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Effect of photosynthetic bacteria on water quality and microbiota in grass carp culture

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Abstract To investigate the effects of photosynthetic bacteria as additives on water quality, microbial community structure and diversity, a photosynthetic purple non-sulfur bacteria, *Rhodopseudamonas palustris*, was isolated and used to remove nitrogen in the aquaculture water. The results of water quality showed that the levels of ammonia nitrogen, nitrite nitrogen, total inorganic nitrogen and total nitrogen in the treatment group were significantly lower ($p < 0.05$) than the nitrogen levels of the controls in an extended range. A 454-pyrosequencing analysis revealed that at the level of phylum, *Proteobacteria* and *Firmicutes* were dominant in the control group respectively, compared to the dominance of the phyla *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* in the treatment group. The relative abundance of phyla *Bacteroidetes* and *Actinobacteria* in treatment witnessed an increase than that in the control. The results also indicated that the treatment group enjoyed a higher microbial diversity than that of the control group. Based on the oxygen requirement and metabolism, the authors observed that the water supplementation with photosynthetic bacteria could significantly decrease ($p < 0.05$) the number of nitrite reducer and anaerobic bacteria. Therefore, the results suggested that adding photosynthetic bacteria to water improves the water quality as it changes the microbial community structure.

Keywords Photosynthetic bacteria · Water quality · Microbial community structure · Grass carp · 454-Pyrosequencing

Introduction

As one of the world's fastest growing agricultural sectors, aquaculture contributed nearly half of the total food fish (FAO 2011). China, the world's largest producer of aquatic animals currently, shares a total production of 38.3 million tons in 2010, with over 70.2 % pond yield of the total inland aquaculture. However, with development of the industry, concerns are evoked about the possible effects of ever-increasing aquaculture waste both on productivity inside the aquaculture system and on the ambient aquatic ecosystem (Cao and Wang 2010). He and Wu (2003) showed that only 13.9 % nitrogen and 25.4 % phosphorous in the fish diets are utilized by aquatic animals, leaving the rest accumulates in the water or deposits into the sediment. Nutrients such as nitrogen and phosphorus were found to lead to eutrophication or algae bloom, excessive loss of oxygen resources, disease outbreak, low productivity, and undesirable changes in aquatic system (Cao et al. 2007; Jang et al. 2004). In addition, nitrogen compounds such as ammonium and nitrite can be toxic to aquatic animals at sufficiently high concentration, while nitrate may cause 'blue baby syndrome' potentially threating public health (Nora'aini et al. 2005). Therefore, a series approach aiming to improve

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aquaculture waste management should be adopted to make aquaculture a sustainable farming practice.

With the demand for more environment-friendly aquaculture practices (Gatesoupe 1999), probiotics have recently attracted extensive attention in the culture of aquatic organisms (Sahu et al. 2008; Wang et al. 2008; Nayak 2010). The use of photosynthetic bacteria has been common practice in many fish or shellfish hatcheries and farms in China (Suzer et al. 2008; Qi et al. 2009), as well as in wastewater treatment since the 1960s (Lu et al. 2010). As shown by Nagadomi et al. (1999), filtration systems packed with both alginate- and polyvinyl alcohol (PVA)-immobilized gel beads of *Rhodobacter sphaeroides* S could remove a relatively high level of COD during the purification of an aquarium for carp breeding. Nagadomi et al. (2000) used porous ceramic immobilized photosynthetic bacteria, (*Rhodobacter sphaeroides* S, *Rb. sphaeroides* NR-3 and *Rhodopseudomonas palustris*) in the batch treatment, observing the removal of COD (89 %), phosphate (77 %), nitrate (99 %) and H_2S (99.8 %) in the latter 48 h. Lu et al. (2010) showed that a new strain of photosynthetic bacteria (PSB), *Rhodobacter sphaeroides*, directly degraded soybean wastewater under natural conditions without extra light or oxygen supply.

