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Characteristics of Bacterial Communities in Cyanobacteria-Blooming Aquaculture Wastewater Influenced by the Phytoremediation with Water Hyacinth

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Abstract: Cyanobacterial blooms often occur in aquaculture wastewater in China. A floating plant, water hyacinth has been widely used to treat this wastewater. Little is known, however, about bacterial community characteristics and the risk of potential pathogens in cyanobacteria-blooming aquaculture wastewater remediated by water hyacinth. In wastewater treated with water hyacinth, we used culture enumeration and high-throughput sequencing to explore the characteristics of bacterial communities, the status of coliform bacteria, and pathogenic bacteria potentially conducive to human disease. Our results indicated that the relative abundance of *Acidobacteria*, *Planctomycetes*, *Actinobacteria*, *Chlorobi*, *Cyanobacteria*, *Proteobacteria*, and phylum OD1 in cyanobacteria-blooming aquaculture wastewater were significantly influenced by water hyacinth. After 30 days, the relative abundance of *Proteobacteria* and phylum OD1 in the water hyacinth treatments increased remarkably, while the relative abundance of the other 5 phyla in treatment was significantly reduced compared with the controls. In 21 major families, the relative abundance of *Comamonadaceae*, *Oxalobacteraceae*, *Rhodocylclaceae*, and an unnamed group from phylum OD1 increased significantly in the water hyacinth treatments compared with the controls. The number of total coliforms in wastewater treated by water hyacinth was significantly elevated and higher than controls during the first 6–18 days, with the maximum reaching 23,800 MPN/L. The level of potential pathogenic bacteria in wastewater treated by water hyacinth significantly reduced compared with the controls after 18 days, but it significantly increased from the initial level. It appears that water hyacinth by itself is not an effective treatment for reducing potential pathogens in aquaculture water.

Keywords: aquaculture wastewater; cyanobacterial bloom; water hyacinth; phytoremediation; bacterial communities; potential pathogenic bacteria

1. Introduction

With the development of large-scale and high-density aquaculture industry in China, aquaculture wastewater of nearly 300 million cubic meters discharges into natural waterbodies every year [1]. The large amount of residual feed and feces make the aquaculture water eutrophic. Therefore, an increasing amount of attention has been paid to the treatment of aquaculture wastewater. Phytoremediation technology has been important due to its low cost and strong ability to remove nutrients. Water hyacinth, a floating aquatic plant, has been widely used in China because of its superior ability to remove nitrogen and phosphorus [2]. However, most studies on the influence of floating plants have focused on the reduction of nitrogen, phosphorus, and chemical oxygen demand in eutrophic water [3,4]. In recent years, specific bacterial communities associated with

water hyacinth, such as sulfate-reducing bacteria [5], nitrifying and denitrifying bacteria [6], and ammonia-oxidizing bacteria [7], have been studied. However, there is little information regarding the overall structure of bacterial communities and the status of potential pathogens in aquaculture wastewater after purification by floating plants. In China, cyanobacterial bloom often occurs in aquaculture wastewater [8]. The problems caused by cyanobacterial bloom are often associated with the toxins that they produce as secondary metabolites and the death of aquatic organisms due to the reduction of dissolved oxygen and transparency. Cyanobacterial bloom also has impacts on bacterial communities [9]. *Microcystis aeruginosa* is the most common and primary bloom-forming cyanobacteria in Chinese eutrophic freshwaters [10]. Some common bacteria associated with *M. aeruginosa* are pathogenic [11]. Therefore, it is necessary to pay attention to the characteristics of bacterial communities and the situation of potential pathogens in *M. aeruginosa*-blooming aquaculture wastewater remediated by water hyacinth. With the rapid development of molecular biological technology, high-throughput sequencing technology has been used in the study of bacterial structure of rivers, lakes, and reservoirs [12–14]. We applied culture enumeration and high-throughput sequencing to quantify the changes in the characteristics of bacterial communities, specific bacteria including coliform bacteria, and pathogenic bacteria potentially conducive to human disease by treating cyanobacteria-blooming aquaculture wastewater with water hyacinth.

2. Materials and Methods

2.1. Strains and Culture Conditions

Aquaculture wastewater was from the fishponds in Jiangsu Academy of Agricultural Sciences (Nanjing, China). Stocking density of grass carp was about 1.6 kg/m^2. Feeding was carried out at the amount of 2.5% fish body weight daily. The water in fishponds was changed every three days. The waste effluent was filtered for use. Its concentration of total nitrogen and total phosphorus were 13.24 $\pm$ 0.83 mg/L and 5.45 $\pm$ 0.98 mg/L, respectively.

