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Investigation of a Farm-scale Multitrophic Recirculating Aquaculture System with the Addition of *Rhodovulum sulfidophilum* for Milkfish (*Chanos chanos*) Coastal Aquaculture

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Abstract: Globally, coastal aquaculture is growing due to the large demand for marine products. Specific impacts caused by coastal aquaculture on the environment include the discharge of culture farm effluents, stress on ground water (the absence of recycling), nutrient pollution, and diseases of cultured animals. Three methods, integrated multitrophic aquaculture (IMTA), recirculating aquaculture system (RAS), and beneficial bacteria for aquaculture, have been developed to solve these problems. In this study, the advantages of IMTA and RAS were integrated to develop a novel multitrophic recirculating aquaculture system (MRAS) to adapt to the farm-scale culturing of milkfish (*Chanos chanos*). The photosynthetic bacteria *Rhodovulum sulfidophilum* was added to enhance the performance of the farm-scale milkfish MRAS. This setting could promote growth of beneficial bacteria, such as the nitrogen cycle-associated microbial community and the anoxygenic phototrophic Acidobacteria community. The ammonia level was reduced, and the total phosphorous level was stable in the water recycled in the MRAS. The cyanobacteria, algae, *Vibrio*, *Escherichia*, and other potential pathogenic bacteria communities were inhibited in the MRAS. This study provides an effective design of a water recycling aquaculture system. Milkfish, Asian tiger shrimp (*Penaeus monodon*), Asian hard clam (*Meretrix lusoria*), and seaweed (*Gracilaria* sp.) can be cultured and simultaneously produced in the system.

Keywords: multitrophic recirculating aquaculture system; coastal aquaculture; beneficial bacteria; nitrogen cycle

1. Introduction

Globally, coastal aquaculture is growing due to the increasing demand for marine products for human consumption. Specific impacts of coastal aquaculture that are very common and that have severe consequences on the environment include the discharge of culture farm effluents, stress on ground water (the absence of recycling), nutrient pollution, and diseases of cultured animals [1].

Three methods, integrated multitrophic aquaculture (IMTA), recirculating aquaculture system (RAS), and beneficial bacteria for aquaculture, have been generally used to solve these problems. The design of IMTA provides byproducts (including waste) from one cultured species as inputs

(e.g., fertilizers and foods) for another [2,3]. The advantage of IMTA is the ability to create balanced systems to make fish farming more sustainable using a combinatorial aquaculture (e.g., fish and shrimp) with inorganic extractive (e.g., seaweed) and organic extractive (e.g., shellfish) components. RAS was designed for the use with fish production where water exchange is limited. The major goal of RAS is the reduction of ammonia toxicity and nutrient pollution using filtration and/or biofiltration to maintain water quality to provide a suitable habitat for fish [4,5]. The main benefit of RAS is the ability to reduce the demand of fresh, clean water, and simultaneously maintain a healthy environment for fish.

There are three types of beneficial bacteria used in aquaculture. The first are bacteria that can maintain water quality, such as nitrifying bacteria [6,7]. The second are bacteria for bioaugmentation that are involved in the removal of organic pollutants from water [8–10]. The third are gut probiotics for cultured animals [11,12]. The photosynthetic bacteria *Rhodovulum sulfidophilum* have previously been used as a beneficial bacterium for aquaculture to improve the larval survival of marble goby *Oxyeleotris marmorata* [13,14].

In this study, the advantages of IMTA and RAS were integrated to develop a novel multitrophic recirculating aquaculture system (MRAS) for the farm-scale culture of milkfish (*Chanos chanos*). The objective of this study was to investigate the effects of MRAS on water quality maintenance for farm-scale milkfish culture. The photosynthetic bacteria *Rhodovulum sulfidophilum* were used as a beneficial bacterium to test its effect on the farm-scale milkfish MRAS. The chemical compositions and the structure of the microbial communities in the farm-scale milkfish MRAS were investigated and revealed.

2. Materials and Methods

2.1. Sediment Sampling and Sampling Site

Sediment and water were sampled from a farm-scale milkfish MRAS at the Mariculture Research Center, Fisheries Research Institute, Tainan, Taiwan, in August 2017. The latitude and longitude of the sampling site are 23°07'20.3" N and 120°04'47.8" E. Three sediment and water samples were collected from each culture pond at three different local positions of the culture pond.

2.2. Experimental Design

The design of a farm-scale MRAS for the culture of milkfish is shown in Figure 1A. Each unit was composed of one fish pond, one hard clam pond, and one seaweed pond. Milkfish (*Chanos chanos*) and Asian tiger shrimp (*Penaeus monodon*) were cocultured in the fish pond. The effluent of the fish pond flowed into the hard clam pond in which the Asian hard clam (*Meretrix lusoria*) was cultured. The effluent of the hard clam pond flowed into the seaweed pond in which the seaweed (*Gracilaria* sp.) was cultured. Finally, the effluent of the seaweed pond flowed into the fish pond. The cultivated species (milkfish, shrimp, hard clam, and seaweed) were continuously added, grown, and harvested in the MRAS. Therefore, milkfish, shrimp, and hard clam of different ages were present together simultaneously in the MRAS. The milkfish, hard clam, and seaweed ponds were 2500, 800, and 1700 m² in area and 1.8, 1.5, and 0.5 m in depth, respectively. For each unit, water was circulated between the three ponds (in the sequence of fish, clam, and seaweed ponds) daily from 12:00 to 18:00 at a rate of about 84 m³ per hour. Two units were used for this study. In Unit a, 100 L of photosynthetic bacteria *Rhodovulum sulfidophilum* culture (10⁵ CFU/ml) was added to the fish pond every two weeks. Unit b was used as a control unit without the addition of *Rhodovulum sulfidophilum*. The sampling timelines are shown in Figure 1B. The first sampling was performed on 14 April 2016. In total, five times of sampling for milkfish and hard clam ponds and three times of sampling for seaweed ponds were performed during a period of five months. Samples of water and sediment were collected for analysis of water chemical compositions and microbial communities.

