dominant fungi are mainly *Rozellomycota*, *Penicillium*, *Aspergillus*, *Cladosporium*, *Sordariaceae*, *Apiotrichum*, and *Dimorphospora* (Figure 6C,D). Melatonin treatment had a significant effect on the relative abundance of bacterial and fungal communities at the genus level. Compared with the control, melatonin increased the relative abundance of *Azospirillum*, *Neisseriaceae*, *Paludibacter*, *Legionella*, and *Desulfovibrio* in the bacterial community in the CWs, and the relative abundance of *Azospirillum* and *Paludibacter* increased significantly (Figure S2), while *Hydrogenophaga* abundance decreased. Melatonin treatment increased the relative abundance of the fungal genera *Aspergillus* and *Apiotrichum* compared to the control.

Table 1. Alpha diversity based on OTU level (Student' *t*-test and FDR value).

	Treatment	Richness Index			Diversity Index		Coverage
		Sobs	Ace	Chao	Shannon	Simpson	
Bacteria	CK	1818 ***	2235.7 ***	2203.8 ***	5.3004 ***	0.020321	0.98911
	MT	1056	1407.9	1399.1	3.9023	0.080602 ***	0.9925
Fungi	CK	358.25 **	377.81 **	375.95 **	3.0495	0.11156 *	0.99945
	MT	111.25	113.15	113.56	3.4637 *	0.061382	0.99995

Note: "*", "**", "***" indicates significance at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively.

3.3.2. Correlation between Contaminants Concentration and Microbial Communities

Mantel test based on the unweighted-uniface distance showed significant correlations between the genus-level composition of bacterial and fungal communities in the CWs and pollutant contents in the effluent (Table S1). The correlation heat map assessed the relationship between the microbial genera in the CWs and the levels of TN, NH_4^+-N, NO_3^--N, TP, and COD_{Mn} (Figure 7). The results showed that the composition of the bacterial community was significantly correlated with the variation of nitrogen content and COD_{Mn} in the wastewater, but no significant correlation was found with TP content. The bacterial genera associated with environmental factors were divided into two main categories (Figure 7A), one in which the abundance at the genus level was positively correlated with nitrogen concentration and negatively correlated with TP and COD_{Mn}, and the other in which it was negatively correlated with nitrogen content and positively correlated with TP and COD_{Mn}. The bacterial genera including *Hydrogenophaga*, *Sulfurimonas*, *Magnetospirillum*, *Sulfuritalea*, and *Symbiobacterium*, which were positively correlated with ammonia nitrogen content, and *Falkowbacteria*, *Azospirillum*, *Paludibacter*, and *Solitalea*, were negatively correlated with ammoniacal nitrogen content. Different fungal genera were significantly correlated with the environmental variables NH_4^+-N, NO_3^--N, and COD_{Mn}, and it is noteworthy that the fungal community at the genus level was sensitive to changes in ammonia and nitrate nitrogen contents but not significantly correlated with the total nitrogen content (Figure 7B).

3.3.3. Prediction of Functional Composition in the Microbial Community

The effect of melatonin treatment on the functional groups of bacterial and fungal communities in the CWs were inferred from FARPROTAX and FUNGuild (Figure 8A); melatonin significantly altered the functional abundance of the bacterial community in the CWs. The predicted results showed a lower abundance of bacteria involved in aerobic chemoheterotrophy and dark hydrogen oxidation and a higher abundance of nitrogen fixation and ureolysis under melatonin treatment compared to the control in CWs. The functional abundance of the fungal community in CWs mainly included saprotroph, pathotroph, and symbiotroph (Figure 8), and compared with the control, the FUNGuild analyses

showed that melatonin did not significantly change the main functional abundance of fungal communities.