M. aeruginosa (FACHB-912) was purchased from Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). This strain was cultivated in batch cultures using 1/10 modified Hoagland's medium (pH 7.0, adjusted with NaOH) and then amplified in the filtered waste effluent for about 7 days. When the density of *M. aeruginosa* reached about 3.8 $\times$ 10^6 cells/Ml, the amplified culture could be used in co-existence experiments.

Water hyacinth (*Eichhornia crassipes*) was collected from a concrete breeding pool at Jiangsu Academy of Agricultural Sciences (Nanjing, China), cleaned of debris, washed with tap water, soaked in 0.01% Potassium Permanganate and then washed with distilled water. The plants with similar height (about 30 cm) were selected and pre-incubated in aseptic 1/10 modified Hoagland's medium for 7 days before used in co-existence experiments.

The incubator was maintained at 28 °C with a constant relative humidity of 75% and illuminated by cool-white fluorescent lamps (40 μmol photons/m^2/s) in 12 h diurnal cycles.

2.2. Co-Existence Experiments

Treatments (T) were prepared in the amplified cultures of *M. aeruginosa* (10 L) in flat-bottomed glass containers with pretreated *E. crassipes* (about 160 g) added. Controls (C) were prepared also in amplified cultures of *M. aeruginosa* without *E. crassipes*. All co-existence experiments were conducted with three independent replicates in a non-sterile environment and the culture solutions were stirred once a day. Over a period of 30 days, water samples were collected at 6-day intervals to determine the total bacteria and the total coliforms in water. The total number of bacteria in water is related to the degree of organic pollution and is often used as an index to evaluate the degree of water pollution. Coliform groups have a certain indicative function to the hygienic condition and safety of a water source. Furthermore, water samples were filtered through a Millipore membrane (0.22 μm pore size). The filtered membrane was stored at −80 °C and used for subsequent DNA extraction and

high-throughput sequencing. Further analysis of bacterial community's characteristics and potential pathogenic bacteria's level were carried out. The bacterial community structure might respond to the water pollution, and the potential pathogenic bacteria in the discharged water pose a potential threat to ecological safety and human health in receiving water.

2.3. General Enumeration of Bacteria

Quantitation of total bacteria in water was determined by the plate count method [15]. Numbers of coliforms in the water were analyzed by a multiple-tube fermentation technique [16].

2.4. DNA Extraction and High-Throughput Sequencing

The total genomic DNA was extracted from water samples using a PowerWater® Sterivex™ DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of DNA was determined via spectrophotometer analysis. The quality of DNA was checked by agarose gel electrophoresis. The V3-V4 hypervariable region of the 16S rRNA gene was amplified by PCR, and sequence data was analyzed by Illumina MiSeq platform at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China) PCR was performed using Q5™ High-Fidelity DNA Polymerase (NEB, Ipswich, MA, USA) with forward primer 338F (5′-barcode+ACTCCTACGGGAGGCAGCA-3′) and reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′). PCR amplification followed a 3-min denaturation at 95 °C. Twenty-seven amplification cycles were performed as follows: denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s. A final extension was performed at 72 °C for 5 min. DNA libraries for sequencing were prepared using TruSeq Nano DNA LT Library Prep Kit (Illumina, San Diego, CA, USA). Qualified libraries were then sequenced on the Illumina MiSeq, using MiSeq Reagent Kit V3 (600 cycles) for a 2 × 300 bp paired-end reads.

2.5. Statistical Analysis

The V3-V4 sequences were clustered at 97% identity by using the Qiime software platform. The information of each operational taxonomic unit (OTU) was obtained by database comparison [17]. The relative bacterial abundances were calculated by the abundance of bacterial OTUs at specific taxonomic ranks (family and phylum). Alpha and beta diversity indices were computed at a sequence depth of 35,654 sequences from the sample with the lowest valid sequences. The ACE richness estimator and Shannon diversity index were calculated with Mothur software [18]. The normality of data was investigated with a Shapiro–Wilk test and the homogeneity of variance was examined with Levene's test. Significant differences between control and treatment were determined via ANOVA (see Supplementary Materials). Differences were considered significant at $p < 0.05$.

