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ORIGINAL PAPER

Bacterioplankton community analysis in tilapia ponds by Illumina high-throughput sequencing

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Abstract The changes of microbial community in aquaculture systems under the effects of stocking densities and seasonality were investigated in tilapia ponds. Total DNAs were extracted from the water samples, 16S rRNA gene was amplified and the bacterial community analyzed by Illumina high-throughput sequencing obtaining 3486 OTUs, from a total read of 715,842 sequences. Basing on the analysis of bacterial compositions, richness, diversity, bacterial 16S rRNA gene abundance, water sample comparisons and existence of specific bacterial taxa within three fish ponds in a 4 months period, the study conclusively observed that the dominant phylum in all water samples were similar, and they included; Proteobacteria, Cyanobacteria, Bacteroidetes, Actinobacteria, Planctomycetes and Chlorobi, distributed in different proportions in the different months and ponds. The seasonal changes had a more pronounced effect on the bacterioplankton community than the stocking densities; however some differences between the ponds were more likely caused by feed coefficient than by stocking densities. At the same time, most bacterial communities were affected by the nutrient input except phylum Cyanobacteria that was also affected by the feed control of tilapia.

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Keywords Bacterioplankton community · Illumina high throughput sequencing · Tilapia ponds

Introduction

Aquaculture is currently the fastest growing sector of animal food production (Klinger and Naylor 2012), and its contribution to the total world fish production was 54.6 % in 2012 (FAO 2014).In developing countries, aquaculture is also considered to be a relatively easy entry point for practicing fishery productions particularly for small-scale producers, where pond culture forms the largest proportion of freshwater fisheries (Mischke 2013).

As an important component in aquatic ecosystem, aquatic microorganisms play a major role in the aquaculture ponds, particularly with respect to its productivity, nutrient cycling, and water quality (Moriarty 1997). Literature reviewed, suggests the microorganisms roles might be affected by many factors close to the aquaculture operations, such as degree of intensifications (Sakami et al. 2008), changes of some water parameters (Zhou et al. 2013), the using of herbicides (Pesce et al. 2011), water exchanges (Sakami et al. 2003), and so on. An in-depth knowledge of the mechanism of the impacts of these factors helps to understand the role of some specific microorganism communities in the biogeochemical cycles. Studies of microbial communities have been done by conventional culture method, however, it has proven difficult to be used due to variable number and species of microbes detected depending on the culture conditions used (Spanggaard et al. 2000) and the existence of a large number of non-culturable bacteria (Giovannoni et al. 1990). This study will use Pyrosequencing, a next generation sequencing and high-throughput analytical method

for studying microbial diversity (Liao et al. 2013) that has been widely used to analyze microbial diversity of environment samples (Ma et al. 2013; Wang et al. 2013; Staley et al. 2013; Huang et al. 2014; Tiirik et al. 2014; Jiang et al. 2013), and amplification of the bacterial 16S rRNA gene has resulted in extremely deep sequencing of bacterial communities (Staley et al. 2014). Step by step, this method has been proven good and most recently is used to understand bacterial community diversities and the factors that may have impacts on the bacterioplankton community in aquaculture ponds thus obtaining more valuable information.

China is the largest producer of Tilapia in the world (Hao 2012), and tilapia has become the sixth most commonly farmed freshwater aquaculture species in China after Silver carp (*Hypophthalmichthys molitrix*), Bighead carp (*Hypophthalmichthys nobilis*), Grass carp (*Ctenopharyngodon idella*), Common carp (*Cyprinus carpio*) and Crucian carp (*Carassius carassius*) (Chen et al. 2007). However, the faster growth rate of tilapia was based on the larger amount of nutrition and energy inputs, which might make the water quality and the bacterial community in water change easily. Thus, it is of great significance to investigate the bacterioplankton communities in tilapia ponds and even their responses to breeding intensity. A recent study (Del'Duca et al. 2015) of the bacterial community within the pond water, sediment and guts of tilapia (*Oreochromis niloticus*) obtained results showing that the bacterial community found in juvenile tilapia intestines was closer to that in water. In another study carried out by us showed that the bacterial community in intestine of adult tilapia fish was closer to that in sediment. Besides, a study on bacterial communities in tilapia brackish water ponds indicated that both pond water and the sediment had effect on the intestinal bacterial community of tilapia (Al-Harbi and Uddin 2005). Although various studies report interactions between tilapia and its environment or tilapia and other species living for an entire breeding cycle, the responses of bacterial community in water to different stocking densities in the whole cultural season have not been reported. Thus, the aim of this study was to investigate this by Illumina high throughput sequencing in order to have an understanding of bacterial community status in this type of water environment.