Multi-trophic Recirculating Aquaculture System (MRAS) Unit a : addition of *Rhodovulum sulfidophilum*

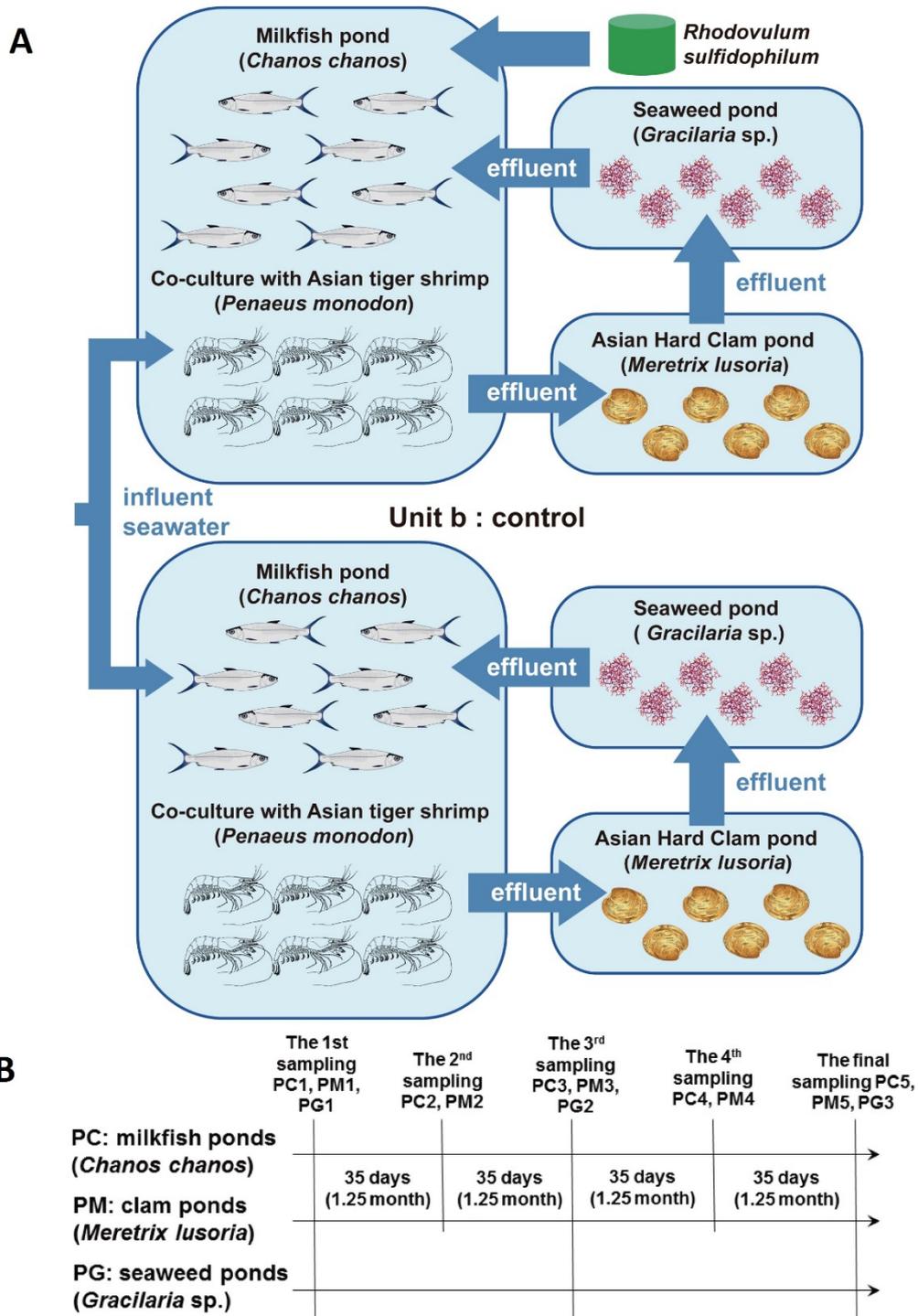


Figure 1. Experimental design and sampling timelines. (A) The design of the milkfish multitrophic recirculating aquaculture system. Unit a: The addition of *Rhodovulum sulfidophilum*; Unit b: The control without the addition of *Rhodovulum sulfidophilum*. (B) Sampling timelines. PC1-5: Five times of sampling of milkfish pond, PM1-5: Five times of sampling of hard clam pond, and PG1-3: Three times of sampling of seaweed pond. PC: Milkfish pond, PM: Hard clam pond, and PG: Seaweed pond.

2.3. Analysis of the Chemical Compositions of Water from MRAS

Water samples from MRAS were initially passed through a 1.20- μm glass–fiber membrane and then refiltered through a 0.45- μm nylon membrane. Chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP), and NH_4^+ were measured using test kits and the Merck spectroquant Nova 60 instrument.

2.4. Next Generation Sequencing and Data Analysis

Total DNA from water and sediment samples of MRAS were extracted using the PowerSoil DNA Isolation Kit (QIAGEN, Hilden, Germany). The 16S rRNA gene sequences of the V5–V8 variable regions were amplified using the 5' primer containing an Illumina adaptor (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3') and a 16S rRNA gene-specific sequence (5'-CCTACGGGNBGCASCAG-3'). The sequence of the 3' primer contained an Illumina adaptor (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3') and a 16S rRNA gene-specific sequence (5'-GACTACNVGGGTATCTAATCC-3'). The PCRs were performed as previously described [10]. Next-generation sequencing (NGS) was performed at the Genome Center of the National Yang-Ming University, Taiwan using the MiSeq platform (Illumina, Inc., San Diego, California, USA), and the NGS data analysis was performed as described previously [15]. Briefly, sequences that passed the chimera check were applied to the classifier software of the Ribosomal Database Project (<https://rdp.cme.msu.edu/>) for phylogenetic assignment [16]. Specific microbial communities (such as the nitrogen cycle) were identified by integration of NGS data and the bacterial/archaeal list of the Kyoto Encyclopedia of Genes and Genomes (KEGG) modules [17]. The proportion of each phylotype (operational taxonomic unit, OUT/genus) in each sample is defined as the formula: Proportion of phylotype for each sample = number of sequences of the phylotype/total number of sequences of the sample. Microbial composition differences between two sets of samples (with and without the addition of *Rhodovulum sulfidophilum*) were identified using the Mann–Whitney U test by the wilcox.test function of R [18]. A *p*-value less than 0.05 was considered to be statistically significant. Data of microbial communities are provided as in Table S1.

3. Results

3.1. Chemical Compositions in the Water of the MRAS

The nitrogen, phosphorus, and chemical oxygen demand were examined to assess the water quality of the farm-scale milkfish MRAS. As shown in Figure 2, the concentrations of ammonia, total nitrogen, and chemical oxygen demand (COD) in Unit b were reduced in the water from the fish pond to the hard clam pond. These results indicate that the design of the MRAS is effective for water recycling and sustainability for farm-scale coastal aquaculture of milkfish.