Non-metric multidimensional scaling (NMDS) analysis was carried out. The distance between samples was calculated based on the evolution and abundance information of each sample sequence. According to the taxonomic information of OTU, the potential pathogens were counted based on genus information summarized in the literature [19,20].

3. Results

3.1. Bacterial Community Structure in Cyanobacteria-Blooming Aquaculture Wastewater Influenced by Water Hyacinth

All samples were sequenced on the MiSeq platform yielding a mean of 39,767 ± 2382 reads per sample after quality filtering. The numbers of reads for each sample group are shown in Table 1. A total of 37 phyla, 79 classes, 119 orders, 136 families, and 141 genera were identified in cyanobacteria-blooming aquaculture wastewater treated by water hyacinth.

Ten bacterial phyla with relatively higher abundance were compared between the controls and water hyacinth treatments. There was no significant difference in the relative abundance of *Bacteroidetes*,

Firmicutes, and *Verrucomicrobia* between the controls and treatments over time (Figure 1). After 30 days, in both controls and treatments, the average relative abundance of *Bacteroidetes* decreased from 20.46 ± 2.70% to 4.26 ± 0.60%, the average relative abundance of *Firmicutes* increased from 0.04 ± 0.01% to 2.54 ± 0.28%, and the average relative abundance of *Verrucomicrobia* remained at 7.40 ± 0.25%. There was a significant difference in the relative abundance of *Acidobacteria*, *Planctomycetes*, *Actinobacteria*, *Chlorobi*, *Cyanobacteria*, *Proteobacteria*, and phylum OD1 between the controls and treatments over time (Figure 1). After 30 days, the relative abundance of *Acidobacteria* in the controls rose from 0.31 ± 0.02% to 1.94 ± 0.30%, while that in the treatments finally remained at 0.46 ± 0.10% ($p < 0.05$). The relative abundance of *Actinobacteria* in the controls increased from 19.46 ± 2.19% to 29.47 ± 2.09%, while that in the treatments decreased finally to 10.63 ± 0.51% ($p < 0.05$). The relative abundance of *Chlorobi* in the controls increased from 0.11 ± 0.03% to 3.76 ± 0.94%, whereas that in the treatments eventually remained at 0.29 ± 0.06% ($p < 0.05$). The relative abundance of *Planctomycetes* in the controls increased from 4.76 ± 0.89% to 10.86 ± 0.61%, whereas that in the treatments eventually declined to 2.45% ± 0.39% ($p < 0.05$). The relative abundance of *Cyanobacteria* in the controls decreased from 23.40 ± 0.96% to 0.31 ± 0.10% and then rose to 1.74 ± 0.20%, while that in the treatments continued to decrease to 0.06 ± 0.02% at the end of the study ($p < 0.05$). The relative abundance of *Proteobacteria* in the controls increased from 23.24% ± 1.46 to 27.51 ± 0.64%, which was still significantly lower than the final abundance in the treatments about 61.27 ± 3.97% ($p < 0.05$). The relative abundance of phylum OD1 in the controls increased from 0.01% ± 0.01 to 0.47 ± 0.05%, which was still significantly lower than the final abundance in the treatments about 3.33 ± 0.68% ($p < 0.05$).

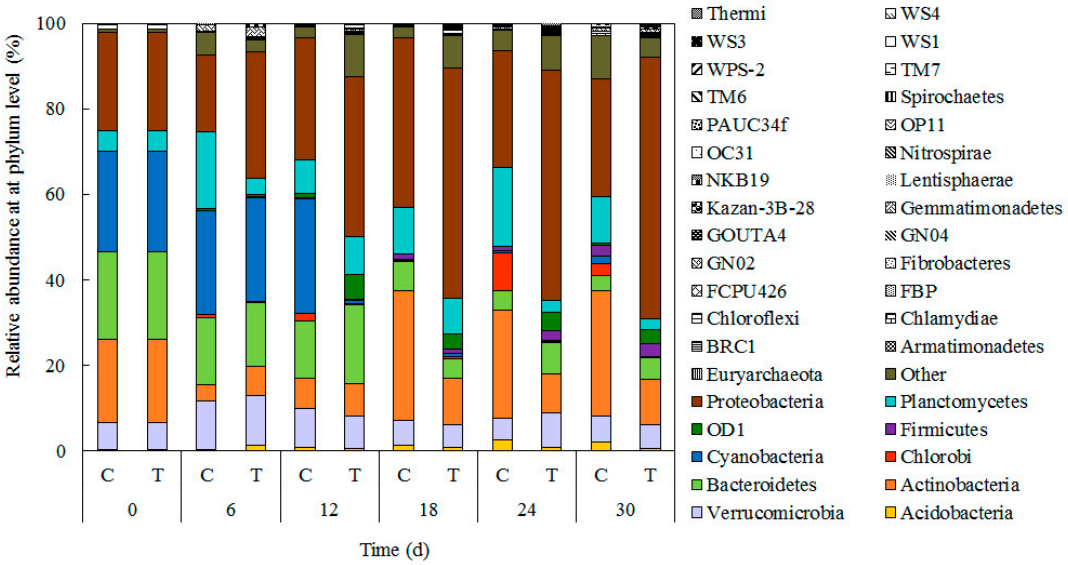
Table 1. The number of reads per each sample groups.