Materials and methods

Sample collection

The water samples were collected on 30 May, 29 July, 16 September and 10 October, 2014 from three ponds located in Yixing, Jiangsu, China. The area of each pond was all approximately 1330 m^2, initially filled with water to a depth of 0.7 m for each until 7 July, when more water was added making the final depth 2 m. The Genetically improved farmed tilapia (GIFT) (*Oreochromis niloticus*) were cultured from fry (released on 23 May) in these three ponds with the stocking densities of 2400 fish/pond (pond 1), 3000 fish/pond (pond 2), and 3600 fish/pond (pond 3) respectively. During the first month of the experiment, no feed were put into the ponds. After 24 June, the fish were fed on formula feed, widely used in the culture of freshwater fish species in China, and the fish were fed four times a day to apparent the satiation (08:00, 11:00, 14:00, and 16:00 h). During the breeding period, no water was changed and no fishery drugs were used. At harvest, survival rates and feed coefficients were performed under formula (1) and (2) below.

$$\text{Survival rate } (\%) = \text{the numbers of adult fish harvested from the pond/the numbers of fry stocked in the pond} \times 100\,\% \quad (1)$$

$$\text{Feed coefficient } (\%) = \text{the whole weight of bait put in the pond/the whole weight of the fish harvested from the pond} \times 100\,\% \quad (2)$$

Water samples were collected from three places of each pond at the depth of 50 cm below the surface. An equivalence mixture of the water samples from each pond was made, and then 200 mLs of the mixture was obtained in triplicate filtered through a 0.2-μm (47 mm diameter) pore size hydrophilic polyethersulfone membrane to obtain the microbe samples for our analysis that were stored at −80 °C until further analysis. At the end of the experiment, four samples per pond were obtained in triplicate denoted by labels of MW11, MW12 and MW13; where MW represented May water samples, One (1) represents pond 1 and the last Figs. 1, 2 and 3 represent the triplicate samples. This labeling was similarly used for July water samples-JW, September samples-SW and October samples-OW. The other ponds were also labeled in a similar way i.e. May water samples for Pond 2 was denoted by MW21, MW22 and MW23 and the others respectively.

DNA extraction, bacterial 16S rRNA gene amplification and pyrosequencing analysis

DNA was extracted in duplicate from each membrane filter by PowerWater DNA Isolation Kit (MO BIO, USA), according to the manufacturer's instructions using a microcentrifuge (MO BIO, USA), quantified and stored at −20 °C for further uses.