A better understanding of microbial communities can help us design the wastewater treatment system, as well as enrich the theory of microbial ecology (Oerther et al. 2001; DeAngelis et al. 2011). Other than conventional molecular biological techniques, the second-generation high-throughput sequencing, such as 454-pyrosequencing, can elucidate the characters of microbial community more completely and accurately (Roesch et al. 2007; Hu et al. 2012). Recently, 454-pyrosequencing was applied to study the composition of microbial communities found in human gut microbiota (Dethlefsen et al. 2008; Zoetendal et al. 2012), fish intestine (Wu et al. 2012), soil (Lumini et al. 2010; Lin et al. 2012), ocean (Sogin et al. 2006; Roberts et al. 2012), and wastewater treatment systems (Kim et al. 2011; Hu et al. 2012). However, studying of the microbial communities in aquaculture water with the assist of this technology is not well documented. In this study, 454-pyrosequencing was applied to investigate the microbial community structure and diversity of microorganisms in grass carp cultures supplementation with photosynthetic bacteria.

Materials and methods

Bacteria preparation

Being isolated from a pond of grass carp in the Zhejiang province of China, the photosynthetic bacteria, purple non-sulfur bacteria *Rhodopseudomonas palustris* was preserved at the Key Laboratory of Molecular Animal Nutrition Zhejiang University, Hangzhou, in Zhejiang, China. Further information on isolation and identification can be seen in supplementary material. The photosynthetic bacteria were cultured on malate basal medium (Van Niel 1971) for 24 h at 30 °C, and then fresh cells (10^9 cfu/ml) were harvested. The collected pellet was dried and kept in a sterilized container at room temperature. The purity and viability of the bacteria were checked routinely according to Nikoskelainen et al. (2003) and Li et al. (2012).

Experimental design

The experiment was performed in Dafan fishery cooperatives in the Shaoxing Country Zhejiang Province with grass carp that were obtained from a Shaoxing freshwater fish farm. In total, 114 healthy fish of average weight 14.58 ± 0.8 g were raised in buckets and randomly divided into two groups such that three replicates were available for the control and treatment groups. Each bucket was supplied with 340 L of water from the Dafan river of the Shaoxing Country Zhejiang Province (30°03′38″N, 120°40′52″E), which kept unchangeable during the course of the experiment and was continuously oxygenated with an air compressor (ACO-318, Zhejiang Sunsun Industry Co. Limited). The water temperature remained constant at approximately 29 °C. The control group was fed a basal diet, not being exposed to photosynthetic bacteria; though also fed a basal diet, the treatment group was exposed to a probiotic preparation of 1×10^{11} cfu/m^3 per week. According to Li et al. (2012) and Zhang et al. (2013), the probiotic preparation was added into the water twice. The fish were hand-fed 2 % of their body weight twice each day (at 09:00 AM and 17:00 PM). The ingredients and nutritional composition of the basal diet were described in previous study (Zhang et al. 2013). During a 15-day experimental period, different physico-chemical parameters such as water temperature were measured daily, whereas ammonia nitrogen, nitrite nitrogen, nitrate nitrogen, total inorganic nitrogen and total nitrogen were estimated at three-day intervals according to our previous study (Zhang et al. 2013). The total inorganic nitrogen is the sum of the ammonia, nitrite and nitrate nitrogen.

454-Pyrosequencing

The rearing water was well mixed, collected (500 mL each) on the 15th day, which was filtered through a 0.22-μm membrane and a stainless steel filter holders (Sartorius, Germany) in order to isolate the microbes. The microbes on the 0.22-μm membrane were stored at −20 °C for further study. The protocol of DNA extraction and PCR

