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Using Natural and Artificial Microalgal-Bacterial Granular Sludge for Wastewater Effluent Polishing

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Abstract: Marimo is a type of microalgal-bacterial granular sludge (MBGS) that exists in natural water bodies. For the first time, this paper explored the feasibility of marimo in real wastewater effluent polishing, focusing on nutrient removal as compared with MBGS. The results showed that the color of marimo gradually darkened during a 21-day experiment, and the chlorophyll content increased significantly. Although marimo and MBGS showed fairly similar removal performance in terms of NO_3^--N and TN, marimo exhibited better phosphate removal as compared to MBGS. Marimo and MBGS contained different algae but the same bacterial phylum of *Proteobacteria*, including denitrifiers. In addition, marimo had a higher relative abundance of nitrite reductase than MBGS, suggesting that the denitrification process might also happen in addition to assimilation. This study is expected to initiate the application of marimo for wastewater effluent polishing and reclamation, shedding light on nature-based wastewater self-purification technology in the era of carbon neutrality.

Keywords: marimo; microalgal-bacterial granular sludge; algae-bacteria symbiosis; wastewater reclamation; metagenomics; nitrite reductase

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1. Introduction

Recently, the world has been facing various water crises, of which the shortage of water resources and water pollution are the most prominent [1,2]. On the one hand, the global demand for water resources is constantly expanding, and a growing number of cities are facing water scarcity issues. According to statistics, the population of cities facing water scarcity worldwide will reach 2.065 billion by 2050 [3–5]. On the other hand, wastewater from cities, agriculture, medicine, and industries is rich in nitrogen and phosphorus, which are the main factors leading to the eutrophication of water bodies [6,7]. The huge discharge volume of effluent from wastewater treatment plants and unreasonable discharge standards are also intensifying the water crisis due to water pollution. Therefore, reclaimed water has been developed as a new water resource. In 2019, China's unconventional water resources reached 9 billion cubic meters, of which over 80% came from reclaimed water [8]. Reclaimed water can simply be derived from the advanced treatment of effluent from wastewater treatment plants, which can not only save water resources but also alleviate pollution of the water environment.

However, there are some difficulties in developing environment-sustainable wastewater reuse technology. Advanced wastewater treatment technology often has complex processes, which makes its management more cumbersome, and maintenance becomes more expensive [9,10]. In addition, the issues of energy consumption and the accompanying greenhouse gas emissions during the treatment process cannot be ignored [11–13]. Some common biological methods for treating wastewater will generate greenhouse gases, which is a major challenge faced by current wastewater treatment technology [14]. Therefore, it is crucial to seek for a simple and economically sustainable method for effluent polishing.

The main purpose of advanced wastewater treatment is to remove nitrogen and phosphorus [15]. As an aggregate formed by microalgae and bacteria, the symbiotic relationship between them provides a guarantee for wastewater treatment [16,17]. Microalgal-bacterial granular sludge (MBGS) has been developed for municipal wastewater treatment with greater environmental and economic sustainability as compared to conventional activated sludge [18,19]. However, it has not been reported for effluent polishing. *Aegagropila linnaei*, along with its bacteria in the phycosphere, is a kind of natural MBGS that exists in natural water bodies, and it is also commonly called "marimo" [20]. Marimo can achieve self-sustaining growth by absorbing external nutrients, thereby achieving the goal of purifying water quality [20]. Additionally, it possesses a special type of zoospore, whose presence helps it maintain an aggregated form while absorbing nutrients [21]. Meanwhile, microalgae usually produce lipids, which can potentially serve as biofuels [22,23]. Considering marimo can grow in lake water with low nutrient concentrations, it can be deduced that it may also adapt well to wastewater effluent. It seems that marimo has the advantages of low energy consumption and environmental sustainability when applied to advanced wastewater treatment. However, until now, this kind of nature-based technology has not been reported. Therefore, the development of this low-cost and natural-based technology is particularly crucial.

Consequently, this paper investigated the granular characteristics, removal performance, and changes in the microbial structure and function of marimo and MBGS for effluent polishing of wastewater treatment plants. This study is expected to provide a theoretical basis for the application of natural and artificial microalgal-bacterial granular sludge in effluent polishing and wastewater reclamation. In addition, this study intends to shed light on nature-based wastewater self-purification technology under the era of carbon neutrality.

2. Materials and Methods

2.1. Wastewater Effluent

The wastewater effluent used in this experiment came from the effluent of the Chongxian Wastewater Treatment Plant, Hangzhou, Zhejiang. The effluent mainly contained 7.3 mg/L total nitrogen (TN) mainly as nitrate nitrogen (NO_3^--N), 0.04 mg/L total phosphorus (TP), and 12.0 mg/L chemical oxygen demand (COD). The pH of the effluent was approximately 7.2.