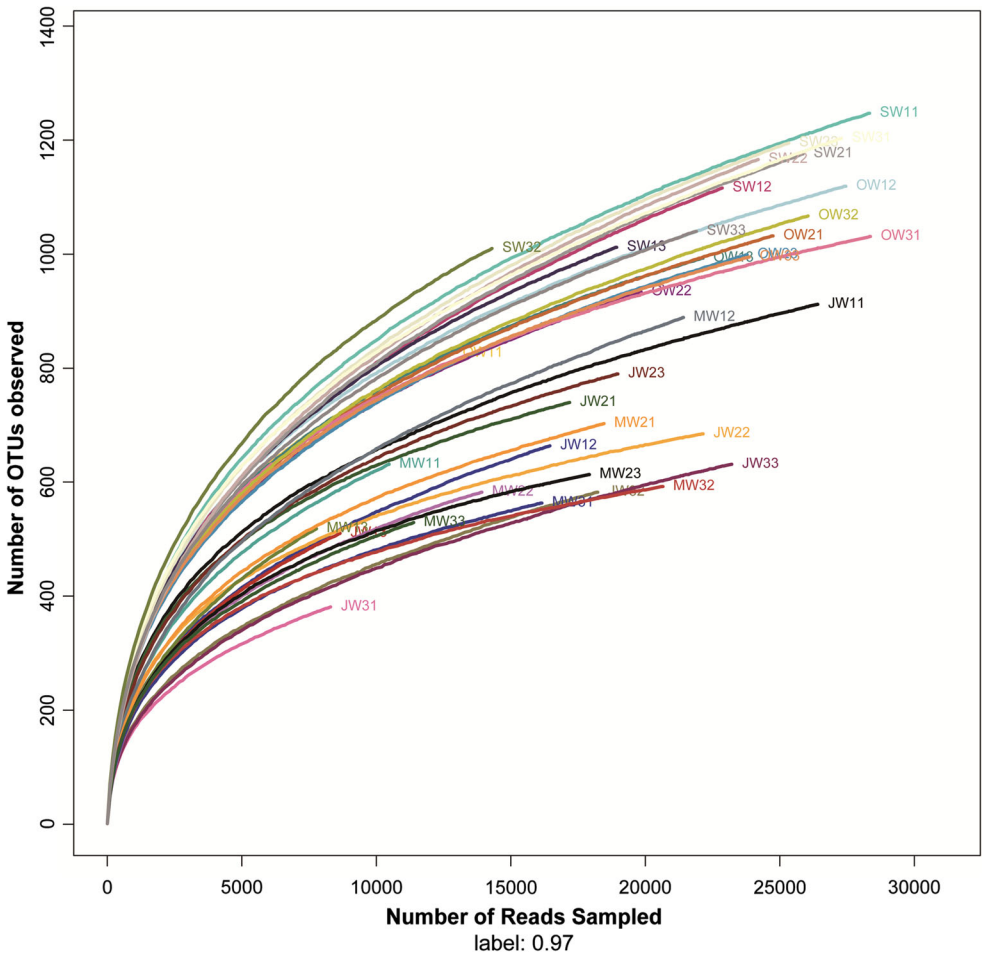


Fig. 1 Rarefaction analysis of the different water samples

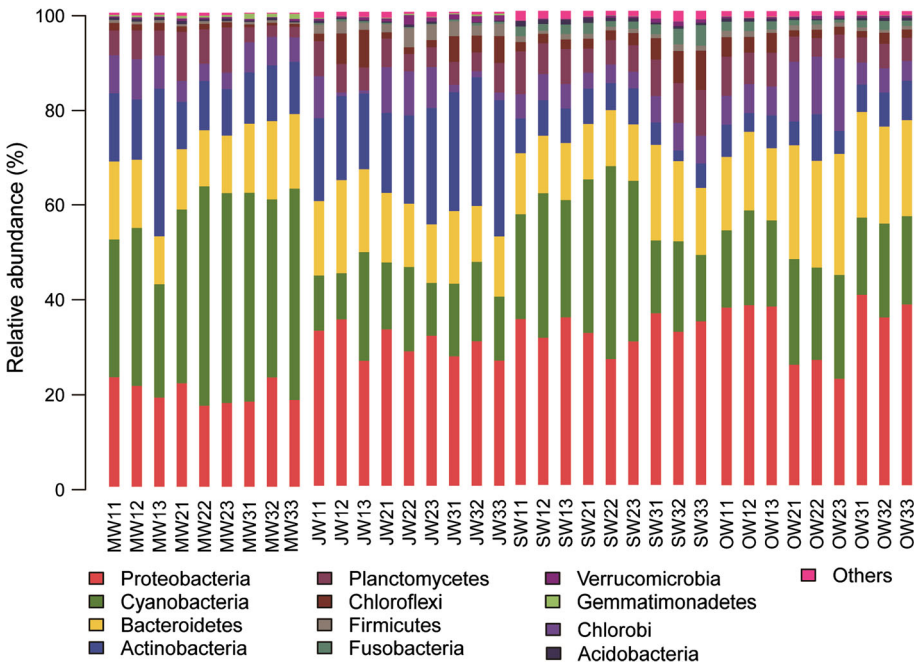


Fig. 2 Bacterial composition of the different communities. Relative read abundance of different bacterial phyla within the different communities. Sequences whose relative abundance was lower than 1 % were assigned as "others"