Fig. 1 Effect of probiotics as water additives on the water quality in grass carp culture. The probiotic preparation was added into water on the 0 day and 7th day, marking with *down arrow*. Each group at each time point has three replicates. *Asterisk* indicated significant differences ($p < 0.05$) compared with treatment group at each time point
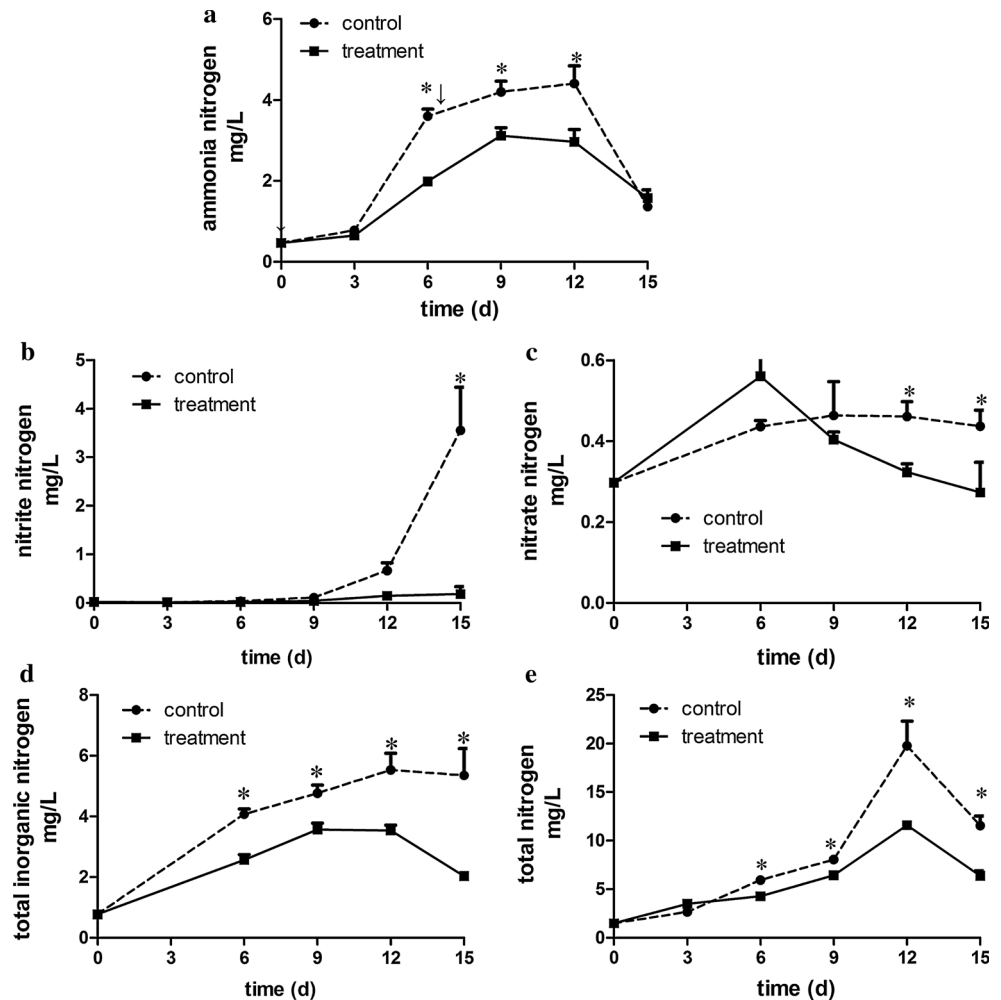
amplification performed in this study followed our previous study (Zhang et al. 2013).

The V3 region of the 16S rRNA gene was amplified by the primers 341F (5′-CCTACGGGAGGCAGCAG-3′) and 534R (5′-ATGAGCTGATTACCGCGGCTGCT-3′), and the 5′ terminus of each forward primer contained an 8-bp barcode sequence to tag specific samples The PCR products in which process, a length of approximately 200 bp were excised from an agarose gel (1 % in TBE buffer) containing Goldview (Beijing Zoman Biotechnology Co. LTD) and purified with an Axyprep™ DNA gel extraction kit (Axygen Scientific Inc, USA). The DNA concentration and quality were assessed with a UV–vis spectrophotometer (NanoDrop ND1000, USA). Equal amounts of three samples containing different sample-specific barcode sequences were pooled to provide a total quantity of 50 ng. Afterwards, the pooled DNA was subsequently sent to Tongji-SCBIT Biotechnology Co. Ltd. for 454-pyrosequencing.