Sample	Valid Sequences	Sample	Valid Sequences
C0	40,114 ± 1224	T0	40,114 ± 1224
C6	41,332 ± 2231	T6	38,333 ± 2264
C12	42,015 ± 1989	T12	38,563 ± 2005
C18	41,142 ± 2388	T18	37,364 ± 1908
C24	40,805 ± 1868	T24	38,579 ± 2179
C30	38,876 ± 2121	T30	39,961 ± 1645

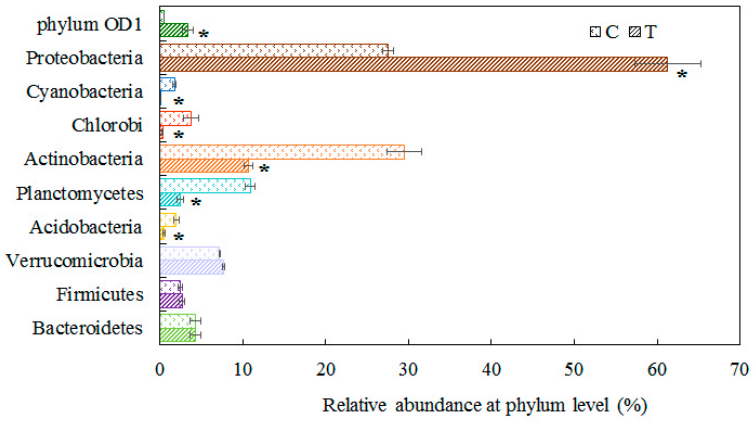
Notes: C means control; T means Treatment; the numbers behind the C/T represent the processing time.

The taxonomic differences between the controls and the treatments at the family level were further analyzed. Twenty-one major families (with relative abundance more than 1%) were investigated (Figure 1c). After 30 days, there was no significant difference in the relative abundance of *Cytophagaceae* (*Bacteroidetes*), *Cryomorphaceae* (*Bacteroidetes*), *Flavobacteriaceae* (*Bacteroidetes*), *Sphingobacteriaceae* (*Bacteroidetes*), *Chitinophagaceae* (*Bacteroidetes*), *Saprospiraceae* (*Bacteroidetes*), *Rhizobiaceae* (*Proteobacteria*), *Cerasicoccaceae* (*Verrucomicrobia*), and R4-41B (*Verrucomicrobia*) between the controls and the treatments. Significant differences between the controls and the treatments were detected in the relative abundance of C111 (*Actinobacteria*), *Actinomycetaceae* (*Actinobacteria*), *Mycobacteriaceae* (*Actinobacteria*), *Solirubrobacteraceae* (*Actinobacteria*), *Microcystaceae* (*Cyanobacteria*), an unnamed family (phylum OD1), *Gemmataceae* (*Planctomycetes*), *Pirellulaceae* (*Planctomycetes*), *Comamonadaceae* (*Proteobacteria*), *Oxalobacteraceae* (*Proteobacteria*), *Rhodocyclaceae* (*Proteobacteria*), and *Opitutaceae* (*Verrucomicrobia*). After 30 days, the relative abundance of C111 in the controls maintained at 1.00 ± 0.32%, while that in the treatments finally decreased to 0.37 ± 0.04% ($p < 0.05$). The relative abundance of *Actinomycetaceae* in the controls decreased from 4.54 ± 0.56% to 3.57 ± 0.34%, while that in the treatments eventually declined to 0.31 ± 0.09% ($p < 0.05$). The relative abundance of *Mycobacteriaceae* in the controls rose from 2.15 ± 0.29% to 15.82 ± 1.70%, which was significantly higher than the final abundance in the treatments about 5.88 ± 0.62% ($p < 0.05$). The relative abundance of *Solirubrobacteraceae* increased from 1.91 ± 0.26% to 3.69 ± 0.09%, whereas that in the treatments finally declined to 0.17 ± 0.09% ($p < 0.05$). The relative abundance of *Microcystaceae* in the controls decreased from 23.37 ± 0.96% to 0.29 ± 0.09% and then rose to 1.63 ± 0.18%, whereas that in the treatments continued to decrease to 0.024 ± 0.008%