2.2. Experimental Setup

The marimo and MBGS used in this experiment were derived from the internet and our previous experiment [24], respectively. The granule sizes of marimo and MBGS were about 7.1 and 7.0 mm, respectively. The 5-min sludge volume index (SVI_5) values of marimo and MBGS were about 20.9 and 41.8 mL/g, respectively, and the initial volatile suspended solids (VSS) of marimo and MBGS were about 3.9 and 2.5 g/L, respectively. The hydraulic retention time (HRT) was set as 4 h. The experimental device was a continuous flow system shown in Figure 1, with an effective volume of 340 mL and a water depth of 5 cm for the runway pool. The influent flowed into the lower inlet of the device and discharged from the 5 cm high outlet. The experiment was conducted under a fixed artificial light source for 12 h/12 h of light/dark cycles, with an indoor temperature of 20 °C and a light intensity of 180 μmol/m^2/s. The effluent samples were collected from one light cycle and one dark cycle per day and passed through a 0.45 μm filter for further analysis. At the beginning and end of the experiment, granule samples were collected for physicochemical analysis and metagenomics sequencing.

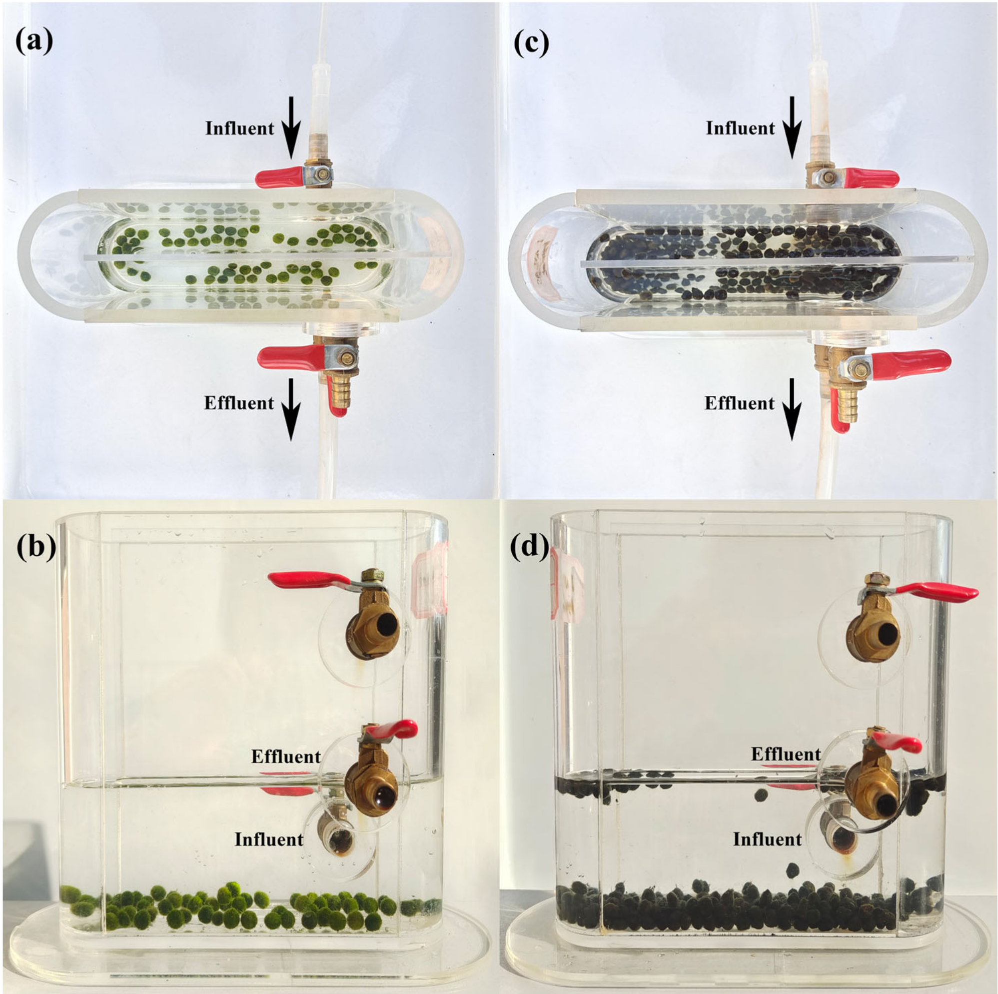

Figure 1. Experiment setup for marimo (**a**,**b**) and MBGS (**c**,**d**) for wastewater effluent polishing.