454-Pyrosequencing data analysis

All analyses described in the current study were performed with version 1.6 of the QIIME (Caporaso et al. 2010) followed the website (http://qiime.org/tutorials/tutorial.html#picking-operational-taxonomic-units-otus-through-making-otu-table). Besides, a multivariate data analysis was performed by using METAGENassist a web server tool (Arndt et al. 2012) that assigns probable microbial functions based on taxonomy (16S ribosomal subunit) following the protocol described in the previous study (Badri et al. 2013).

Statistical analysis

Data were subjected to independent t test method to determine significant differences through SPSS 17.0 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered to be statistically significant.
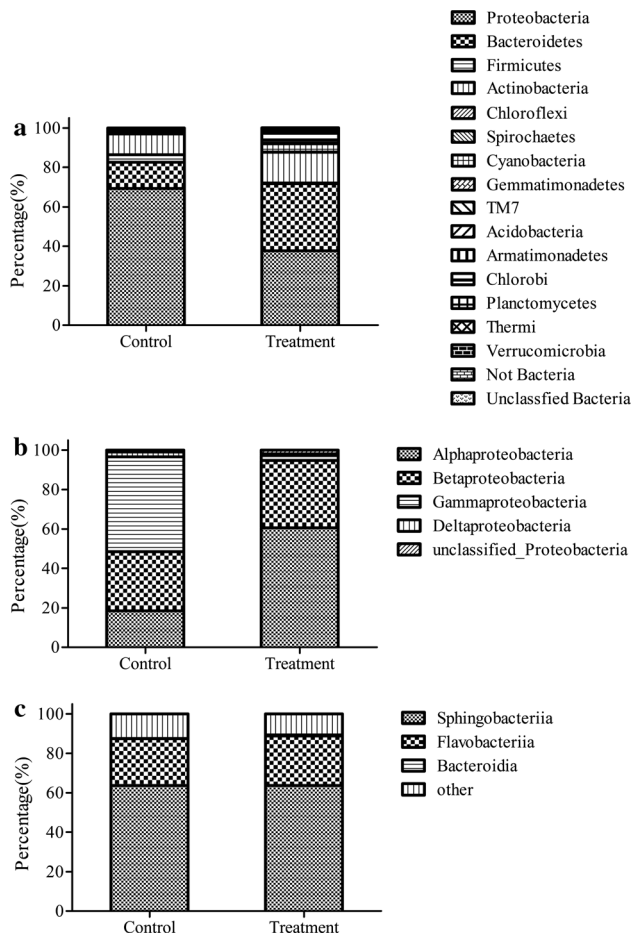
Fig. 2 Bacterial community structures in control and treatment group. **a** Bacterial community structures at phylum level. The abundance is presented in terms of a percentage of the in total effective bacterial sequences in sample. **b** Proteobacteria composition by class in samples from control and treatment. **c** Bacteroidetes composition by class

Results

Effect of photosynthetic bacteria on water quality

The photosynthetic bacteria influenced the water quality in the grass carp culture water (Fig. 1) as the concentration of ammonia nitrogen in the control group was higher than that in the treatment group (Fig. 1a). On the 6th and 9th days, compared with the treatment, the concentration in control increased by 80.92 % ($p < 0.05$) and 34.54 % ($p < 0.05$), respectively (Fig. 1a). As shown in Fig. 1b, from the beginning of the experiment to the 15th day, the concentrations of nitrite and nitrate nitrogen were at a low level, exhibiting no significant differences between the two groups; however, on the 15th day, the nitrite nitrogen content in the treatment group decreased by 94.90 % ($p < 0.05$) compared with the control (Fig. 1b). On the

12th day (Fig. 1c), compared with the control, the nitrate nitrogen content in the treatment group decreased by 29.81 % ($p < 0.05$). The concentration of total inorganic nitrogen and total nitrogen experienced an ascending trend overall, and from 6th to 15th, both of them in treatment group were significantly lower ($p < 0.05$) than those in the control (Fig. 1d, e). Since effects of photosynthetic bacteria on other water quality parameters were shown in Fig. S1. Therefore, the results suggested that the ammonia nitrogen, nitrite nitrogen, total inorganic nitrogen and total nitrogen contents in the treatment group were significantly lower ($p < 0.05$) than those in control in an extended range.