at the end of the study ($p < 0.05$). The relative abundance of *Gemmataceae* in the controls rose to 2.96 ± 0.86%, while the final abundance in the treatments remained at 0.21 ± 0.24% ($p < 0.05$). The relative abundance of *Pirellulaceae* in the controls increased to 6.46 ± 0.54%, which was significantly higher than the final abundance in the treatments, which was about 0.80 ± 0.30% ($p < 0.05$). The relative abundance of *Opitutaceae* in the controls was up to 3.61 ± 0.51%, which was significantly higher than the final abundance in the treatments, which was about 2.59 ± 0.57% ($p < 0.05$). In addition, the relative abundance of only four major families in the treatments was significantly higher than that in the controls. The relative abundance of the unnamed group from phylum OD1 in the controls remained at 0.45 ± 0.05%, while the final abundance in the treatments was significantly elevated to 2.80 ± 0.66% ($p < 0.05$). The relative abundance of *Comamonadaceae* in the controls decreased to 8.92 ± 1.28%, while the final abundance in the treatments significantly increased to 23.14 ± 1.18% ($p < 0.05$). The relative abundance of *Oxalobacteraceae* in the controls decreased to 0.37 ± 0.21%, which was significantly lower than the final abundance in the treatments, which was about 1.63 ± 0.26% ($p < 0.05$). The relative abundance of *Rhodocyclaceae* in the controls decreased from 2.05 ± 0.39% to 0.90 ± 0.27%, while the final abundance in the treatments significantly rose to 24.43 ± 1.55% ($p < 0.05$).



(a)



(b)

Figure 1. *Cont.*

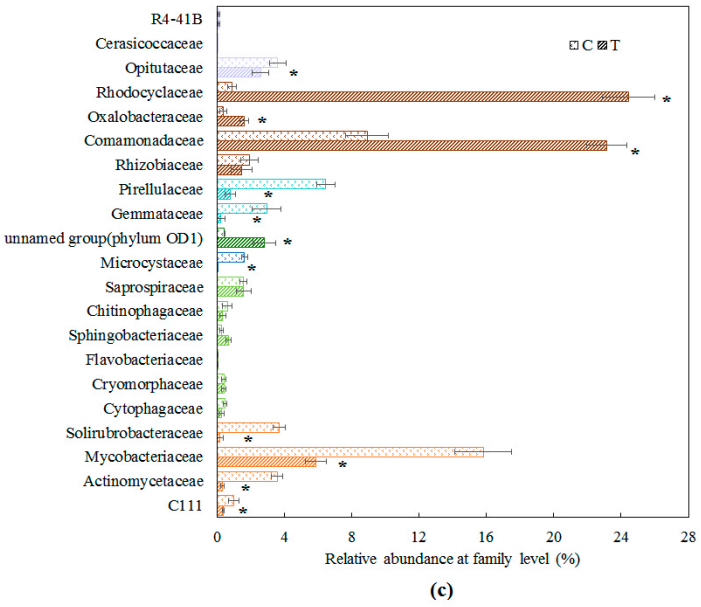


Figure 1. (**a**) Change of bacterial community composition at the phylum level in the controls (C) and water hyacinth treatments (T) over time. (**b**) Comparison of 10 bacterial phyla with relatively higher abundance between the controls (C) and the water hyacinth treatments (T) at day 30; (**c**) Comparison of 21 major families between the controls (C) and the water hyacinth treatments (T) at day 30. Asterisks were marked in histograms to indicate significant differences ($p < 0.05$) between the controls and the treatments. The same colored label showed the same phylum or the family in the same phylum.