As shown in Figure 2A, the ammonia concentration in the water of Unit a (with the addition of *Rhodovulum sulfidophilum*) was less than 1/2 of the ammonia concentration in the water of Unit b. The total phosphorus concentration of Unit a was more stable than that of Unit b (Figure 2C). The total nitrogen concentration and the chemical oxygen demand of Units a and b exhibited no difference (Figure 2B,D). These results indicate that the addition of *Rhodovulum sulfidophilum* enhanced the performance of the MRAS. The major contributions of *Rhodovulum sulfidophilum* addition were ammonia reduction and total phosphorus stabilization.

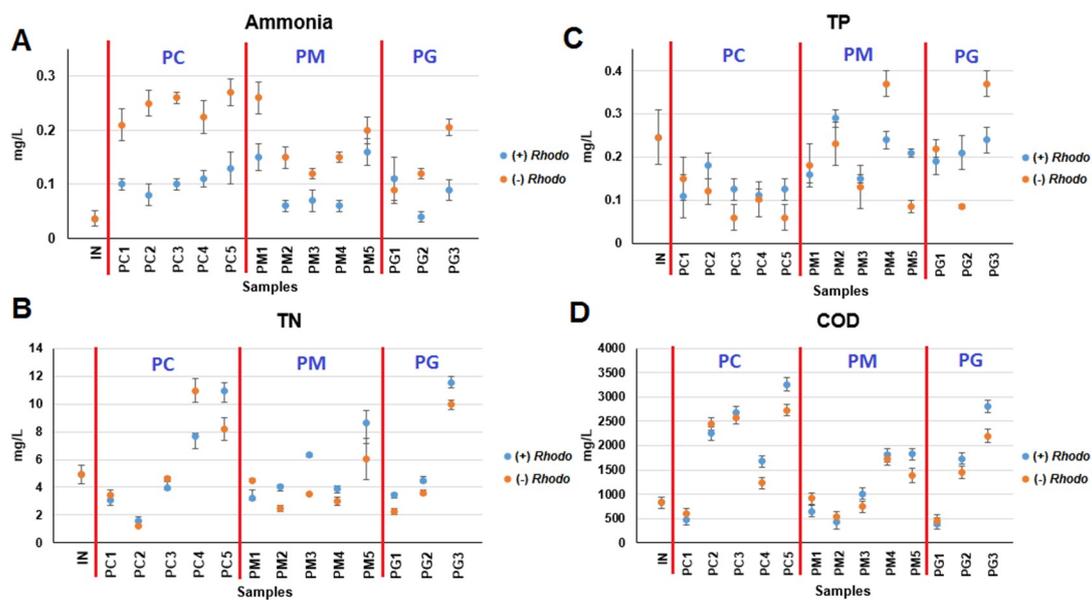


Figure 2. Chemical compositions in the milkfish multitrophic recirculating the aquaculture system. (A) ammonia; (B) total nitrogen, TN; (C) total phosphorus, TP; and (D) chemical oxygen demand, COD. PC: Milkfish pond, PM: Hard clam pond, and PG: Seaweed pond. (+) *Rhodo*: The MRAS with the addition of *Rhodovulum sulfidophilum*; (-) *Rhodo*: The MRAS without the addition of *Rhodovulum sulfidophilum*. Data from three measurements are presented as mean \pm SE.

To further understand why *Rhodovulum sulfidophilum* addition greatly reduced ammonia in the system, the metagenomic approach was used to analyze the changes of the nitrogen cycle associated microbial community in the MRAS. The nitrogen cycle is composed of reactions such as nitrogen fixation, nitrification, denitrification, and nitrate reduction. Overall, and as shown in Figure 3B, the nitrification community containing ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) exhibited a higher proportion in the sediments of the fish ponds and hard clam ponds in Unit a (with *Rhodovulum sulfidophilum* addition). The proportion of the denitrification community is also slightly higher in the sediments of the fish and hard clam ponds of Unit a (Figure 3D). The proportion of nitrogen fixation, dissimilatory nitrate reduction, and assimilatory nitrate reduction communities was higher in the water of the hard clam pond (Figure 4A) and the sediment of the fish and hard clam ponds of Unit a (Figures 4 and 5). Based on Figures 3–5, the proportion of the nitrogen cycle associated microbial community was higher in Unit a. These results indicated that the nitrogen cycle was more active in Unit a, which may have resulted in a lower ammonia concentration in Unit a (Figures 2A and 5C).