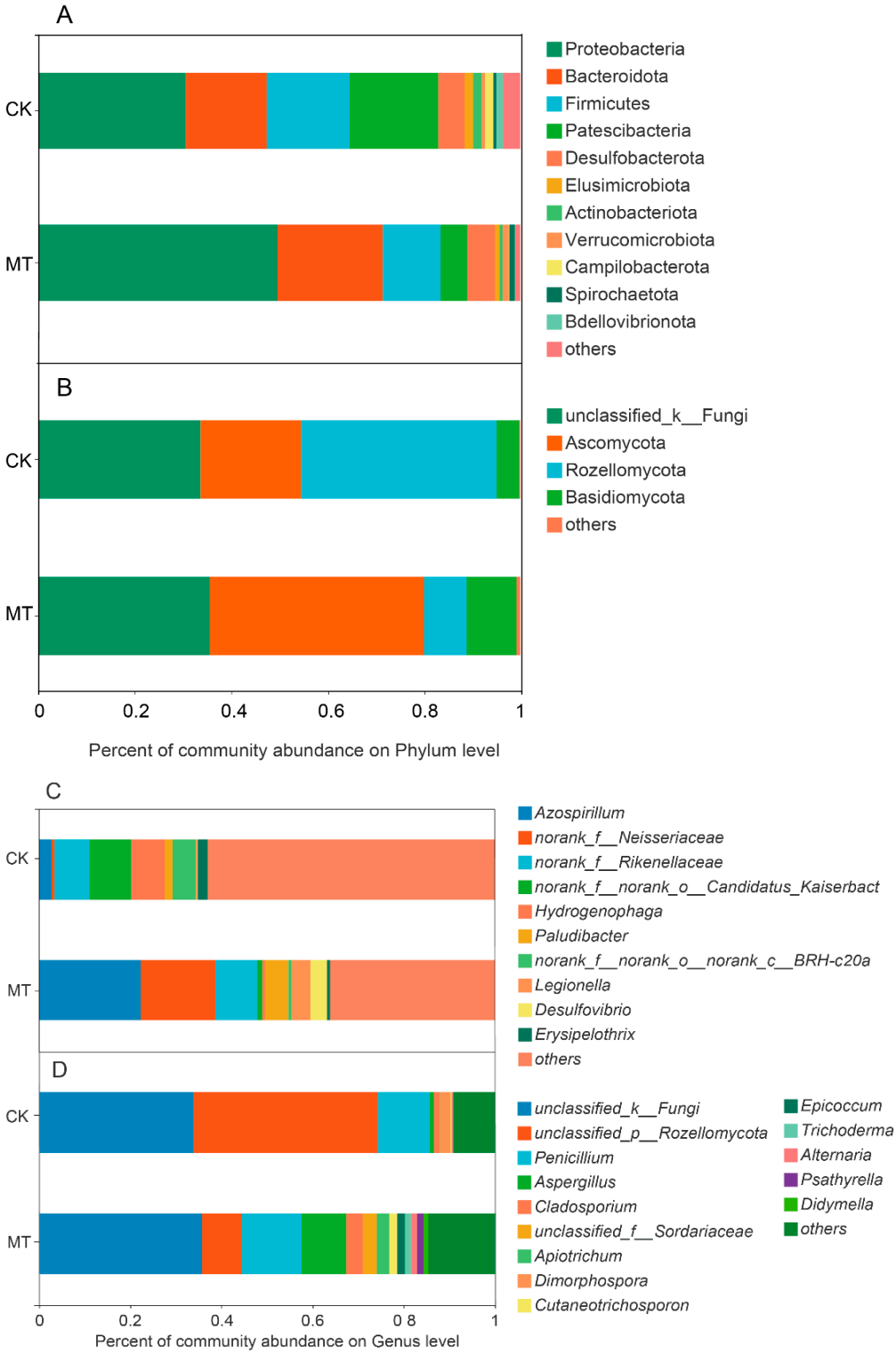

Figure 6. The relative abundance of bacteria (**A**,**C**) and fungi (**B**,**D**) community structure at the phylum and genus level after treatment for 7 days under control (CK) and melatonin (MT) conditions in wastewater.