Effect of photosynthetic bacteria on the community structure

After filtering the low quality reads and trimming the adapters, barcodes and primers, 12, 675 effective reads were there for the samples. Then the authors picked 990 representative set of sequences. After denoising and filtering out chimeras, 974 representative sequences remained for control and treatment group to conduct the downstream analyses. The selected bacterial sequences were assigned to different taxa levels (from genus to phylum) using the RDP Classifier at 50 % threshold.

At the phylum level (Fig. 2a), *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* were dominant in both control and treatment groups. Compared with the control, with a decreasing number of *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* increased in treatment group. Within *Proteobacteria* phyla (Fig. 2b), where *Alphaproteobacteria* and *Betaproteobacteria* were higher, *Deltaproteobacteria* and *Gammaproteobacteria* were lower in treatment group compared to those in control group. While Within *Bacteroidetes* phylum, *Sphingobacteria* in control and treatment groups was in the same level in terms of amount, and *Bacteroidia* and *Flavobacteria* were higher in treatment group than those in the control group (Fig. 2c).

To further compare the microbial communities of two samples from the control and treatment groups, the tree created by MEGAN was shown in Fig. 3. The pie charts beside the leaves of the tree indicate the relative abundance of the microbes in the control and treatment samples. From Fig. 3, it could be seen that the microbial communities of the two groups were quite different from phylum to genus level. There were some genera (such as *Propionibacteriaceae*, *Bacteroides, Fluviicola, Opitutus, Sphingomonas*, etc.) appearing only in the treatment, while many other genera, which were marked by gray color in Fig. 3, exist only in the control. As above, the results suggested that supplementation with photosynthetic bacteria could cause alterations of the microbial diversity in grass carp water.