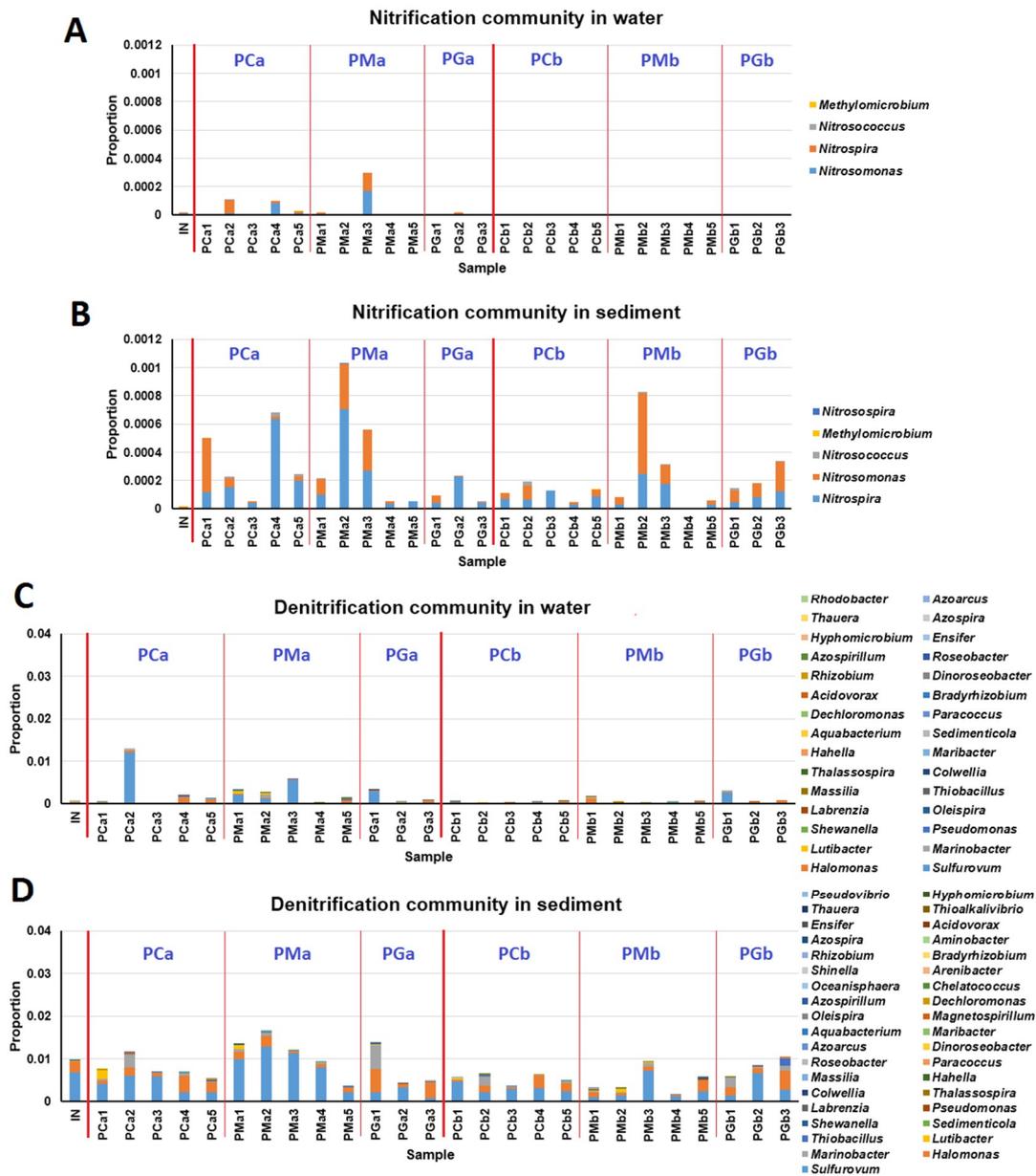


Figure 3. Differences of the nitrifying bacteria community in the milkfish multitrophic recirculating aquaculture system. The proportion of ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) in the water (A) and sediment (B) of the milkfish multitrophic recirculating aquaculture system. The proportion of denitrification bacteria in the water (C) and sediment (D) of the milkfish multitrophic recirculating aquaculture system. The *p*-values of comparison between overall proportions of the microbial community of Units a and b by the Mann–Whitney U test are 0.014, 0.544, 0.034, and 0.336 for (A–D), respectively. IN: Gateway of seawater influent, PC: Milkfish ponds, PM: Hard clam ponds, PG: Seaweed ponds, a: Unit a and b: Unit b. The Y-axis represents the proportion (%) of 16S sequences of each bacterial/archaea genus in the next-generation sequencing data of each sample.

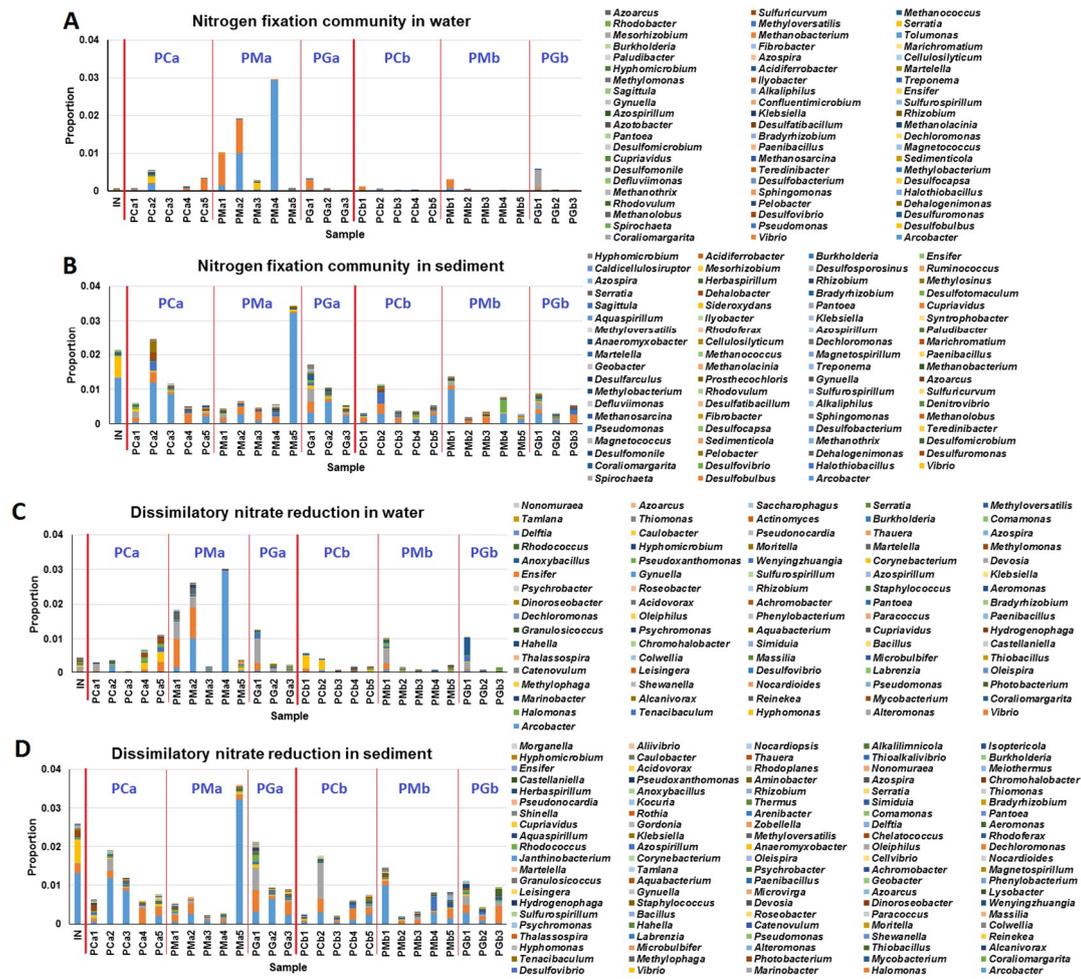


Figure 4. The nitrogen fixation community in the water (A) and sediment (B) of the milkfish multitrophic recirculating aquaculture system. Dissimilatory nitrate reduction community in the water (C) and sediment (D) of the milkfish multitrophic recirculating aquaculture system. The *p*-values of comparison between overall proportions of the microbial community of Units a and b by the Mann–Whitney U test are 0.014, 0.044, 0.026, and 0.389 for (A–D), respectively. IN: Gateway of seawater influent, PC: Milkfish ponds, PM: Hard clam ponds, PG: Seaweed ponds, a: Unit a and b: Unit b. The Y-axis represents the proportion (%) of 16S sequences of each bacterial/archaea genus in the next-generation sequencing data of each sample.