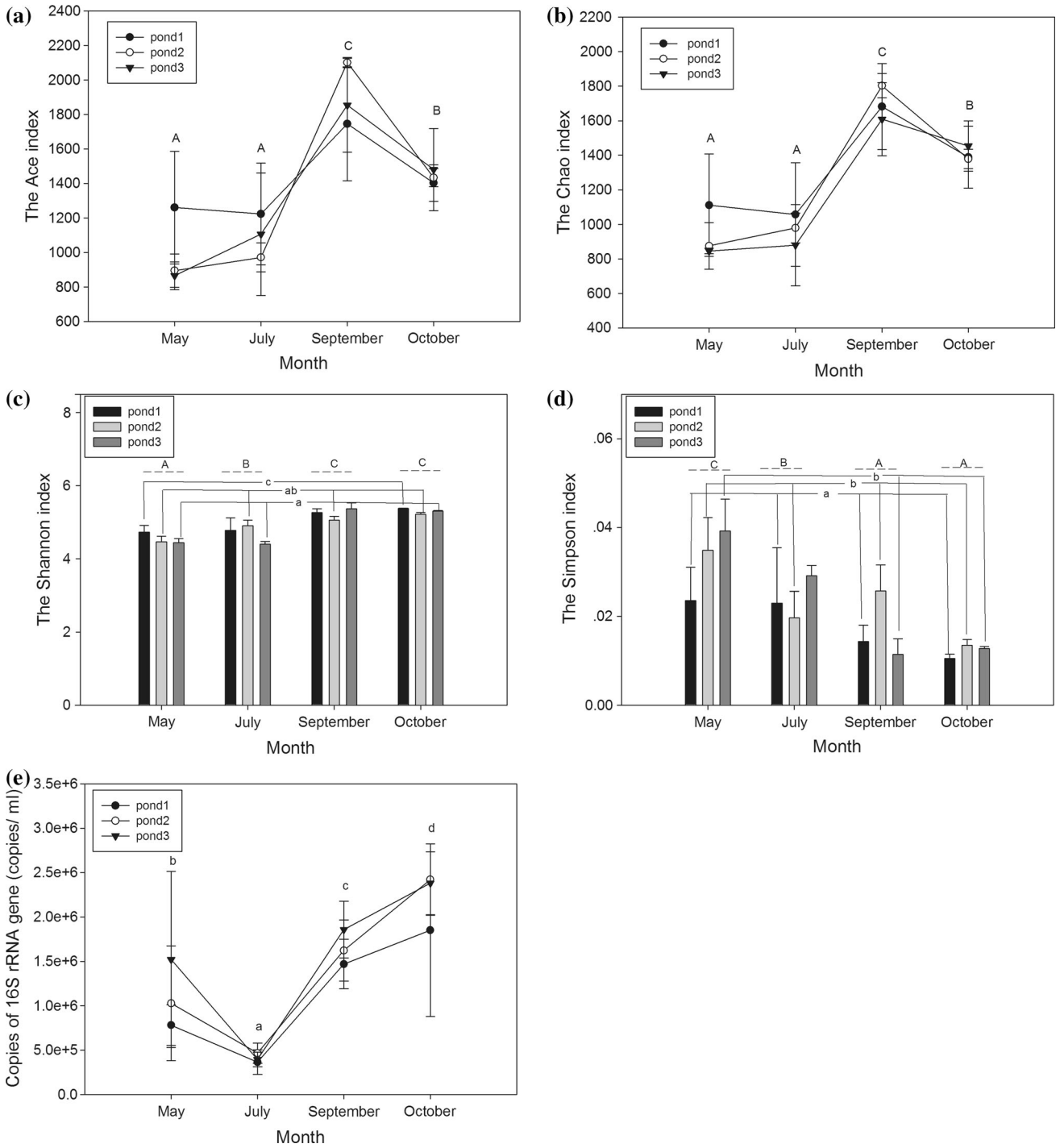


Fig. 3 Richness [the Ace (**a**), Chao (**b**) index], diversity [the Shannon (**c**), Simpson (**d**) index] and 16S rRNA gene copies (**e**) of bacterial communities in pond water. The *letters* upon the *curves* and *bars* show the differences between groups, where the *capital* and *lowercase* forms show the differences between months and ponds respectively. The *same letters* mean no significant differences, while *different letters* mean that there are statistically significant differences between groups at 95 % level. *Higher values* of Ace index and Chao index represent more richness. A *higher value* of Shannon index represent more diversity, while a *higher value* of Simpson index represents less diversity

DNA amplification was performed with the 515F (5′-GTGCCAGCMGCCGCGG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) primers specific for the V3–V4 hypervariable regions of the 16S rRNA gene with different barcodes for the region (Bates et al. 2011). The PCR mixture (final volume 20 μL) contained 4 μL five-fold

FastPfu buffer (TransGen Biotech, China), 0.4 μL (5 μM) each of the primers, 0.4 μL FastPfu polymerase (TransGen Biotech, China), 10 ng template DNA, and 2.5 mM dNTPs. For each sample, three independent PCRs were performed using a GeneAmp® 9700 PCR instrument (ABI, USA). The PCR conditions were as follows: 95 °C for 3 min; 26 cycles of denaturation (95 °C for 30 s), annealing (55 °C for 30 s), and extension (72 °C for 45 s), followed by a final extension of 72 °C for 10 min. The three replicate PCR products were mixed, detected on agarose gels (2 % in TBE buffer), and purified with a DNA gel extraction kit (Axygen, China). After purification, each mixture of amplicons was quantified with a QuantiFluorTM-ST fluorometer (Promega, USA), and pooled in equimolar ratios based on concentration. Amplicons were then used for pyrosequencing with an sequencing primer kit on a Illumina MiSeq PE300 platform at Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China.

Bioinformatics analysis

After pyrosequencing, the raw data was filtered according to barcode and primer sequences using the software of Trimmomatic and FLASH under the following rules: (1) Remove the bases with the trailing quality score being under 20, scan the read with a 50-base wide sliding window, do cutting operations when the average quality score per base drops below 20, and drop the reads below 50 bases long, (2) Merge the reads in pairs into a new one on the basis of their overlap (the minimum length overlapped was 10 bases), (3) Remove the merged reads that the mismatch ratio in overlapping regions larger than 0.2, (4) Remove the reads with the mismatch numbers of primer larger than 2. Then the high-quality sequences were matched with samples according to their barcodes. Sequences with similarities >97 % were clustered into one operational taxonomic unit (OTU) with Usearch (version 7.1 http://drive5.com/uparse). Sequences were assigned to taxonomies with the Ribosomal Database Project classifier (Luo et al. 2013). A BLAST search for taxonomic classification was performed using Qiime via SILVA (version 119 http://www.arb-silva.de) database at 70 % confidence level. Rarefaction analysis of the 36 libraries, analysis of community richness with the Chao 1 estimator (http://www.mothur.org/wiki/chao), abundance-based coverage estimator (ACE) (http://www.mothur.org/wiki/Ace), analysis of community diversity with the Shannon index (http://www.mothur.org/wiki/Shannon), and the Simpson index (http://www.mothur.org/wiki/Simpson), the hierarchical clustering of all libraries was all determined in the Qiime program. The Hierarchical clustering tree was constructed using unweighted pair group method with arithmetic mean (UPGMA).The redundancy analysis (RDA) was carried out using Canoco for Windows version 4.5 software with the Monte Carlo permutations test. The Good's coverage (http//www.mothur.org/wiki/Coverage) was generated in the MOTHUR program. The characterization of microorganismic features differentiating the microbiota specific to different grouping types was performed by linear discriminant analysis (LDA) effect size (LEfSe) method (http://huttenhower.sph.harvard.edu/lefse/) for biomarker discovery, which emphasizes both statistical significance and biological relevance (Ling et al. 2014).

Real-time PCR analysis

The 16S rRNA gene quantitative amplifications of total bacteria were conducted in triplicate using the SYBR Green Real-Time PCR Kit [ABI Power SybrGreen qPCR Master Mix (2X)]. The real-time PCR (qPCR) assays were performed on ABI 7500 qPCR System (America). Primers used in this study were the same with bacterial 16S rRNA gene amplification for pyrosequencing analysis.

Determination of water quality

Nitrate, nitrite and sulfate were measured with a DIONEX ICS 3000 Ion Chromatograph. Ammonia was measured with a Nessler colorimetric assay. Water transparency was assessed by the Behcet's disk method. Total organic carbon (TOC) was assessed using a GE Sievers InnovOx Laboratory TOC Analyzer. The dissolved oxygen and the pH in water were measured with the dissolved oxygen meter and pH meter respectively. The potassium permanganate index (COD) was measured with the method of acidic potassium permanganate oxidation. The total nitrogen and total phosphorus were all measured with the method of alkaline potassium persulfate oxidation.