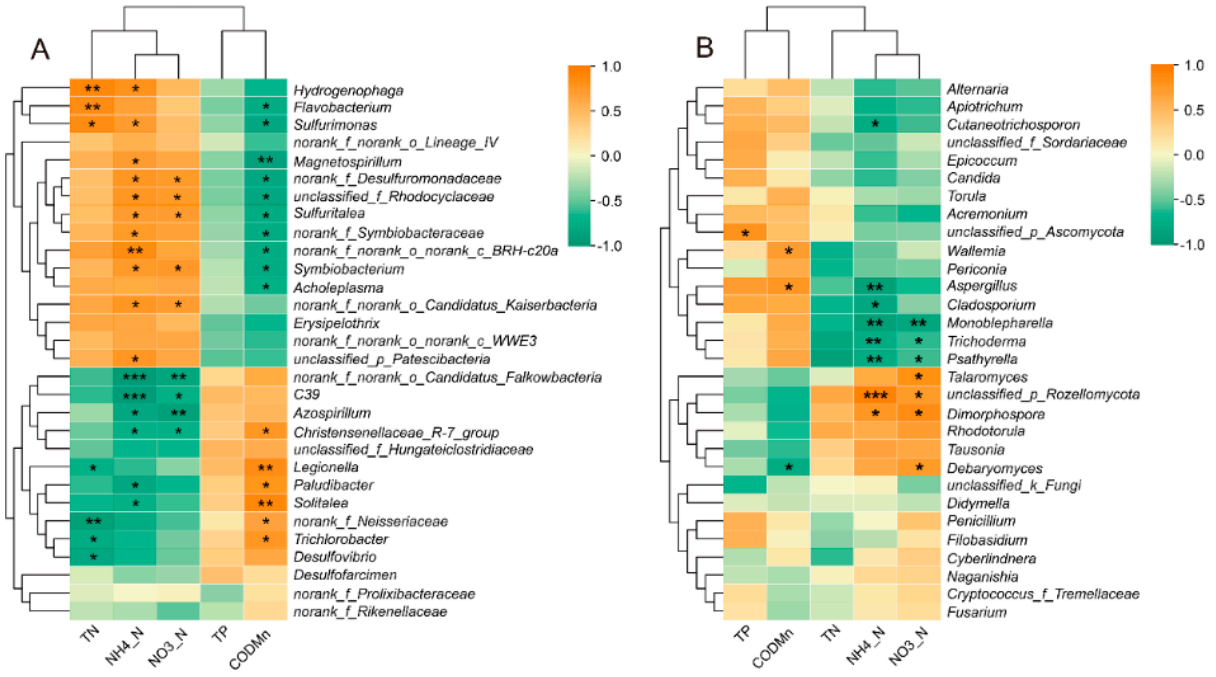

Figure 7. Correlation heatmap of bacteria (**A**) and fungi (**B**) taxa in control and melatonin after treatment for 7 days with the wastewater TN, TP, NH_4^+-N, NO_3^--N, and CODMn, chemical oxygen demand. TN, total nitrogen; TP, total phosphorus; NH_4^+-N, ammonium nitrogen; NO_3^--N, nitrate nitrogen. "*" indicates significance at $p < 0.05$, "**" indicates significance at $p < 0.01$, and "***" indicates significance at $p < 0.001$.