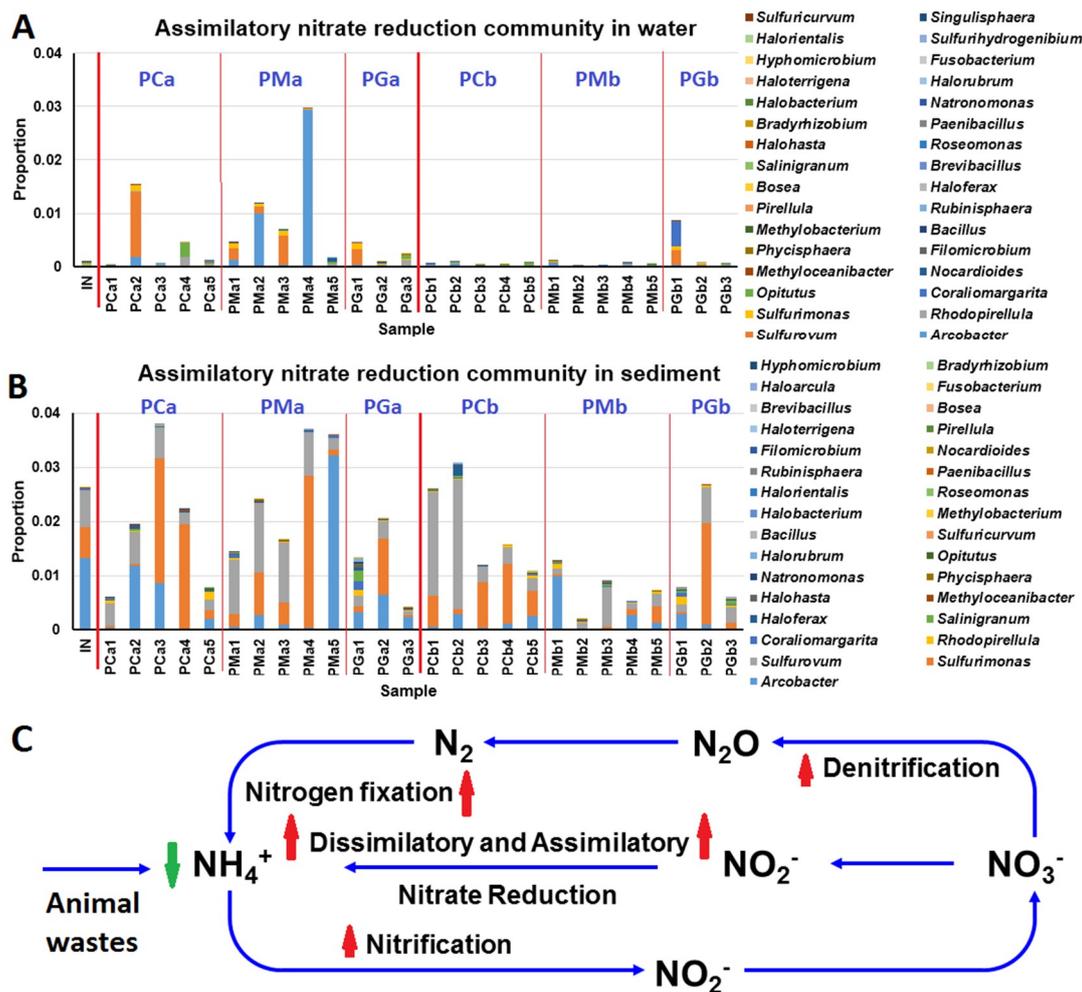


Figure 5. The assimilatory nitrate reduction community in the water (A) and sediment (B) of the milkfish multitrophic recirculating aquaculture system. (C) The nitrogen cycle pathways. Red arrows indicate that the pathway-associated microbial communities increased in the milkfish multitrophic recirculating aquaculture system with the addition of *Rhodovulum sulfidophilum* (Unit b). The green arrow indicates that the ammonia concentration decreased in Unit b. The *p*-values of comparison between overall proportions of the microbial community of Units a and b by the Mann–Whitney U test are 0.003 and 0.113 for (A,B). IN: Gateway of seawater influent, PC: Fish ponds, PM: Hard clam ponds, PG: Seaweed ponds, a: Unit a and b: Unit b. The Y-axis represents the proportion (%) of 16S sequences of each bacterial/archaea genus in the next-generation sequencing data of each sample.

3.2. Analysis of Photosynthetic Bacteria, Cyanobacteria, and Algae Communities

It was previously known that the concentration of ammonia can affect the growth of photosynthetic bacteria, cyanobacteria, and algae in water [19,20]; therefore, the community compositions of these microbes in the MRAS water and sediment were analyzed. As shown in Figure 6A, the proportion of cyanobacteria in the water of Unit b (without *Rhodovulum sulfidophilum* addition) was significantly higher than that of Unit a. Moreover, a higher proportion of algae appears in the hard clam pond water of Unit b (Figure 6C). By contrast, the proportions of anoxygenic photosynthetic bacteria (the genera of purple sulfur bacteria, purple nonsulfur bacteria, green sulfur bacteria, and phototrophic Heliobacteria) were slightly higher in the water of Unit a (Figure 7B). A higher proportion of Acidobacteria (filamentous anoxygenic phototrophs) was found in the hard clam pond sediment of Unit a (Figure 7D). These results suggest that the addition of *Rhodovulum*

sulfidophilum led to an increase in anoxygenic photosynthetic bacteria communities and that the cyanobacteria and algae communities were inhibited.