Statistical analysis

The data were analyzed by two-way ANOVA analysis using the software SPSS 11.5. The level of statistical significance was accepted by as $P < 0.05$.

Results

Tilapia culture results

Table 1 showed the status of the tilapia culture. The results indicated the tilapia yield in the three ponds were 1442, 1905 and 2091 kg for Pond 1, 2 and 3 respectively. The registered survival rate was least in pond 1, and highest in pond 3, and on contrary the feed coefficient in the three ponds registered pond 1 as highest and pond 2 as the lowest.

Table 1 Some results related to the culturing of GIFT tilapia under different stocking densities

	Pond 1	Pond 2	Pond 3
Stocking density (fish/hm^2)	18,000	22,500	27,000
Yield (kg)	1442	1905	2091
Survival rate (%)	80	88	97
Feed coefficient	1.511	1.207	1.262

Changes of bacterial community response to seasons and ponds differentiation

A total of 715, 842 valid reads and 3486 OTUs were obtained from the 36 water samples after the pyrosequencing and filtering operations. They were assigned to 41 different phyla or groups. The rarefaction curves tended to approach the saturation plateau (Fig. 1), indicating that a reasonable number of individual samples had been taken. Good's coverage estimations revealed that 97.17–99.17 % of the species were obtained in all of the water samples, which indicated that the sequencing depth was enough for the community analysis.

As shown in Fig. 2, the dominant phyla in all water samples were similar, they were Proteobacteria, Cyanobacteria, Bacteroidetes, Actinobacteria, Planctomycetes and Chlorobi. The average relative abundance for them was 92.8 %. And they showed different proportions between the different months and ponds.

Comparison of 16S rRNA profiles from all water samples showed that the relative abundance of six main phyla were all affected by season to some extent, among which Cyanobacteria and Actinobacteria were more strongly affected. Cyanobacteria showed the highest relative abundance in May [(37.67 ± 7.81) %], and the second in September [(25.88 ± 9.11) %]. In July [(14.73 ± 4.02) %] and October [(19.18 ± 2.11) %] the value were somehow low. Actinobacteria showed the second highest relative abundance in July [(21.43 ± 4.99) %], however it was in the fourth row in May [(13.56 ± 6.79) %], and in the fifth row in September [(6.11 ± 1.83) %] and October [(6.51 ± 1.84) %]. Compared with the two phyla, the relative abundance of Proteobacteria was stable. It showed the highest relative abundance in most of the sampling times except in May (still in second row).

The relative abundance of Proteobacteria, Cyanobacteria and Chlorobi appeared significantly different between pond 2 and the other two ponds ($P < 0.05$), among which Proteobacteria had the lowest abundance in pond 2, however, Cyanobacteria and Chlorobi had the highest abundance in that pond. The relative abundance of Bacteroidetes and Planctomycetes showed significant differences only between pond 1 and pond 3, among which Bacteroidetes had the lower abundance in pond 1 and Planctomycetes had the higher abundance in pond 1. No differences were found between the three ponds for the abundance of Actinobacteria.

Richness, diversity and abundance of 16S rRNA gene

The bacterial richness of the water samples from the three ponds was indicated by Ace index (Fig. 4a) and Chao index (Fig. 4b). There were no differences ($P > 0.05$) between different ponds for all of Ace and Chao indices, while the bacterial richness was affected by season changes ($P < 0.05$). The bacterial richness was higher during the peak period of breeding than that at the beginning of the culturing season, and the highest value appeared in September.

The bacterial diversity of the water samples from the three ponds was indicated by Shannon index (Fig. 4c) and Simpson index (Fig. 4d). The results showed that the value of Shannon index were significantly different between different seasons and different ponds ($P < 0.05$). The value of Shannon index was higher during the peak period of breeding than that at the beginning of the culturing season, which was also supported by the rarefaction analysis (Fig. 1). And the value was higher in pond 3 than that in pond 1 and pond 2. While there were no significant differences ($P > 0.05$) between different ponds for the value of Simpson index, although values of Simpson index in pond 2 and pond 3 were higher than that in pond 1. On the other hand, the value of Simpson index was also affected by season changes. With the development of the culture, the values decreased.

The dynamic changes of bacterial abundance were reflected by the real-time PCR results of the 16S rRNA gene (Fig. 3e). There were no significant differences ($P > 0.05$) between the gene copies from the three ponds, while the gene copies were affected by season changes ($P < 0.05$). The results showed that the lowest abundance of bacteria appeared in July, and the highest one appeared in October. In overall, the bacterial abundance was higher during the peak period of breeding than that at the beginning of the culturing season.