3.2. Bacterial Richness and Diversity in Cyanobacteria-Blooming Aquaculture Wastewater Influenced by Water Hyacinth

The results of the ACE index showed that the richness of bacterial communities in cyanobacteria-blooming aquaculture wastewater treated by water hyacinth was significantly higher than that of the controls (Figure 2a). The results of the Shannon index showed that the diversity of bacterial communities in cyanobacteria-blooming aquaculture wastewater treated by water hyacinth was significantly higher than that of the controls from Day 6 to Day 24 and then returned to the level of the controls at Day 30 (Figure 2b). However, the results of NMDS intuitively showed that the treatment changed the micro-ecological balance of the cyanobacteria-blooming aquaculture wastewater and its further development (Figure 3).

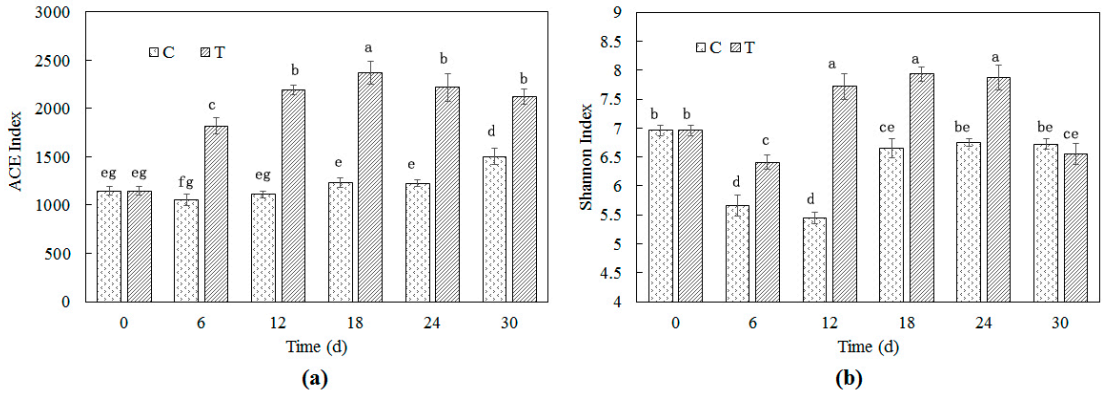


Figure 2. Change of ACE richness index (**a**) and Shannon diversity index (**b**) in bacterial communities of the controls (C) and the water hyacinth treatments (T) over time. Different letters were marked in histograms to indicate significant differences ($p < 0.05$).

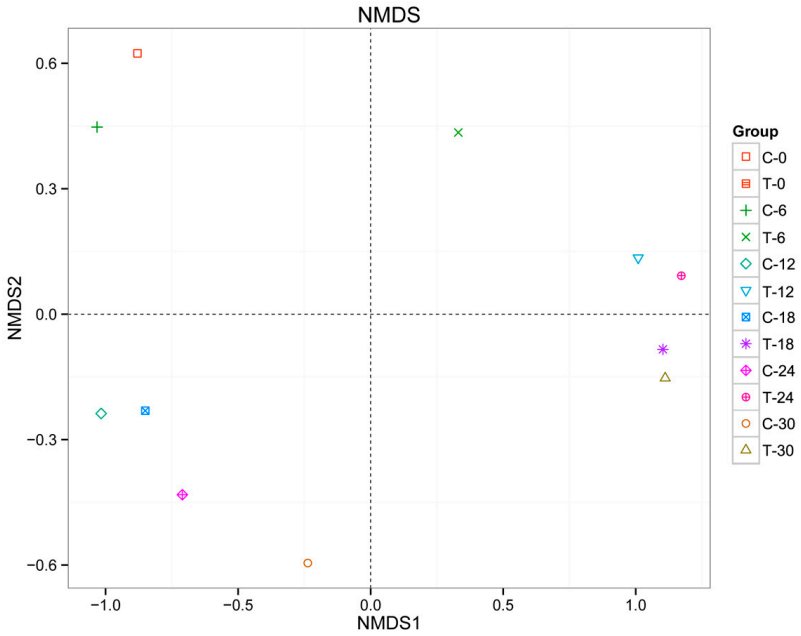


Figure 3. NMDS plots derived from pairwise unweighted UniFrac distances between bacterial communities in the controls (C) and in the treatments (T). The numbers behind the C/T represent the processing time.