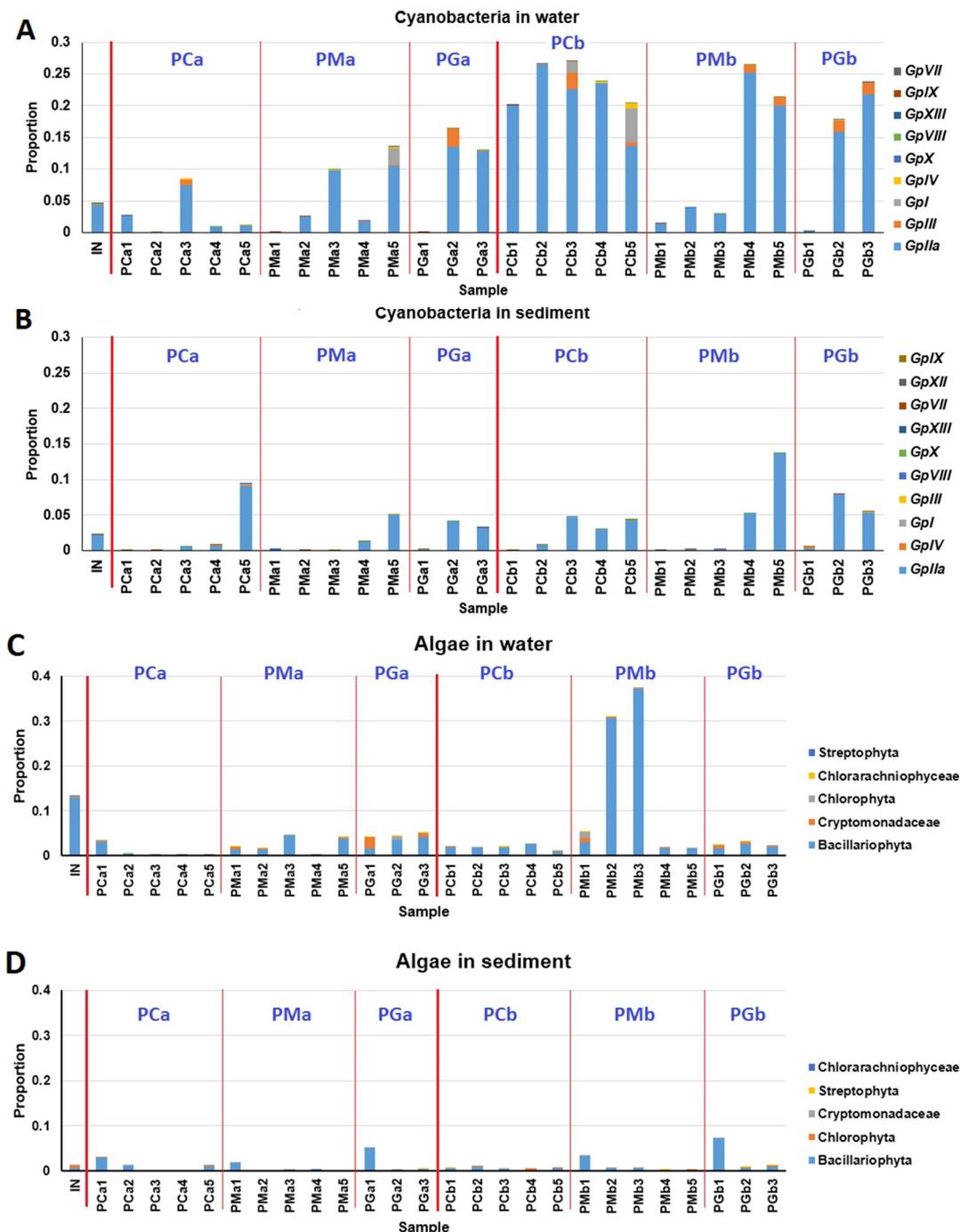


Figure 6. Differences of the cyanobacteria and algae community in the milkfish multitrophic recirculating aquaculture system. Proportion of cyanobacteria (genera) in the water (A) and sediment (B). Proportion of algae (classes) in the water (C) and sediment (D). The p -values of comparison between overall proportions of the microbial community of Units a and b by the Mann–Whitney U test are 0.003, 0.223, 0.336, and 0.153 for (A–D), respectively. IN: Gateway of seawater influent, PC: Fish ponds, PM: Hard clam ponds, PG: Seaweed ponds, a: Unit a and b: Unit b. The Y-axis represents the proportion (%) of 16S sequences of each bacterial/archaea genus in the next-generation sequencing data of each sample.