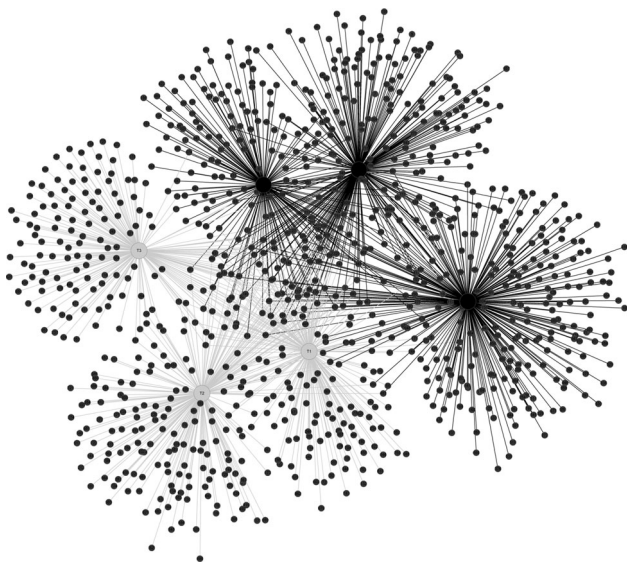**Fig. 3** Sequences from control and treatment were compared by MEGAN 4
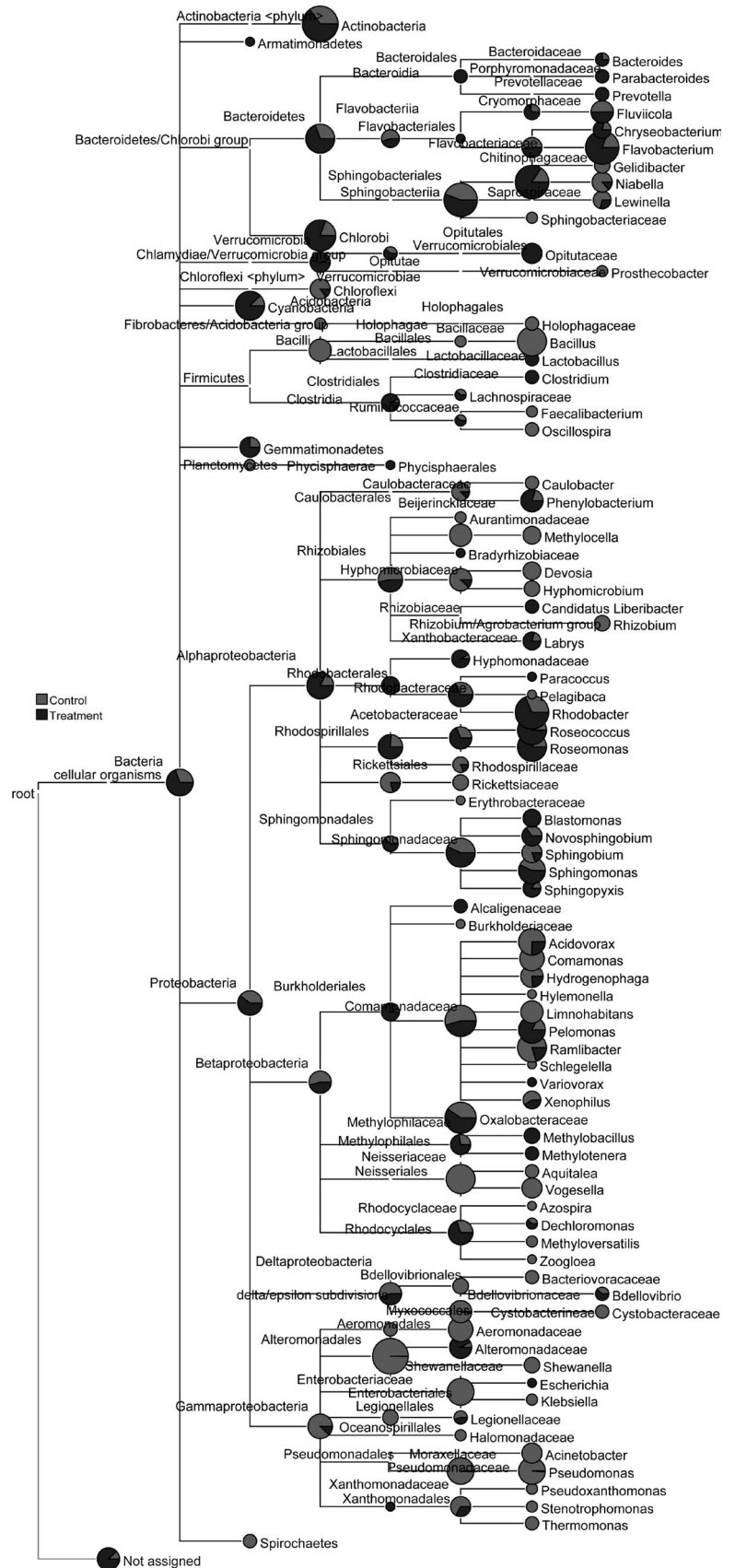
Fig. 4 Network based analysis of the water bacterial communities. *White circles* are representative sequences from each OTU clustered based on the relatedness of the sequences (97 % similarity). Each sample is connected with the OTUs through edges color-coded (*dark* control; *gray* treatment)

Similarity between communities

To visualize the phylogenetic information and process of partition in the operational taxonomic units (OTUs) between samples, the data were summarized in a spring-embedder network (Fig. 4), which consisted in 996 nodes and 1,347 edges. Network analyses indicated a high number of shared OTUs between samples highlighting a co-occurring community (Fig. 4). The authors also used principal component analyses on pair-wise and normalized OTUs between all treatments to identify the main factors driving community composition differences. Based on principal components analysis, two different clusters were formed for the control and treatments at the phylum and genus level (Fig. 5). The second principal component (4.8 and 13 %) revealed that the controls distinguished from their respective treatments. This pattern was recapitulated by hierarchical clustering using Ward method (Fig. S2). The results indicated that there was not only difference in control and treatment group, but also shared some OTUs.