2.3. Analytical Methods

The NO_3^--N, TN, TP, COD, and SVI_5 were determined by standard methods [25]. The content of chlorophyll (Chl) was determined by acetone extraction [26]. The pH values were measured using the STARTER3100 pH meter (Ohaus, Parsippany, NJ, USA), and the dissolved oxygen (DO) concentration was measured using the YSI5100 DO meter (Yellow Springs, Ohio, USA). The light intensity and temperature were measured using a light quantum meter (TES-1339P, Taibei, Taiwan) and a thermometer (MITIR TP-677, Wenzhou, China), respectively. Microalgae were examined using optical microscopy (RX50, SOPTOP, Zhejiang, China). The total genome DNA was extracted by using a FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) for metagenomic sequencing, and the metabolic pathways were analyzed using the KEGG. The original sequence data can be obtained in NCBI with login no.PRJNA967691.

2.4. Statistical Analysis

Data were statistically analyzed using SPSS software (IBM SPSS Statistics 27), and the results were tested for statistical differences using one-way analysis of variance (ANOVA). The difference analysis results are statistically significant at $p < 0.01$.

3. Results and Discussion

3.1. Granular Characteristics and Changes in Chl Content

The granules of marimo and MBGS were recorded at the beginning and end of the experiment, respectively. As shown in Figure 2, compared to MBGS, the granule color of marimo significantly darkened as the experiment progressed, transitioning from light green to dark green, which was ascribed to the significant increase in its Chl content (Figure 3b). As can be observed in Figure 2c, marimo had a basic structural unit consisting of filamentous cells wrapped in a sheath, resembling a straw bag or teardrop [21]. The main microorganisms were eukaryotic algae of *Chlorophyta*. In MBGS, there were multitudes of *Cyanobacteria* and a small amount of green algae (Figure 2d). As shown in Figure 3a, the granule sizes of marimo and MBGS increased by 0.5 mm and 0.8 mm, respectively. This suggested that marimo had a more compact structure than MBGS during the cultivation process [20]. In addition, the SVI_5 of marimo decreased by 10.3% (Figure 3b), indicating an improvement in its settling performance. In the experiment, it was intuitively observed that MBGS continuously floated up during the experiment, and until the end of the experiment, most granules of MBGS were in a floating state, while the granules of marimo always remained precipitated at the bottom of the reactor. The main factor causing MBGS to float was the generation of dissolved oxygen [27], with the growth of SVI_5, while marimo's excellent settling performance could be ascribed to its compact structure. Its excellent settling performance will be an advantage of the application of marimo in wastewater treatment, especially in continuous flow system [28].

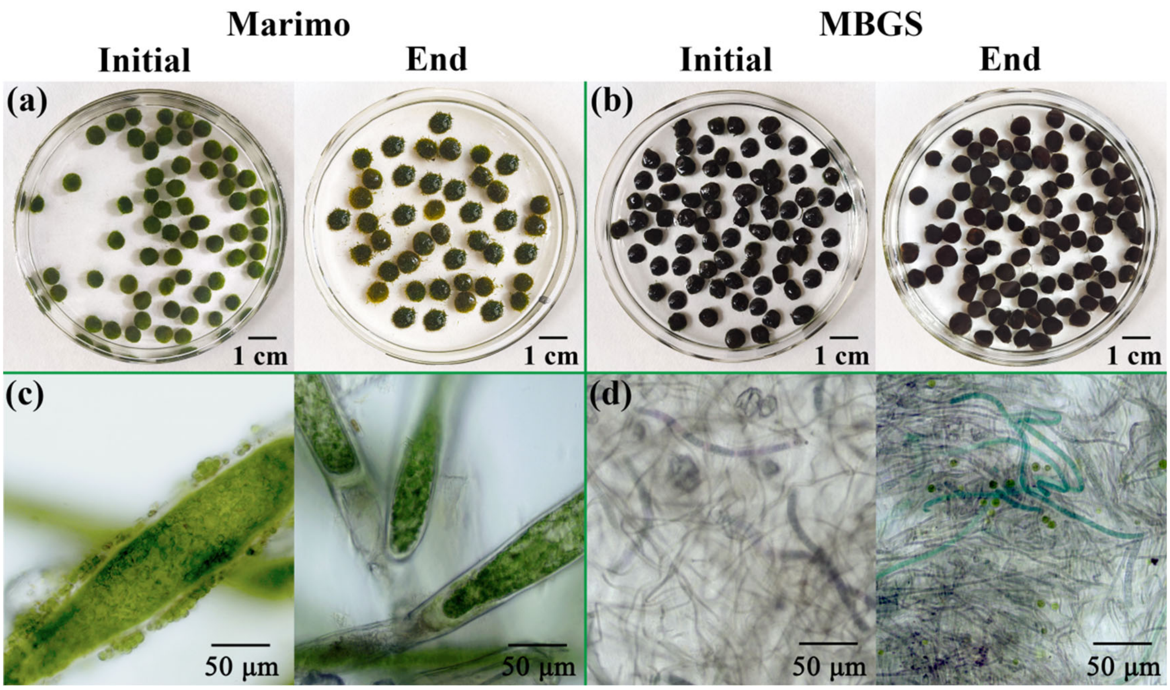

Figure 2. The morphology and micro images of marimo (**a**,**c**) and MBGS (**b**,**d**) at the beginning and end of the experiment.