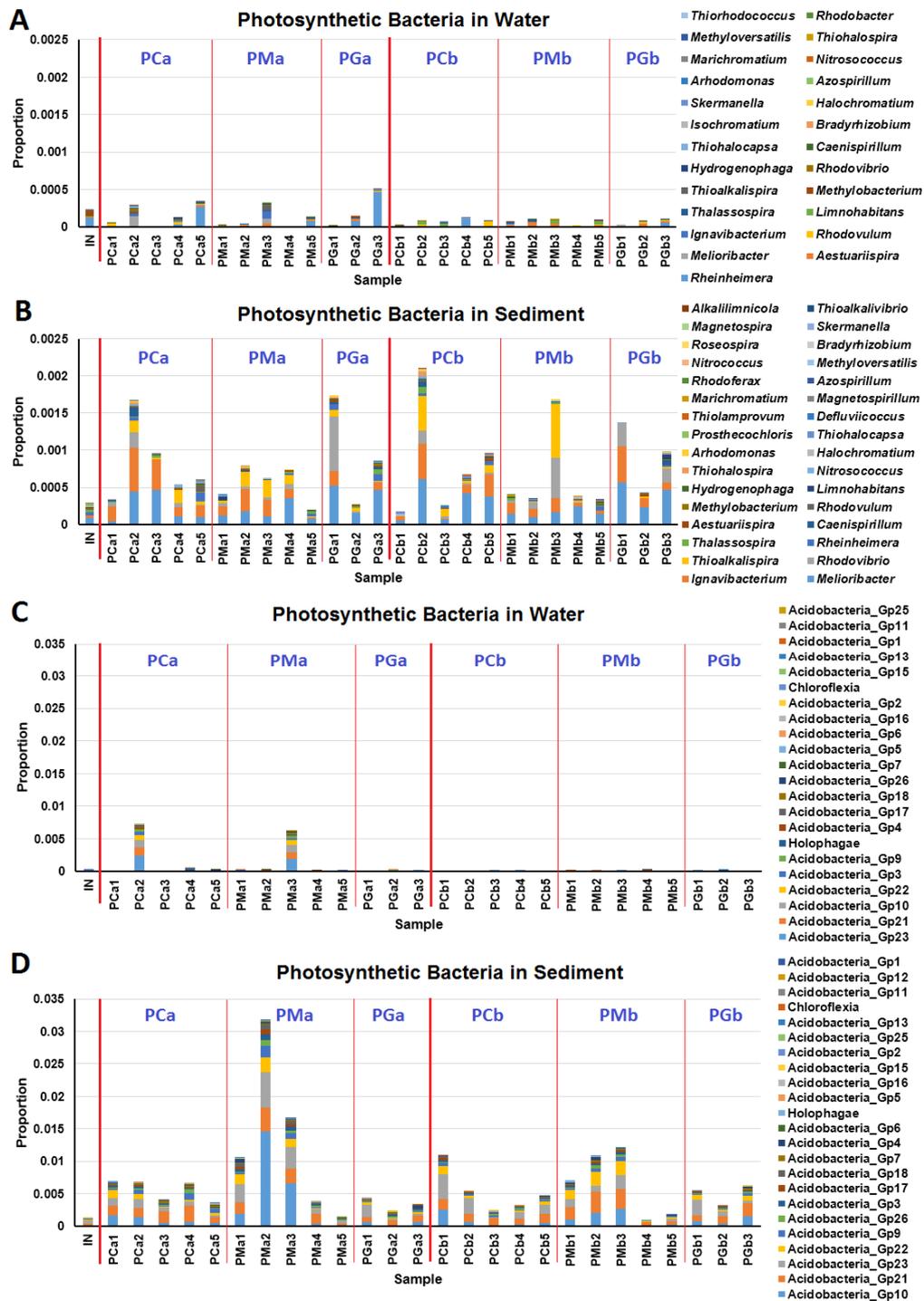


Figure 7. Differences of the community of photosynthetic bacteria in the milkfish multitrophic recirculating aquaculture system. The proportion of anoxygenic photosynthetic bacteria (the genera of purple sulfur bacteria, purple nonsulfur bacteria, green sulfur bacteria, and phototrophic Heliobacteria) in the water (A) and sediment (B). The proportion of photosynthetic bacteria (classes of filamentous anoxygenic phototrophs and phototrophic Acidobacteria) in the water (C) and sediment (D). The *p*-values of comparison between overall proportions of the microbial community of Units a and b by the Mann–Whitney U test are 0.724, 0.879, 0.034, and 0.762 for (A–D), respectively. IN: Gateway of seawater influent, PC: Fish ponds, PM: Hard clam ponds, PG: Seaweed ponds, a: Unit a and b: Unit b. The Y-axis represents the proportion (%) of 16S sequences of each bacterial/archaea genus in the next-generation sequencing data of each sample.

3.3. Analysis of Potential Pathogenic Bacterial Communities

Five bacterial genera (*Escherichia*, *Shigella*, *Vibrio*, *Clostridium*, and *Enterococcus*) with potential pathogens [21,22] and water quality [23] indicators were examined. As shown in Figure 8, the proportion of *Escherichia*, *Shigella*, and *Vibrio* was higher in the water of Unit a. However, the proportion of *Escherichia*, *Shigella*, and *Vibrio* was decreased in the sea weed pond. By contrast, the proportion of *Escherichia/Shigella* was higher in the sediment of Unit b. The overall proportions of these five bacterial genera were less than 1.4% and 1.8% in the water and sediment, respectively. The bacterial genera with potential pathogens in the systems were also examined (Figure 9). The proportions of potential pathogenic bacteria were higher in the water of Unit a and decreased in the sea weed pond. The overall proportions of potential pathogenic bacteria were less than 1% and 0.3% in the water and sediment, respectively. These results indicate that a combination of MRAS with the addition of *Rhodovulum sulfidophilum* could effectively inhibit the growth of potential pathogenic bacteria, and the quality of recycling water was maintained very well.

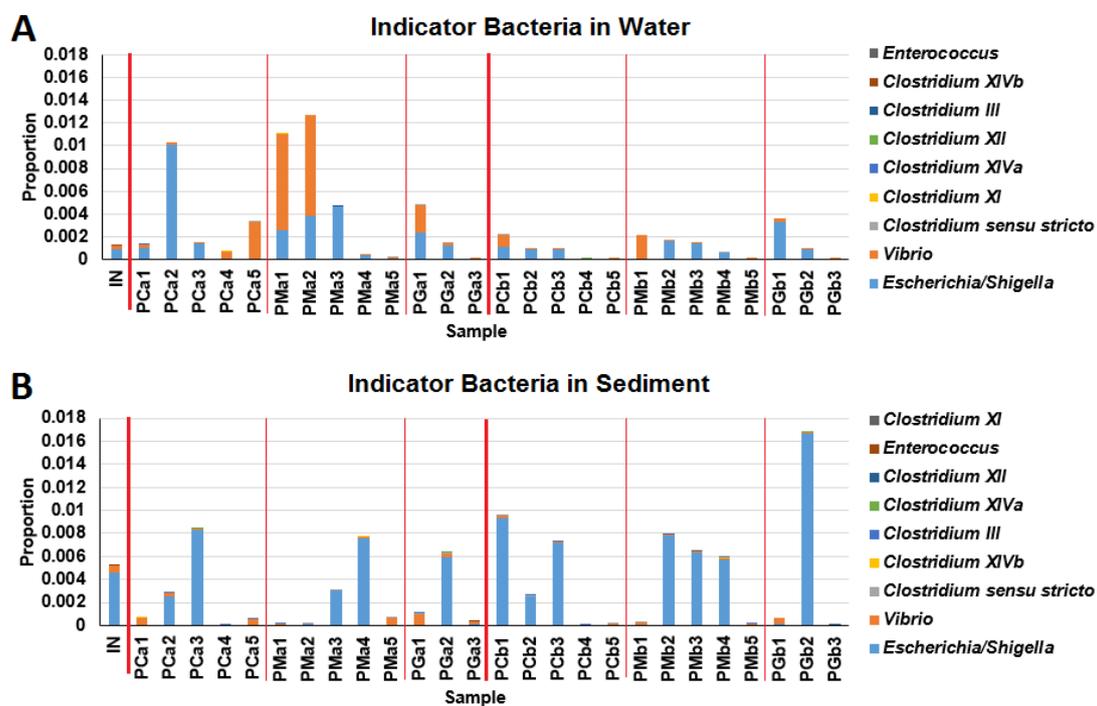


Figure 8. Differences of the proportion of *Escherichia/Shigella* and *Enterococcus* in the water (A) and sediment (B) of the milkfish multitrophic recirculating aquaculture system. The *p*-values of comparison between overall proportions of the microbial community of Units a and b by the Mann–Whitney U test are 0.091 and 0.724 for (A,B), respectively. IN: Gateway of seawater influent, PC: Fish ponds, PM: Hard clam ponds, PG: Seaweed ponds, a: Unit a and b: Unit b. The Y-axis represents the proportion (%) of 16S sequences of each bacterial/archaea genus in the next-generation sequencing data of each sample.