Similarity analysis of the water samples on the basis of bacterial compositions

Through the hierarchical cluster analysis, the similarity tree was obtained as Fig. 4a. The results showed that the bacterial communities in the water of three ponds collected in the same month were more similar to each other than that collected in the same pond. Further comparison of them between the 4 months showed that the bacterial

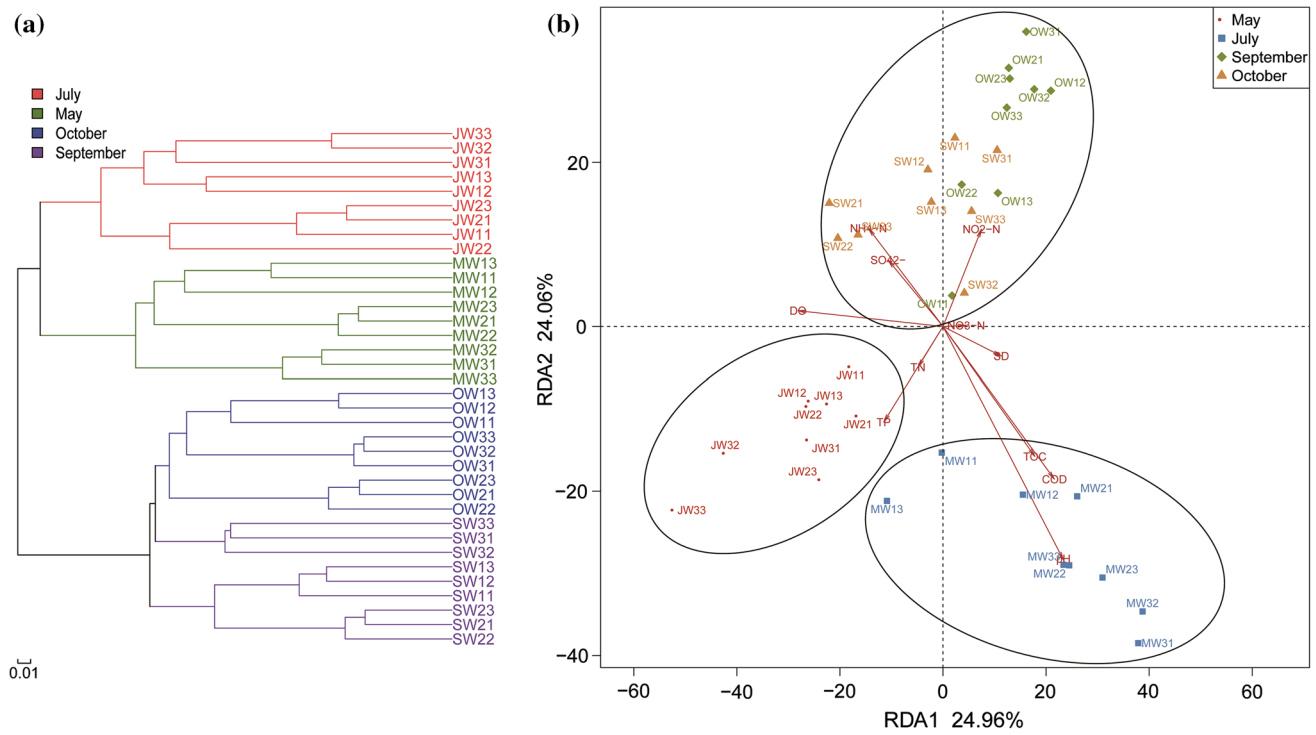


Fig. 4 Cluster analysis (**a**) and redundancy analysis (RDA) (**b**) of the bacterial 16S rRNA gene extracted from the three ponds. The samples collected in the same month were assigned to the *same color* groups both in (**a**) and (**b**). The *red arrow* showed the parameters of water quality

communities from the samples collected in May were similar to those in July, and the September microbes were similar to those in October.