Usually, the removal performance of microalgae is closely related to the intensity of its photosynthesis. As shown in Figure 3b, due to the presence of a large amount of *Cyanobacteria* in MBGS, which only contained chlorophyll-a (Chl-a) [29], the content of Chl-a in MBGS was much higher than that in marimo during the initial stage of the experiment. On the contrary, the main algae in marimo were eukaryotic algae of *Chlorophyta*, which resulted in a higher chlorophyll-b (Chl-b) content than MBGS in the early stage of the experiment. At the end of the experiment, both of Chl-a and Chl-b contents in marimo and MBGS increased (Figure 3b), suggesting the increase in photosynthesis efficiency.

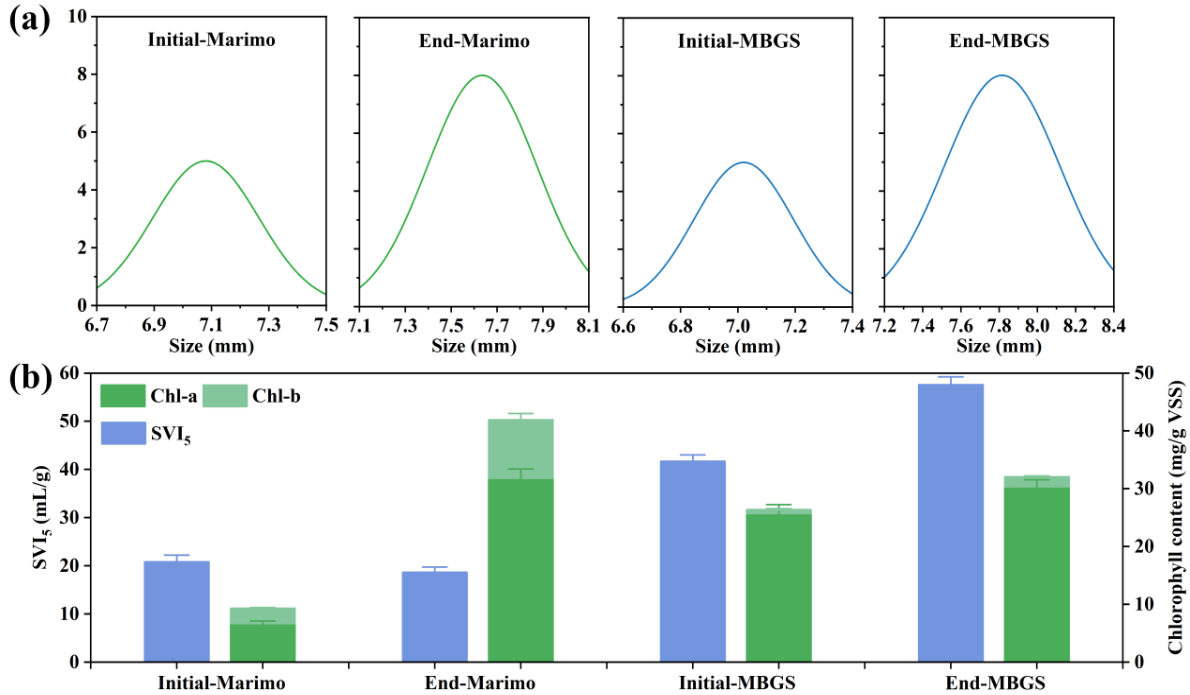

Figure 3. The granule size (**a**), SVI_5 value and Chl content (**b**) of marimo and MBGS at the beginning and end of the experiment.

3.2. Reactor Performance

An intuitive comparison of the removal rates between marimo and MBGS was conducted. As shown in Figure 4a,b, there were significant differences in the removal rates of NO_3^--N and TN between marimo and MBGS during the day (ANOVA, $p < 0.01$). In addition, it could be seen that the concentration changes in NO_3^--N and TN were quite similar. The concentrations of NO_3^--N and TN in the treated wastewater had decreased by an average of less than 2 mg/L (Figure 5a,b). The nitrogen component of the effluent of the wastewater plant was mainly NO_3^--N, and the removal of NO_3^--N usually depends on microbial denitrification as well as assimilation [30,31]. Usually, the optimal C/N ratio for aerobic denitrification is around 7.5; it is not conducive to NO_3^--N removal in an environment with a low C/N ratio [32,33]. In this study, the C/N ratio in the wastewater used in the experiment was around 0.62. In addition, the high DO environment might also hinder the reduction of nitrate nitrogen. Usually, DO values below 0.7 ± 0.1 mg/L is necessary for denitrification [34,35], but aerobic denitrification can also happen at DO concentrations of around 4 mg/L [36]. As shown in Table 1, the average DO concentration in this study was around 5.4 mg/L. Therefore, based on the above analyses and taken the biofilm property of bio-granules into account, it is speculated that both marimo and MBGS could remove nitrite from wastewater mainly through microbial assimilation but denitrification might also happen, which was also verified by the subsequent microbial and metagenomics analyses.