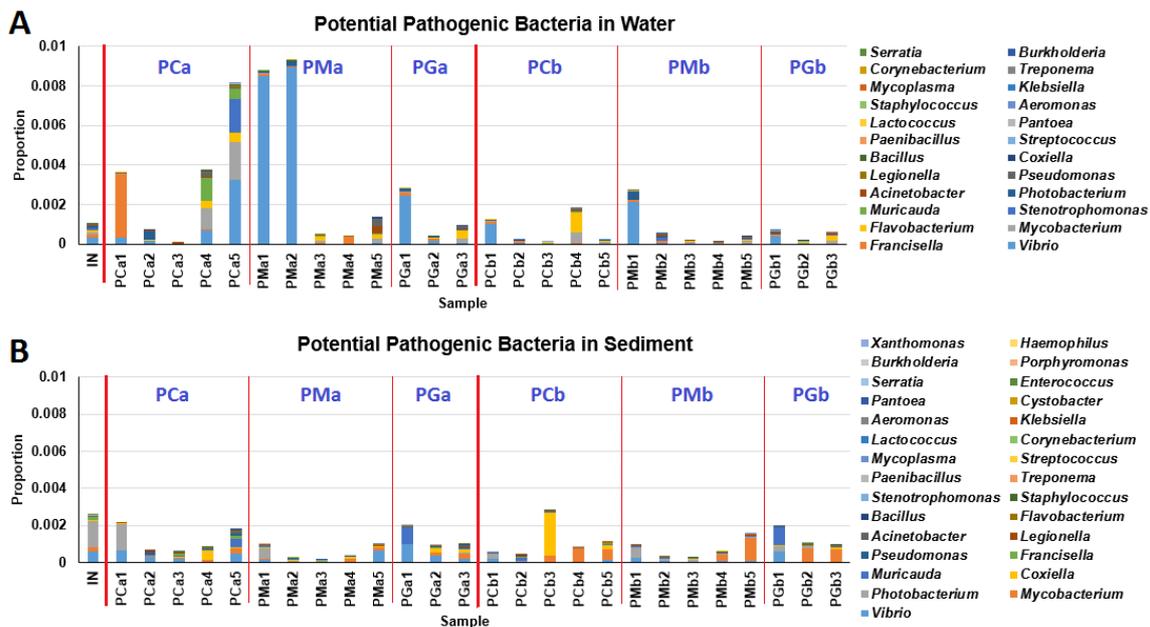


Figure 9. Differences of the potential pathogenic bacteria community in the water (A) and sediment (B) of the milkfish multitrophic recirculating aquaculture system. The p -values of comparison between overall proportions of the microbial community of Units a and b by the Mann–Whitney U test are 0.019 and 0.959 for (A,B), respectively. IN: Gateway of seawater influent, PC: Fish ponds, PM: Hard clam ponds, PG: Seaweed ponds, a: Unit a and b: Unit b. The Y-axis represents the proportion (%) of 16S sequences of each bacterial/archaea genus in the next-generation sequencing data of each sample.

4. Discussion

Intensive land-based fish and shrimp farms produce wastewater high in organic matter (e.g., feed residues, feces, and algal detritus) which is discharged in the effluent and disperses in the environment, causing eutrophication in coastal regions [24–26]. In arid regions, where fresh water is scarce, aquaculture wastewater is an alternative water resource. Irrigation with aquaculture wastewater may affect the soil microbial community composition and functional diversity. Consequently, the changes in the soil environment would be significant [27]. Reducing aquaculture impacts on the environment is still a scientific challenge [28].

The design of IMTA has several advantages to address the aquaculture impacts on the environment. However, the application of IMTA is mainly for inshore and offshore open water fields [29–31] that are not suitable for coastal aquaculture of milkfish. The design of RAS is suitable for milkfish. However, the facility for filtration and/or biofiltration after culture harvest produces new wastes and problems. Therefore, Shinn-Lih Yeh (from the Mariculture Research Center, Fisheries Research Institute, Taiwan) designed a MRAS that integrated the advantages of IMTA and RAS and is suitable for coastal aquaculture of milkfish on land fields. The MRAS, using the advantages of RAS, not only largely reduced the demand of fresh, clean water but also reduced the amount of wastewater and consequently reduced the aquaculture pollutions on the environment. Moreover, the MRAS, using the advantages of IMTA, raised the water quality after culture harvest. The facility for filtration and/or biofiltration in RAS is replaced by a hard clam pond and a sea weed pond in the MRAS. No additional waste needs to be discarded and processed for the MRAS after culture harvest.

The NGS data in this study indicated that the overall levels of *Vibrio*, *Escherichia*, and other potential pathogenic bacteria were lower than 2% in the farm-scale milkfish MRAS. Milkfish (*Chanos chanos*), Asian tiger shrimp (*Penaeus monodon*), Asian hard clam (*Meretrix lusoria*), and seaweed (*Gracilaria* sp.) were cultured and simultaneously produced in the MRAS.

The application of *Rhodovulum sulfidophilum* further enhanced the performance of the MRAS. The addition of *Rhodovulum sulfidophilum* promoted the growth of the nitrogen cycle-associated

microbial community and reduced the concentration of ammonia. Reduction of the ammonia concentration may reduce the growth of the cyanobacteria and algae communities and prevent the occurrence of cyanobacteria and alga blooming and eutrophication. It seems likely that the addition of *Rhodovulum sulfidophilum* also promotes the growth of other beneficial bacteria, such as phototrophic Acidobacteria. Photosynthetic bacteria have been used as beneficial bacteria in aquaculture and affect the water quality and microbiota of the culture pond [32,33]. Growth of these anoxygenic phototrophs may also contribute to the prevention of the growth of cyanobacteria and algae communities. A low ammonia concentration, low cyanobacteria and algae communities, and low coliform and pathogenic bacteria simultaneously provide a healthy environment for fish, shrimp, and clam growth in the MRAS.

5. Conclusions

This study provides an effective design of a water recycling aquaculture system. The system can be used on coastal land fields and greatly reduces the demand for aquaculture water resources, aquaculture wastewater discharge, and marine pollution and is conducive to the sustainable use of water resources.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2071-1050/11/7/1880/s1>. Table S1: Lists of microbial communities in Figures 3–9.

Author Contributions: Conceptualization, S.-L.Y.; Formal analysis, D.-L.K.; Funding acquisition, B.-V.C. and W.-L.C.; Investigation, C.-S.L., Y.-T.C. and D.-L.K.; Methodology, C.-S.L., Y.-T.C. and S.-L.Y.; Project administration, W.-L.C.; Supervision, C.-W.Y.; Writing—original draft, B.-V.C. and C.-W.Y.; Writing—review & editing, B.-V.C. and C.-W.Y.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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