Taxonomic to phenotype mapping

The OTUs from taxonomy were assigned to phenotype mapping by employing METAGENassist webserver tool (Arndt et al. 2012) based on oxygen requirement, metabolism, etc. On account of the analyses, the authors noticed that the water supplementation with photosynthetic bacteria could significantly decrease ($p < 0.05$) the number of
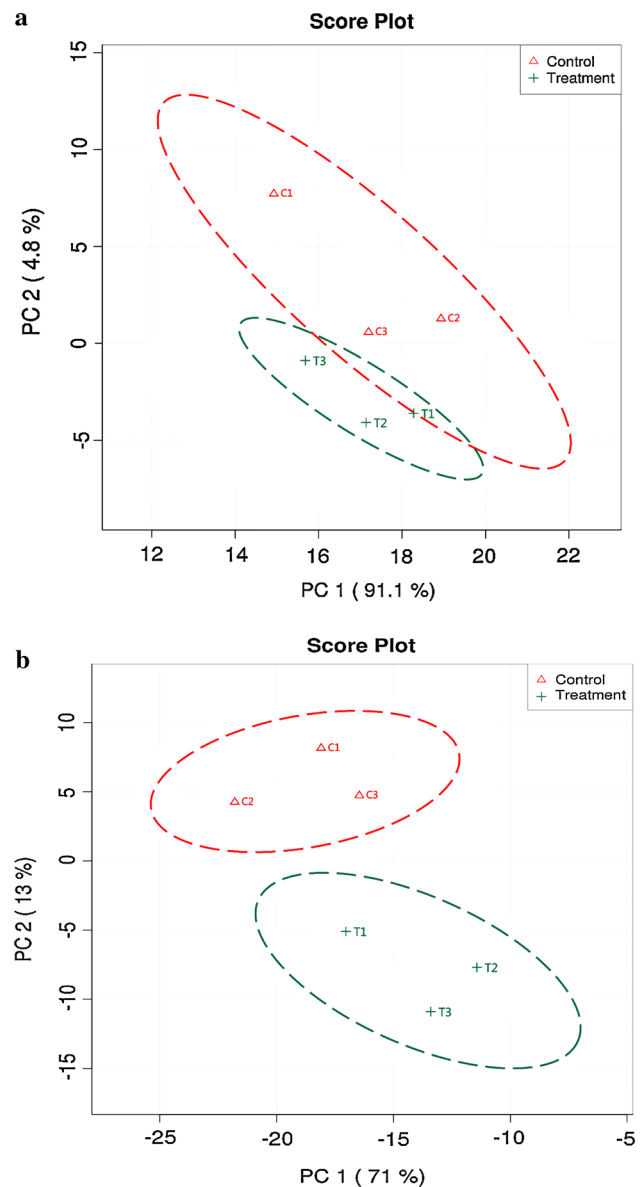

Fig. 5 Water microbiome sequencing data of treatments and controls analyzed by principal component analyses at phyla levels (**a**) and genus levels (**b**)

nitrite reducer and anaerobic bacteria (Fig. 6). Nevertheless, there was no difference ($p > 0.05$) in relative abundance of ammonia oxidizer and aerobic bacteria between control and treatment groups (Fig. 6). In a word, the results showed that photosynthetic bacteria could change the phenotype mapping in grass carp water.