Contrary to the results of NO_3^--N and TN, Figure 4c indicated that there was no significant difference in the removal rates of TP between marimo and MBGS (ANOVA, $p > 0.05$); the average removal rates of TP during the day were 16.1% and 16.5%, which were 18.1% and 16.2% at night, respectively. The influent concentration of TP was as low as around 0.04 mg/L because the raw wastewater in this experiment came from the wastewater treatment plant treated by chemicals. However, after advanced treatment by marimo, the concentration of TP became even lower than before. In addition, it could also be seen that the performance of marimo in advanced wastewater treatment of phosphorus was comparable to MBGS during the day and even slightly better than MBGS at night. Under the condition of light/dark cycles, MBGS was found to remove phosphorus mainly through cell assimilation and polyphosphate accumulation [37]. Considering that the HRT in this study was only 4 h, it can be assumed that by extending the

HRT, the phosphorus removal efficiency could be further improved when treating effluent with a higher phosphorus concentration.

Table 1. Dissolved oxygen concentrations in influent and effluent of marimo and MBGS systems during the day and night.

Time	Dissolved Oxygen Concentration (mg/L)							
	Light				Dark			
	Influent		Effluent		Influent		Effluent	
	Marimo	MBGS	Marimo	MBGS	Marimo	MBGS	Marimo	MBGS
1	6.74	6.74	13.00	15.40	9.51	10.71	6.54	4.46
2	6.49	5.45	16.40	16.75	11.53	11.71	5.80	4.04
3	5.95	5.07	15.47	16.54	10.79	11.33	4.40	3.47
4	5.53	5.06	18.20	20.00	12.20	13.10	4.43	4.52
5	5.22	5.27	18.58	19.07	11.81	12.05	4.99	3.69
6	5.43	4.78	9.50	17.97	7.31	11.54	6.12	4.26
7	5.73	4.80	14.40	14.57	9.81	9.90	3.17	5.12
8	4.31	5.28	16.07	17.50	10.84	11.55	4.56	5.06
9	5.32	5.57	15.33	13.82	10.43	9.68	4.72	3.75
10	5.22	4.74	14.37	10.11	9.50	7.37	3.05	1.46
11	4.32	3.52	14.57	16.64	9.98	11.02	3.25	3.27
12	4.35	4.36	15.12	20.59	10.45	13.19	5.20	5.74
13	5.32	5.59	15.01	17.12	10.15	11.20	4.59	6.24
14	4.97	5.79	14.23	17.34	10.46	12.02	5.05	6.12
15	5.24	5.78	14.78	16.73	10.20	11.18	4.22	8.73
16	4.95	7.21	16.70	16.84	11.20	11.27	6.73	9.78
17	6.22	7.74	15.30	16.69	10.48	11.18	4.91	9.10
18	5.28	7.38	19.85	16.46	12.60	10.90	4.85	7.54
19	5.01	6.36	18.79	14.55	11.94	9.82	5.15	9.14
20	5.39	7.38	18.74	13.04	11.89	9.04	6.97	7.26
21	6.30	6.45	17.96	16.36	11.90	11.10	4.58	9.59

Moreover, the average concentrations of NO_3^--N in the effluent of marimo and MBGS were 6.19 and 5.54 mg/L, respectively (Figure 5a), and the average concentrations of TN in effluent were 6.29 and 5.66 mg/L, respectively (Figure 5b). Excessive nitrogen emissions often bring some negative environmental impacts, such as nitrate and ammonia emissions causing pollution to water bodies, while nitrogen oxides can cause air pollution [38]. The NO_3^--N and TN concentrations in this experiment were reduced to some extent. As shown in Figure 5c, the average concentrations of TP in the effluent of marimo and MBGS were both around 0.02 mg/L. Phosphorus is considered to be a limiting element for preventing eutrophication [6]. In this study, the extremely low concentration of phosphorus allows the treated wastewater to be returned to natural water to maintain its healthy state. Due to the influent COD concentration being less than 15 mg/L in this experiment, only the COD data from the first ten days were measured, indicating that COD were also reduced by 25% to 40% by marimo and MBGS during the light/dark cycles, respectively.