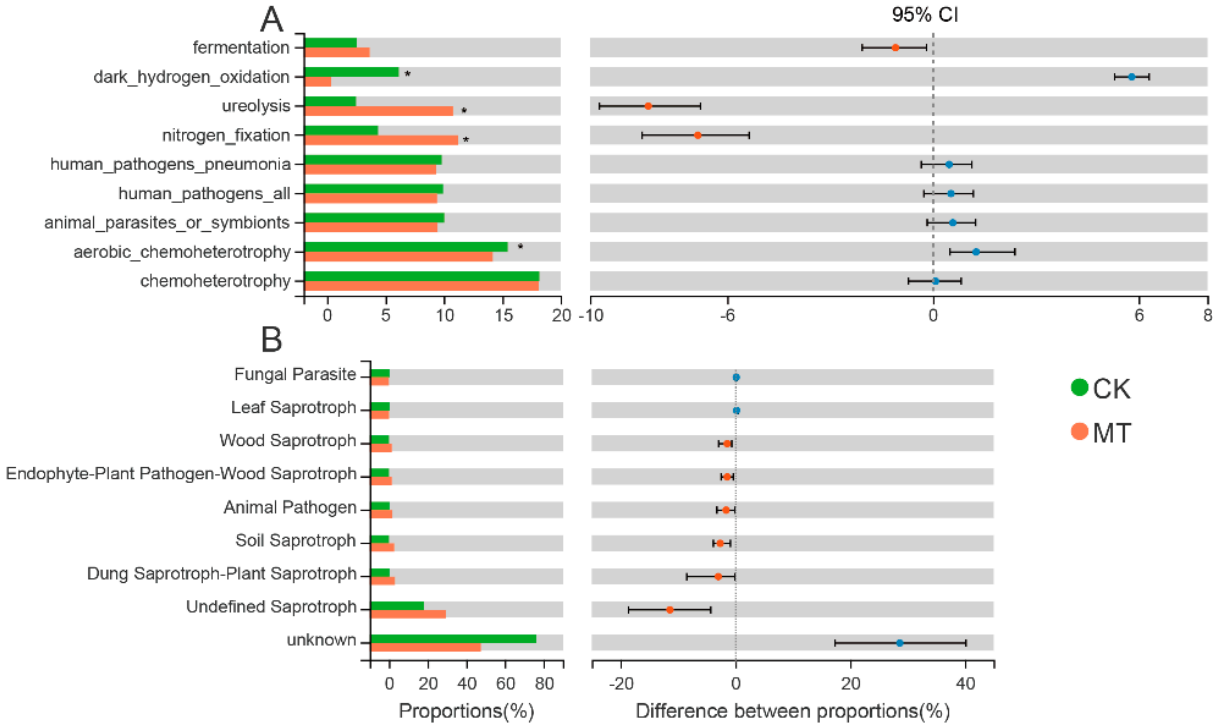

Figure 8. Wilcoxon rank-sum test bar plot. The relative abundances of potential bacterial (**A**) and fungal functional groups (**B**) under control (CK) and melatonin (MT) conditions in wastewater predicted by FARPROTAX and FUNGuild, respectively. "*" indicates significance at $p < 0.05$.

4. Discussion

Plants are an important component of constructed wetland systems. As a typical wetland plant with wide adaptability and high tolerance to adversity, *Phragmites australis* is one of the effective purification plants that can remove organic and inorganic pollutants from wastewater [27]. It has been found that the growth performance of plants, including photosynthetic capacity and transpiration characteristics, were closely geared to the denitrification effect of constructed wetlands [28]. Melatonin can be extensively involved in plant development and stress tolerance, and our results indicated that melatonin treatment stimulated gas exchange, including increased An and g_s of *Phragmites australis* leaves, and the photosynthetic capacity was affected by melatonin also found in maize and wheat [29,30]. Liu et al. suggested that melatonin can improve plant photosynthetic efficiency by reducing chlorophyll degradation and promoting chlorophyll synthesis [31]. Wetland plants can secrete oxygen through the roots, thus creating an aerobic environment in the rhizosphere and promoting the nitrification of microorganisms, which is conducive to nitrogen removal in wastewater [32]. In addition, the results here showed that melatonin could affect the carbohydrate synthesis and metabolism of *Phragmites australis*. Melatonin treatment increased the UGPase activity and decreased Susy activity of leaves, which is involved in sucrose biosynthesis, which is consistent with our result that sucrose content under melatonin treatment was not significantly different from the control. The invertase (cytInv, vacInv) activities under melatonin treatment were increased compared to the control, promoting the catalytic breakdown of sucrose to fructose in leaves. Additionally, this is also consistent with the results of a significant increase in soluble sugar content in leaves under melatonin treatment. Soluble sugar contents in plants are highly sensitive to environmental stress [33]. Artificial sewage stress caused oxidative damage in wetland plants, while soluble sugars' accumulation can benefit the osmotic homeostasis of plant cells, suggesting that melatonin treatment can improve the plant tolerance to sewage stress in relation to the soluble sugar accumulation [34]. In addition, compared with the control, the HXK activity of leaves was also increased under melatonin treatment, and HXK could catalyze the phosphorylation of hexose and promote the glycolytic pathway of glucose. Previous studies have also reported that melatonin can improve the stress tolerance of plants by regulating sugar metabolism [35,36]. Combined with our results, melatonin can promote the activity of carbohydrate metabolism enzymes in common reed, the accumulation of soluble sugar in leaves, and energy metabolism.