Discussion

The present findings revealed that supplementation with photosynthetic bacteria could significantly reduce

Fig. 6 Taxonomic to phenotypic mapping based on the metabolism (**a**) and oxygen requirement (**b**)
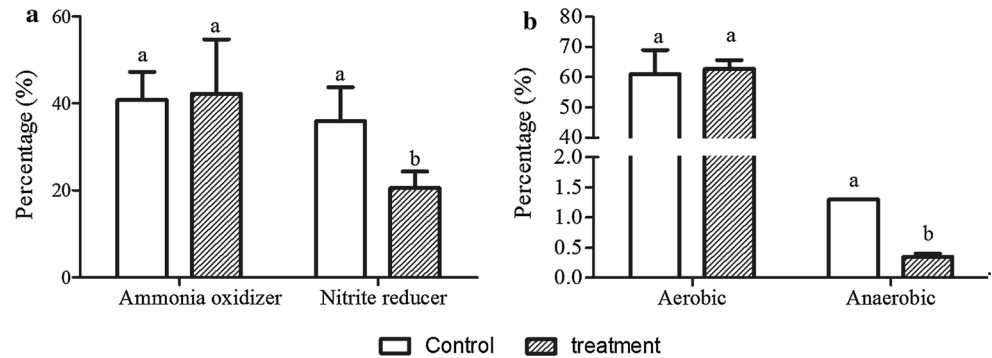
$(p < 0.05)$ the levels of ammonia nitrogen, nitrite nitrogen, total inorganic nitrogen and total nitrogen in grass carp water, which may be a result of the ability of photosynthetic bacteria to remove nitrogen compounds, since it has been reported that photosynthetic bacteria can take up nitrite nitrogen and nitrate nitrogen (Tosques et al. 1997; Sabaty et al. 1999). Additionally, in the study of Takabatake et al. (2004) about the influence of carbonate on ammonia removal by photosynthetic bacteria, they showed ammonia nitrogen was removed involving the uptake of carbon source and growth. While photosynthetic bacteria can use small organic molecules (Pfennig 1967) and reduce the levels of nitrogen-containing inorganic substances (Pfennig and Truper 1974), contributing to reduction in the source of the ammonia nitrogen. On the other hand, addition of the photosynthetic bacteria in water improves the water quality, which explains changes in microbial community diversity (Zhang et al. 2013).

Besides, a 454-pyrosequencing analysis revealed that the composition of the bacterial communities in the culture water was highly variable between the control and treatment groups. Actinobacteria contribute to the breakdown and recycling of organic compounds (Goodfellow and Haynes 1984), which perform other yet undefined ecological roles (Jensen and Lauro 2008). Members of the Bacteroidetes phylum are abundant in aquatic habitats when assessed by fluorescent in situ hybridisation and in some 16S rRNA gene libraries (O'Sullivan et al. 2004). Xia et al. (2007) have reported members of Bacteroidetes presented in a significant amount in activated-sludge WWTPs by using fluorescent in situ hybridisation (FISH) analyses. They also found Bacteroidetes are one of the main components of the Enhanced biological phosphorus removal (EBPR) microbial communities with highest probability to have an important function, but their detailed ecophysiology is unclear. Therefore, the increased number of bacteroidetes and actinobacteria observed in the treatment group may contribute to the improvement in water quality over the control. Miura et al. (2007) revealed that adequate dynamics of the bacterial community or flexibility to adapt

to changes in environments are important for the stable performance of both the conventional membrane bioreactors (CMBRs) and hybrid MBRs (HMBRs). In addition, microbes can help improve the water quality as the bacteria has the ability to participate in the metabolism (Hu et al. 2012). As reported, there was a significant correlation between denitrification potential rates and microbial community patterns (Peralta et al. 2010; Song et al. 2011). Thus, there may be a correlation between microbial community and the improvement of water quality.

The results also demonstrate that the addition of photosynthetic bacteria to the water can increase the microbial diversity, possibly because the composition of microbial communities can be altered by husbandry practices and environmental conditions that stimulate the proliferation of selected bacterial species (Balcazar et al. 2006). Additionally, photosynthetic bacteria can convert the organic molecules into nutrients (Pfennig 1967), remove nitrogen (Johansson and Howard 1976; Masters and Madigan 1983) and reduce the levels of nitrogen-containing inorganic substances (Pfennig and Truper 1974), which can improve water quality and create a better living environment for microorganism. However, further study is needed to confirm whether probiotics as water additives have a beneficial effect on the health of aquatic animals when improving water quality.

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