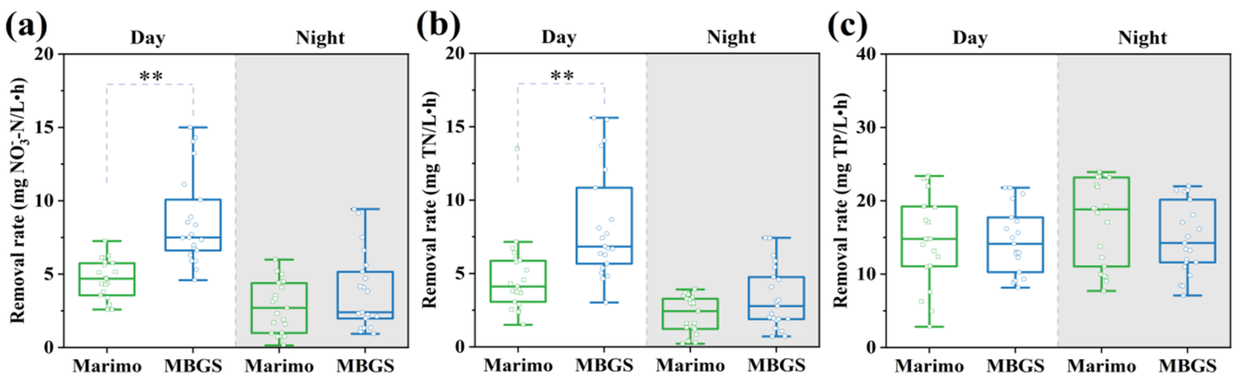

Figure 4. Differences in removal rates of NO_3^--N (**a**), TN (**b**), and TP (**c**) between marimo and MBGS during the day and night. ** symbols for $p < 0.01$.

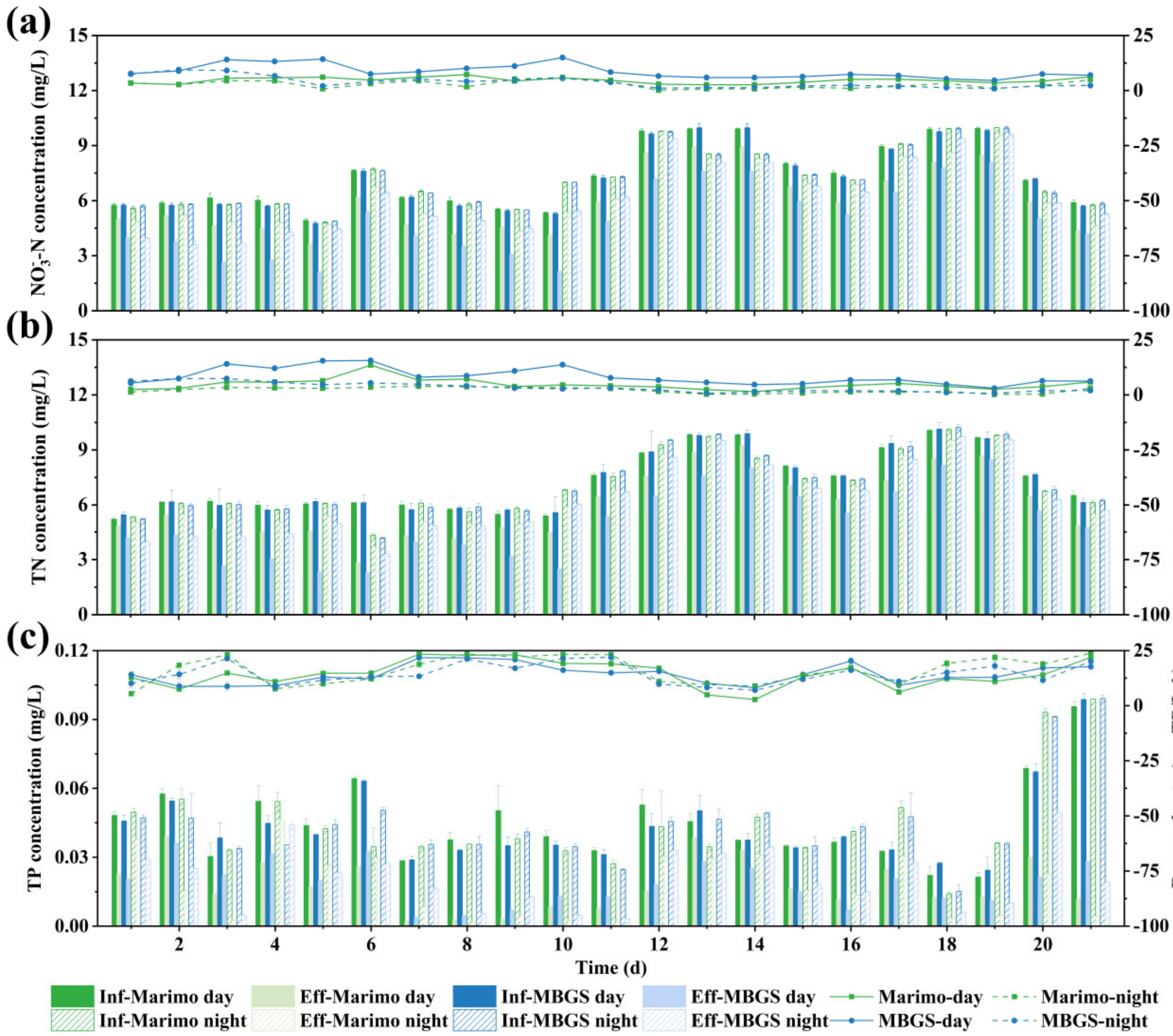

Figure 5. NO_3^--N (**a**), TN (**b**), and TP (**c**) influent and effluent concentrations (left axis) and removal rates (right axis) by marimo and MBGS.