The removal of nitrogen and phosphorus from wastewater in constructed wetlands occurs mainly through three pathways: plant uptake, substrate uptake, and microbial decomposition [2]. The xylem of plants transports water and mineral nutrients absorbed by the root to the shoot and plays a key role in the transport of nutrients and signals [37]. The composition of nutrients in xylem sap might reflect the nut availability in the soils and could be used to determine the content of absorbable nutrients by plants in wastewater [38,39]. Our results showed that *Phragmites australis* could effectively absorb nitrogen and phosphorus from wastewater, and the phosphate and anion contents in xylem sap were significantly higher under melatonin treatment than those in control, which is consistent with the results of Chen et al. that show that melatonin treatment increased phosphorus content in alfalfa under high nitrogen stress [40]. Phosphorus is not only an important component of plants, but also provides the anionic equivalent of the xylem. Although melatonin promoted phosphorus uptake in common reeds, the total phosphorus content in the effluent was not affected by melatonin, which may be due to the fact that phosphorus absorbed by plants is mainly inorganic phosphorus in wastewater and the total phosphorus in the CWs includes inorganic and organic phosphorus [41]. Thus, melatonin increased the uptake of inorganic phosphorus in *Phragmites australis* but did not affect total phosphorus in the effluent. Nitrogen pollutants in wastewater are mainly in the form of organic nitrogen and ammonia nitrogen, and nitrogen removal in wetlands mainly occurs by plant uptake and microbial decomposition [42]. Our results showed that there was no significant difference in nitrogen content in the xylem sap of common reeds under the two treatments, but the NH_4^+-N

concentration in the effluent under melatonin treatment was significantly lower than that in the control. The organic nitrogen in the wastewater is first decomposed into ammonia nitrogen by wetland microorganisms, and the ammonia nitrogen is converted into nitrite and nitrate nitrogen by aerobic nitrifying bacteria and finally removed by denitrification and plant absorption [43]. The current results suggested that melatonin may promote the removal of ammonia nitrogen in wastewater by microbes in CWs. These findings were in good agreement with previous studies, indicating that plants and substrates can only absorb a small fraction of nitrogen, and most of the nitrogen was removed by nitrification and denitrification of microorganisms in CWs [44]. Combined with our results, melatonin can reduce the content of inorganic phosphorus in wastewater by improving the absorption capacity of wetland plants but does not affect nitrogen uptake.

The microbial community plays an important role in reducing contaminants in constructed wetlands. However, the composition and structure of microbial communities in CWs are still rarely known. The coverage index for 16S/ITS rRNA gene sequences was greater than 0.98 in two treatments, indicating that there was satisfactory coverage for all the microbial samples. We found that melatonin had a significant effect on the alpha diversity of both bacterial and fungal communities in the CWs. This is consistent with previous findings that melatonin can affect the alpha diversity of soil microorganisms [45]. Interestingly, Li et al. found that melatonin can reduce the richness of bacteria in silage but increase its diversity [46]. The present study found that melatonin treatment decreased the richness of bacterial and fungal communities in CWs, as well as bacterial diversity, while increasing fungal diversity. This suggests that melatonin can affect microbial alpha diversity and that its alterations to the microbiome are influenced by environmental factors.

In the two CWs, *Proteobacteria*, *Bacteroidiota*, *Firmicutes,* and *Patescibacteria* were the main bacterial phyla with a high relative abundance, which is consistent with previous findings that these bacterial phyla are the main composition in the microbial community of constructed wetlands [47–49]. Additionally, the main fungi in CWs belonged to the phyla *Ascomycota*, *Rozellomycota,* and *Basidiomycota*. *Ascomycota* is the major fungi phylum in water ecosystems and is the dominant fungal phylum for the wetland [50]. The application of melatonin altered the relative abundance of dominant bacterial and fungal phyla in CWs. Melatonin increased the relative abundance of phyla *Proteobacteria* and *Bacteroidetes* and decreased *Firmicutes* and *Patescibacteria* in CWs. Combined with our results, *Proteobacteria* was the most predominant bacterial phyla, and the increase in its abundance under melatonin treatment might be due to the melatonin-induced decreased diversity of the bacterial community. *Proteobacteria* and *Firmicutes* play crucial roles in the denitrification process, and the abundances of these two phyla were changed under melatonin treatment, which may affect the denitrification in CWs [51]. The relative abundance of *Ascomycota* was elevated under melatonin treatment, while in CWs, the relative abundance of *Rozellomycota* decreased. Several studies have shown that *Ascomycota* was strongly negatively affected by melatonin in soils [17], which is inconsistent with our findings, and this may be due to the effects of melatonin on microbes influenced by environmental factors.