3.3. Specific Bacteria and Disease-Related Genes in Cyanobacteria-Blooming Aquaculture Wastewater Influenced by Water Hyacinth

3.3.1. Total Bacteria

Quantitation results of total bacteria indicated that the number of total bacteria in the wastewater treated by water hyacinth for 6 days increased and was significantly higher than that in the controls. However, there was a marked increase in the number of total bacteria in the controls at Day 12, which might be related to the decline of cyanobacteria and nutrient release. After 18 days, the total bacterial number in the wastewater treated by water hyacinth continued to be significantly higher than that in the controls (Figure 4a).

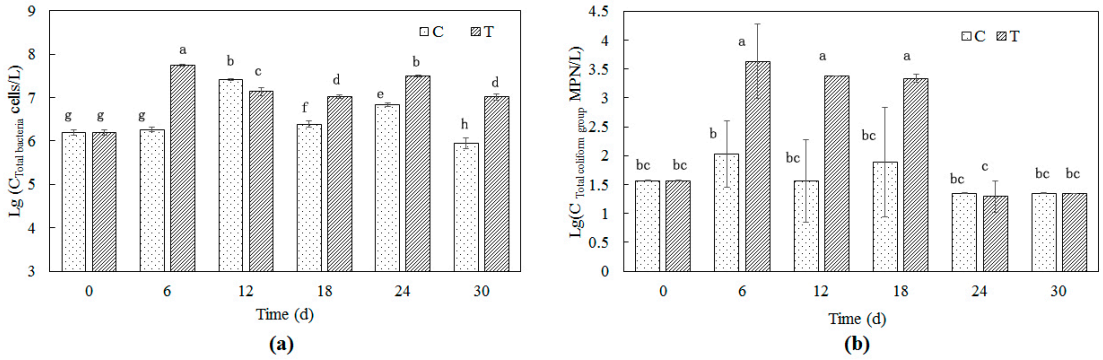


Figure 4. *Cont.*

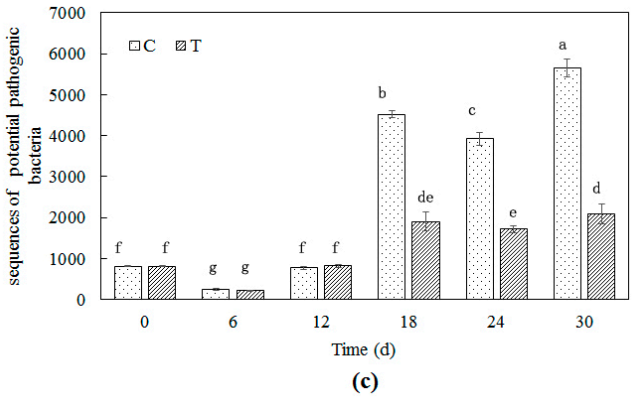


Figure 4. Change of total bacterial number (**a**), total coliform levels (**b**) and potential pathogenic bacteria levels (**c**) in the controls (C) and the water hyacinth treatments (T) over time. Different letters were marked in histograms to indicate significant differences ($p < 0.05$).

3.3.2. Total Coliform Group

The total coliform group is an important indicator in the hygienic standard of drinking water. As shown in Figure 4b, the number of total coliforms in cyanobacteria-blooming aquaculture wastewater treated by water hyacinth for 6–18 days was significantly higher than in the controls and then returned to the control level after 24 days. It is possible that the potential risk of intestinal epidemic was enhanced during the early treatment of cyanobacteria-blooming aquaculture wastewater by water hyacinth.

3.3.3. Potential Pathogenic Bacteria

Based on the statistics of potential genus of pathogenic bacteria, aquaculture wastewater used in this experiment contained 11 genera of potential pathogenic bacteria. They belonged to *Mycobacterium*, *Bacillus*, *Clostridium*, *Rickettsia*, *Arcobacter*, *Helicobacter*, *Enterobacter*, *Legionella*, *Pseudomonas*, *Treponema*, and *Leptospira*. *Mycobacterium* with the highest abundance accounted for 87.27 ± 2.89% of the total pathogenic bacteria. As shown in Figure 4c, potential pathogenic bacteria both in the controls and in the treatments significantly increased from the initial level after 18 days. However, the level of pathogenic bacteria in cyanobacteria-blooming aquaculture wastewater treated by water hyacinth was significantly lower compared with the controls 18 days later.