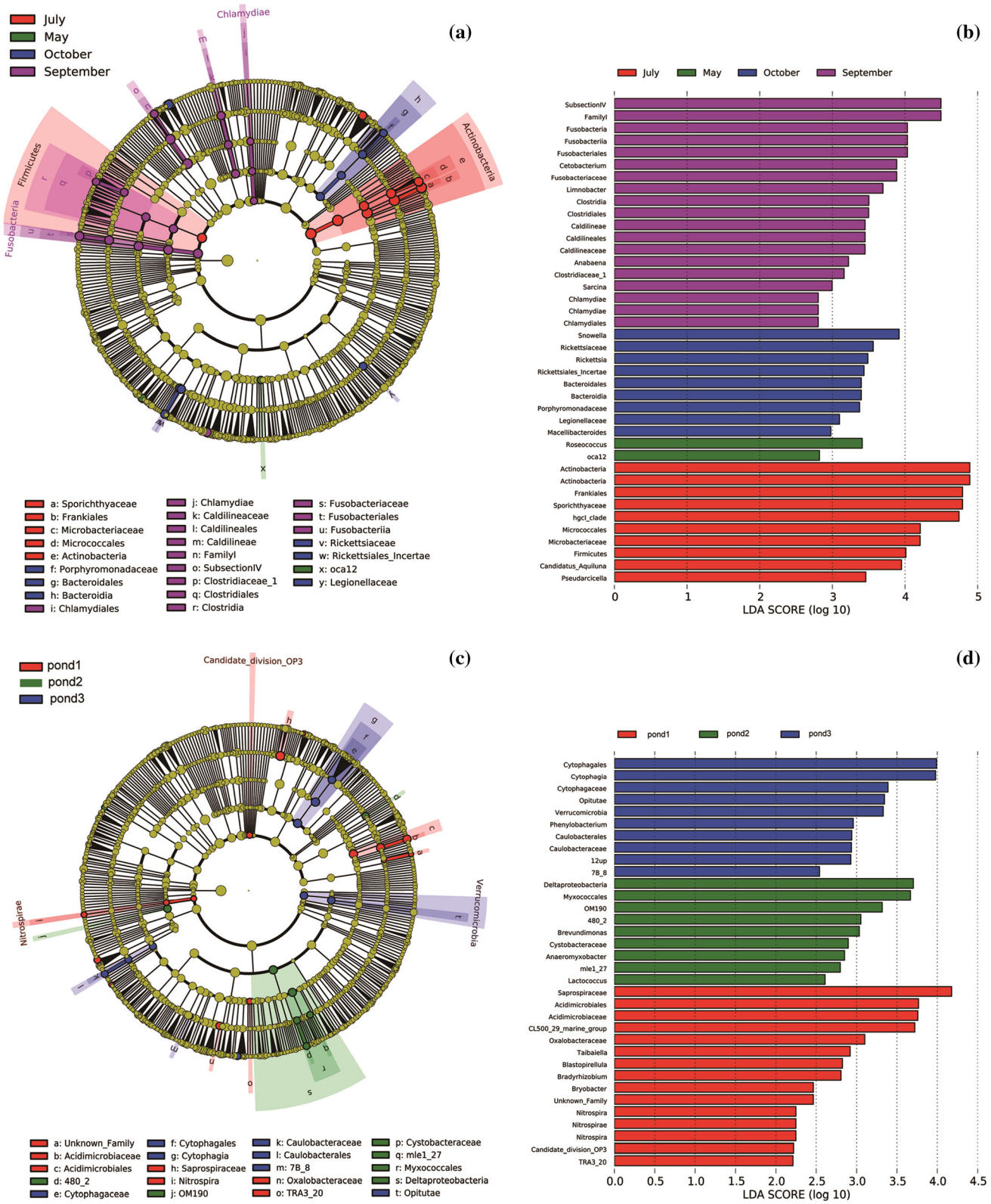
The sample grouping results showing more seasonal change effects than pond differentiation were also by redundancy analysis (RDA) (Fig. 4b), even though the stocking densities in the three ponds were different. Figure 4b also showed that the water samples collected in September and October were more similar than those of any other 2 months. The RDA also indicated that pH, TOC and COD were more associated with the bacterial community of pond water in May; the TN and TP were more associated with the bacterial community of pond water in July; ammonia nitrogen and sulfate were more associated with the bacterial community of pond water on September; and the nitrite nitrogen was more associated with the bacterial community of pond water in October.

Specific bacterial taxa in the different months and different ponds

The above studies showed that the bacterial communities in pond water were strongly affected by season changes. And differences of Shannon index values could also be detected. To identify the specific bacterial taxa associated with season and pond changes, we did the comparisons within the 4 months and within the three ponds using LEfSe. The cladograms representative of the structure of the microbiota on different months and in different ponds were shown in Fig. 5. The greatest differences in taxa between different months (Fig. 5a, b) and between different ponds (Fig. 5c, d) were displayed.

The results showed that the genus of *Roseococcus* and the order of *oca12* were the specific bacterial taxa in May; the phylum of Actinobacteria, the class of Actinobacteria, the order of *Frankiales*, the family of *Sporichthyaceae* and the genus of *hgcI_clade*, et al. were the specific bacterial taxa in July; the *family I* (phylum Cyanobacteria), the phylum of Fusobacteria, the class of Fusobacteriia, the order of *Fusobacteriales* and the genus of *Cetobacterium*, et al. were the specific bacterial taxa in September; the genus of *Snowella* and *Rickettsia*, the family of *Rickettsiaceae* and *Rickettsiales_Incertae*, and the order of *Bacteroidales*, et al. were the specific bacterial taxa in October.

Within the three ponds, the family of *Saprospiraceae*, *Acidimicrobiaceae* and *Oxalobacteraceae*, the order of *Acidimicrobiales* and the genus of *CL500_29_marine_group*, et al. were the specific bacterial taxa in pond 1; the class of Deltaproteobacteria and OM190, the genus of *Brevundimonas*, the order of *Myxococcales* and the family of *480_2*, et al. were the specific bacterial taxa in pond 2; the order of *Cytophagales*, the class of Cytophagia and Opitutae, the family of *Cytophagaceae*, and the phylum of Verrucomicrobia, et al.were the specific bacterial taxa in pond 3.