3.3. Microbial Community and Gene Functional Analysis

As shown in Figure 6a, at the phylum level, marimo mainly consisted of *Proteobacteria* at the initial stage of the experiment, followed by *Chlorophyta*. At the end of the experiment, the relative abundance of *Proteobacteria* became higher, with *Proteobacteria* and *Chlorophyta* accounting for more than 75% of the total. The increase in *Proteobacteria* might be due to that the low nutrient conditions were more suitable for their growth [39]. A small amount of *Bacteroidota* was also detected. *Proteobacteria* and *Bacteroidota* can play the role of denitrification in wastewater treatment [40]. In addition, the presence of *Proteobacteria* can play an important role in removing anti-inflammatory and antimicrobial substances [41]. Therefore, it was speculated that *Proteobacteria* acted as one of the main contributors in the wastewater treatment process [42]. The dominant phylum transformed from *Proteobacteria* into *Cyanobacteria* for MBGS, which was consistent with the previous study [43]. At a finer scale, *α-proteobacteria* and *γ-proteobacteria* occupied the main position in MBGS, and by the end of the experiment, the dominant class had passed from *γ-proteobacteria* to *α-proteobacteria*. Interestingly, it could be observed that, at the end of the experiment, marimo and MBGS were quite similar in microbial composition, both containing higher relative abundances of *Burkholdericeae*, *Acetobacteraceae*, *Rhodobacteraceae*, and *Xanthomonadaceae*. Compared with the initial data, there was a significant increase in the relative abundance of *Rhodobacteriaceae* in marimo, which was a kind of denitrification bacteria with the *nirS* gene [44]. In contrast, MBGS had little change in *Rhodobacteriaceae*, which might suggest that the denitrification potential of marimo slightly increased in the later stage of the experiment.