4. Discussion

After years of development, the Chinese aquaculture industry has become pivotal and has a large share in the world. However, the Chinese aquaculture wastewater is mostly accompanied by cyanobacterial bloom now [21]. The discharge standards of wastewater are mainly composed of nitrogen, phosphorus, and other basic physical or chemical indicators. Sufficient attention should also be paid to the characteristics of bacterial communities and the situation of potential pathogenic bacteria in discharged water after purification by aquatic plants.

Our results revealed that bacteria in cyanobacteria-blooming aquaculture wastewater mainly belonged to *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Verrucomicrobia*, and *Planctomycetes* besides *Cyanobacteria*. This was similar to the reported microbial community in the natural lake suffering cyanobacterial bloom [22]. After cyanobacteria-blooming aquaculture wastewater was treated by water hyacinth for 30 days, *Proteobacteria* was found to be the predominant phylum in total bacteria of water with a relative abundance of 61.27 ± 3.97%. As reported, *Proteobacteria* is one of the most common bacterial phylum in aquaculture ponds [23] and is also an important microorganism involved in global carbon and nitrogen and sulfur cycle [24]. Its relative abundance in constructed wetland is always particularly high and up to 50–61% [25]. It was indicated that the flourishing root system of

water hyacinth might also form a purifying environment similar to constructed wetland. Under the impact of water hyacinth, the relative abundance of only four major families was significantly higher than the controls. *Comamonadaceae*, *Oxalobacteraceae*, and *Rhodocylclaceae* are all within *Proteobacteria*. *Oxalobacteraceae* has been reported to be root-associates [26]. *Rhodocyclaceae* was considered important for the formation of aerobic granular sludge biofilms and the nutrient removal [27]. *Comamonadaceae* has been commonly detected in activated sludge wastewater treatment processes and is likely to be important for nutrient removal [28]. These bacteria might coordinate with water hyacinth and improve the removal of nutrients.

Wetland rhizospheres have been extensively studied with respect to microorganism communities [29]. It has been shown that the root exudates from different plants could direct the different development of microbial communities [30]. Organic material from cyanobacteria could also impact bacteria [22]. In our previous study, the growth rate of *M. aeruginosa* could be inhibited by water hyacinth and the algal cell death could be provoked [31]. In this study, it was also found that the reduction of relative abundance of phylum *Cyanobacteria* occurred at a quicker pace due to water hyacinth treatment. Water hyacinth in cyanobacteria-blooming aquaculture wastewater not only introduced the root exudates but also had an inhibitory effect on cyanobacteria. As a result, the bacterial community balance and subsequent development trends in aquaculture wastewater were changed due to the multiple influences of water hyacinth.

Most aquaculture wastewater, after it was treated, would eventually be discharged into natural water, although part of it was recycled. The excellent ability of water hyacinth to remove nutrients from wastewater has been generally acknowledged [2]. However, it is worth investigating whether there are any harmful bacteria for human beings in the water-hyacinth-treated water that has met the nutrients standard. Based on the Chinese water quality standard for drinking water sources (CJ 3020-93), the lowest grade requires that the density of total coliforms be less than 10,000 MPN/L. Our experiments found that the number of total coliforms in water hyacinth treatments was 1.90 ± 0.22 times that of the control at the initial period from Day 6 to Day 18, with the maximum reaching 23,800 MPN/L. This might be explained by the roots of water hyacinth, which could provide habitats and shelters for microbes such as enterobacteria [32]. Moreover, endophytic *Enterobacter* exists in water hyacinth and can promote plants growth [33]. However, coliform groups are a basic indicator of pathogen pollution in a body of water, and they are correlated with intestinal pathogenic bacteria. Therefore, the detention time of wastewater in water hyacinth treatments should be paid attention to.

Compared to the control, the level of potential pathogens in the wastewater decreased significantly after the water was treated with water hyacinth for 30 days in our experiments. However, this level was still significantly elevated by 1.33 ± 0.22 times compared with the initial level. The risk of this elevation needs to be further investigated. These findings show that water hyacinth by itself might not be an effective treatment for reducing potential pathogens in aquaculture wastewater. Further efforts, such as ozone treatment [34], an application of nano-antibacterial materials [35], and biological sand filtration [36], should be made to remove pathogenic bacteria from aquaculture wastewater.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4441/9/12/956/s1.

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Author Contributions: Qing Zhou conceived and designed the experiments; Qing Zhou and Ting Chen performed experiments; Qing Zhou analyzed the data and wrote the paper; Qing Zhou and Shiqun Han revised the paper.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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