Melatonin treatment increased the relative abundance of genera *Azospirillum* and *Paludibacter* in CWs. Bacteria of the genus *Azospirillum* belong to *Proteobacteria*, a group of nonsymbiotic nitrogen-fixing bacteria. *Azospirillum* has been reported to promote plant growth and nitrogen uptake, and the growth-promoting effect of this bacterium on plants can be attributed to its biological nitrogen fixation effect and ability to produce growth hormones [52,53]. *Paludibacter* belongs to the phylum *Bacteroidetes* and is one of the most abundant bacterial genera in wastewater [54]. In addition, melatonin treatment led to a decrease in the relative abundance of the genus *Hydrogenophaga*, a group of hydrogen-oxidizing bacteria, which can use hydrogen as an energy source for chemotrophic growth [55]. Moreover, melatonin increased the relative abundance of *Aspergillus* and *Apiotrichum* in CWs. *Aspergillus* is widespread in multiple habitats and plays an important role in the carbon and nitrogen cycle in ecosystems. Bacteria of genus *Apiotrichum* belong to Basidiomycota, and Chen et al. suggested that it might be related to the decomposition of organic matter in

sewage [56]. The increase in the relative abundance of *Apiotrichum* caused by melatonin may be beneficial to the decomposition of organic pollutants in CWs.

The FARPROTAX analyses showed that melatonin increased the abundance of bacteria involved in ureolysis and nitrogen fixation. Ureolysis is the process by which bacteria degrade urea into ammonia [57,58]. This may be related to the fact that melatonin promoted the photosynthetic properties of plants, thus improving the oxygen environment in the rhizosphere of common reeds. Nitrogen fixation is the biological process by which the inert nitrogen is converted to a usable form of nitrogen for plants and microorganisms [59]. Our results indicated that bacteria affected by melatonin tend to decompose the fixation and depletion of nitrogen. This was consistent with the results of the clustering heatmap, where bacteria at genus levels under melatonin treatment were correlated with inorganic nitrogen in CWs.

5. Conclusions

The current study detected the physiological activity of *Phragmites australis* plant. Microbial enzymes and microbial diversity and structure under melatonin treatment were investigated. Melatonin significantly promoted photosynthetic carbon assimilation and phosphorus content of xylem sap in plants and improved the uptake of NH_4^+-N in CWs but had no effect on TP. In addition, the increase in microbial urease activity was not significant under melatonin treatment, but the functional abundance of the bacterial community was associated with ureolysis, and nitrogen fixation increased under melatonin treatment in CWs. CWs provide an economical and effective method for wastewater treatment. The study here provides convincing evidence that melatonin could improve the wastewater treatment efficiency of CWs by stimulating plant growth and microbial community composition and function, which are vital for developing exogenous additives to improve the sewage purification capacity of CWs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/land11112022/s1, Figure S1: Activities of key carbohydrate metabolism enzymes in the leaves of *Phragmites australis* under control (CK) and melatonin (MT) conditions. Ald, aldolase; UGPase, UDP-glucose pyrophosphorylase; Susy, sucrose synthase; AGPase, ADP-glucose pyrophosphorylase; HXK, hexokinase; PGI, phosphoglucomutase; G6PDH, glucose-6-phosphate dehydrogenase; PFK, phosphofructokinase; PGM, phosphoglucomutase; FK, fructokinase; vacInv, vacuolar invertase; cytInv, cytoplasmic invertase; cwInv, cell wall invertase. ns indicates not significant and "*" indicates significance at $p < 0.05$. Data are expressed as means ± SE (n = 3); Figure S2: Changes in the relative abundance of bacteria (A) and fungi (B) after treatment for 7 days under control (CK) and melatonin (MT) conditions in wastewater at the genus level. Data are expressed as means ± SE (n = 3); Table S1: Mantel test based on the unweighted-unifrac distance between the microbial community at genus level in CWs and pollutant contents in the effluent.

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