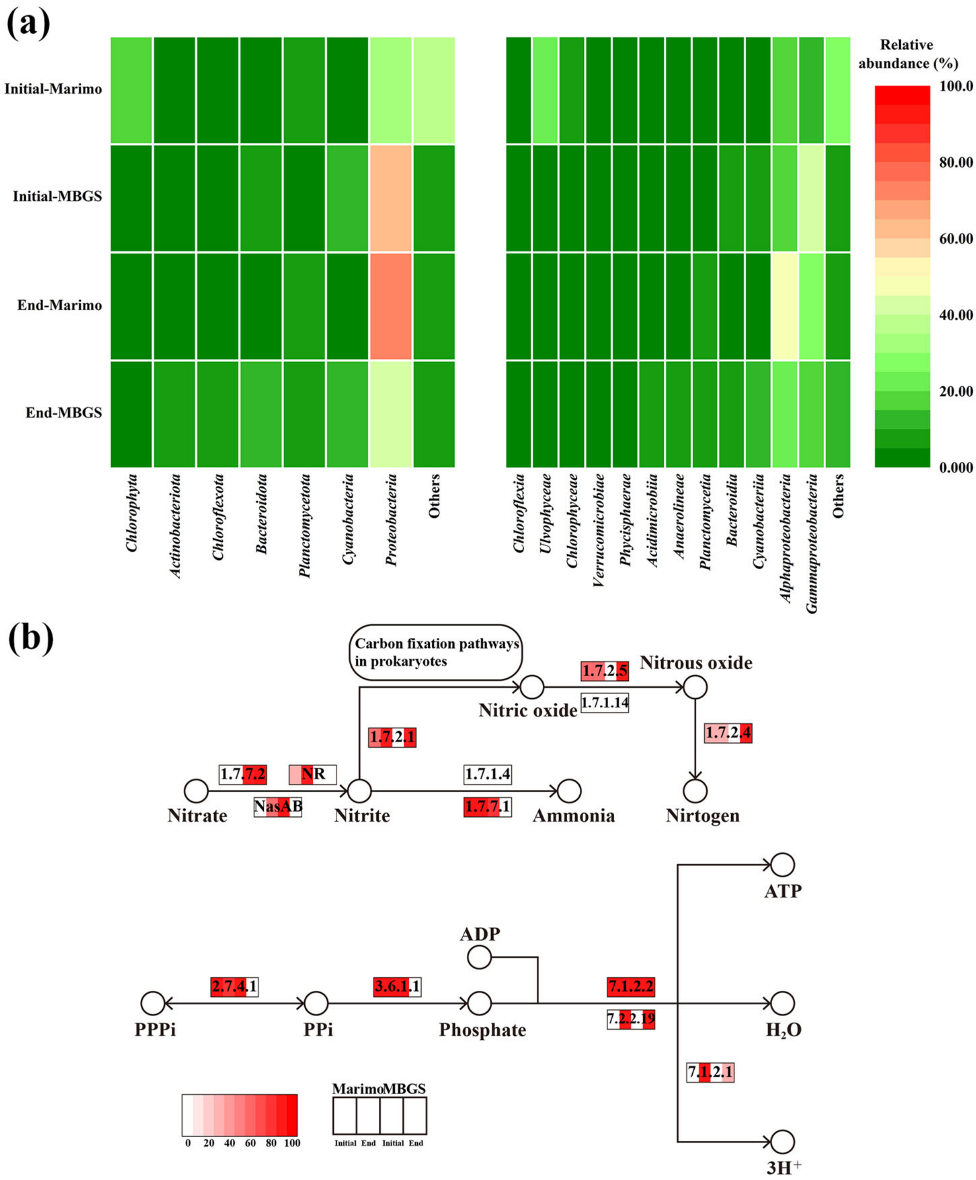

Figure 6. Changes in microbial communities (**a**) and key metabolic pathways of nitrate and phosphate (**b**) of marimo and MBGS.

According to Figure 6b, it was speculated that marimo might also have a mechanism of nitrogen removal through denitrification in addition to assimilation. It could be observed that the abundance of *Pithopora* in marimo was significantly reduced, while *Roseomonas* and *Hydrogenophaga* showed a downward trend. In addition, marimo and MBGS both detected the presence of *Thauera*, which was also a famous denitrification bacterium, and it might be the main contributor to nitrogen removal via N_2 in MBGS [45]. Recently, it was also found that some *Thauera* belong to denitrifying phosphorus-accumulating organisms [46]. Polyphosphate accumulation is usually considered to be the main avenue of biological phosphorus removal [37]. In this experiment, it is speculated that phosphorus-accumulating organisms in marimo might adopt available oxygen as the electron acceptor to decompose polyhydroxyalkanoates and used the energy generated by this reaction to absorb phosphate.

Changes in key metabolic pathways of nitrate and phosphate were further analyzed for marimo and MBGS based on the metagenomics. As shown in Figure 6b, the genes of nitrite reductase (EC 1.7.7.1) contained in marimo had higher relative abundance, while MBGS had higher relative abundance of nitrate reductase (EC 1.7.7.2). In the denitrification pathway, the relative abundances of nitric oxide reductase (EC 1.7.2.5) and nitrous oxide reductase (EC 1.7.2.4) in marimo remained stable, while at the end of the experiment, the relative abundances of both enzymes in MBGS increased. Based on the above results, it can be reasonably speculated that, at the end of the experiment, both of marimo and MBGS could remove nitrogen mainly through assimilation, but the denitrification might also happen due to the present of complete enzymes responsible for denitrification (Figure 6b). In the key metabolic pathway of phosphate, according to Figure 6b, the relative abundances of polyphosphotransferase (2.7.4.1) and diphosphate hydrolase (3.6.1.1) in marimo were higher than those in MBGS, which might explained for why the phosphorus removal performance of marimo was slightly better than MBGS in the later stage of the experiment.

4. Conclusions

The advanced wastewater treatment performances of marimo on NO_3^--N, TN, and TP were first evaluated and compared with MBGS in this study. The results showed that the pollutant removal performance of marimo was close to that of MBGS. Especially in the removal of phosphate, marimo had an even more excellent performance than MBGS. Overall, marimo experienced a slight increase in granule size and a significant increase in Chl content. During the day, marimo had the capacity to remove NO_3^--N, TN, and TP during real wastewater effluent polishing at an HRT of 4 h. Due to the HRT being 4 h in this study, it can be assumed that extending HRT might further promote the pollutant removal. This paper speculated that marimo could remove nitrogen through microbial assimilation, and denitrification might also happen during wastewater effluent polishing. The high abundances of polyphosphotransferase and diphosphate hydrolase in marimo might be the possible reason for its excellent phosphorus removal efficiency. In addition, marimo can simply be acquired from nature and had a certain oxygen production capacity when it was applied for effluent polishing, making it economically and environmentally sustainable for advanced wastewater treatment and reclamation.

Author Contributions: Y.W.: Investigation, Writing—original draft, Data curation, Software. P.S.: Investigation, Writing—original draft, Data curation, Formal analysis. M.L.: Resources. Q.H.: Writing—review and editing. B.J.: Conceptualization, Writing—review and editing, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

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