(a)
July
May
October
September
Chlamydiae
Actinobacteria
Firmicutes
Fusobacteria
a: Sporichthyaceae
b: Frankiales
c: Microbacteriaceae
d: Micrococcales
e: Actinobacteria
f: Porphyromonadaceae
g: Bacteroidales
h: Bacteroidia
i: Chlamydiales
j: Chlamydiae
k: Caldilineaceae
l: Caldilineales
m: Caldilineae
n: Familyl
o: SubsectionIV
p: Clostridiaceae_1
q: Clostridiales
r: Clostridia
s: Fusobacteriaceae
t: Fusobacteriales
u: Fusobacteriia
v: Rickettsiaceae
w: Rickettsiales_Incertae
x: oca12
y: Legionellaceae
(b)
July
May
October
September
SubsectionIV
Familyl
Fusobacteria
Fusobacteriia
Fusobacteriales
Cetobacterium
Fusobacteriaceae
Limnobacter
Clostridia
Clostridiales
Caldilineae
Caldilineales
Caldilineaceae
Anabaena
Clostridiaceae_1
Sarcina
Chlamydiae
Chlamydiae
Chlamydiales
Snowella
Rickettsiaceae
Rickettsia
Rickettsiales_Incertae
Bacteroidales
Bacteroidia
Porphyromonadaceae
Legionellaceae
Macellibacteroides
Roseococcus
oca12
Actinobacteria
Actinobacteria
Frankiales
Sporichthyaceae
hgcI_clade
Micrococcales
Microbacteriaceae
Firmicutes
Candidatus_Aquiluna
Pseudarcicella
0
1
2
3
4
5
LDA SCORE (log 10)
(c)
pond1
pond2
pond3
Candidate_division_OP3
Nitrospirae
Verrucomicrobia
a: Unknown_Family
b: Acidimicrobiaceae
c: Acidimicrobiales
d: 480_2
e: Cytophagaceae
f: Cytophagales
g: Cytophagia
h: Saprospiraceae
i: Nitrospira
j: OM190
k: Caulobacteraceae
l: Caulobacterales
m: 7B_8
n: Oxalobacteraceae
o: TRA3_20
p: Cystobacteraceae
q: mle1_27
r: Myxococcales
s: Deltaproteobacteria
t: Opitutae
(d)
pond1
pond2
pond3
Cytophagales
Cytophagia
Cytophagaceae
Opitutae
Verrucomicrobia
Phenylobacterium
Caulobacterales
Caulobacteraceae
12up
7B_8
Deltaproteobacteria
Myxococcales
OM190
480_2
Brevundimonas
Cystobacteraceae
Anaeromyxobacter
mle1_27
Lactococcus
Saprospiraceae
Acidimicrobiales
Acidimicrobiaceae
CL500_29_marine_group
Oxalobacteraceae
Taibaiella
Blastopirellula
Bradyrhizobium
Bryobacter
Unknown_Family
Nitrospira
Nitrospirae
Nitrospira
Candidate_division_OP3
TRA3_20
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
LDA SCORE (log 10)

◀**Fig. 5** LEfSe identified the most differentially abundant taxa between different months (**a**, **b**) and different ponds (**c**, **d**). In (**a**) and (**b**), *red*, *green*, *blue* and *purple* colors represented bacterial communities of July, May, October and September respectively, however in (**c**) and (**d**), *red*, *green* and *blue* colors represented bacterial communities of pond 1, pond 2 and pond 3 respectively. The *dots* on the two cladograms (**a**, **c**) representing the specific bacterial taxa whose *colors* were the same with that of the corresponding groups played important roles in that group. Only taxa meeting an LDA significant threshold >2 were shown on the *right side* of the figure (**b**, **d**)

Discussion

Information about bacterioplankton community responses to season changes and tilapia ponds differentiation with different stocking densities by Illumina high-throughput sequencing are described here within. The dominant bacteria at phylum level were similar, however their abundance were affected not only by season changes but also by ponds differences. As one of the two phyla that were more affected by season changes, Cyanobacteria showed the higher relative abundance in the early stage of the breeding period than that in the later stage, which might be attributed to the tilapia breeding. With the development of the culture, the larger amount of nutrient input could dramatically increase the abundance of heterotrophic bacteria, which might be the reason as why the relative abundance of Cyanobacteria decreased. However, within the three ponds, pond 3 with the highest stocking density showed lower absolute abundance of Cyanobacteria in the later stage of the breeding period. Combining the results that large amount of nutrient input and high temperature (Wang et al. 2015) can lead to the Cyanobacteria bloom. We could be able to formulate the hypothesis that the abundances of Cyanobacteria were affected by the seasonal variations, at the same time, that could probably be affected by the stocking density. The Nile tilapia feeding stuy, helping the removal of Cyanobacteria from the water column (Zeng et al. 2014) might support this hypothesis. As to the groups among the phylum, the top five dominant ones were belong to Family I, among which only two OTUs could be further classified as *Microcystis aeruginosa* and the genus of *Synechococcus* respectively. Still the pattern they changed was not consistent with that of phylum Cyanobacteria. Thus the mechanism that the culture of tilapia caused effects on Cyanobacteria needs further research. As another more-affected-by-season-changes phylum, Actinobacteria showed the highest abundance in July and the second abundance in May. The abundance of Actinobacteria dropping to a low level at the later stage of the culturing season which would mean, there were strong effects by increasing input of nutrient and high temperature. The results that there were no differences between their abundance under different stocking densities might support this assumption. And as one of the most abundant groups of freshwater bacterioplankton (Allgaier et al. 2007; Glockner et al. 2000; Warnecke et al. 2004), Actinobacteria were thought to be sensitive to conditions leading to Cyanobacterial blooms, usually linked to sustained or pulsed nutrient inputs particularly under high temperatures (Ghai et al. 2014).

Between the three ponds, the abundance of Cyanobacteria and Chlorobi were highest in pond 2, where the feed coefficient was the least. As two main types of photoautotrophic bacteria (Bryant and Frigaard 2006), Cyanobacteria and Chlorobi would not consume organic carbon for their growth. On the other hand, too much organic particles would prevent the light from penetrating the water. So that might be the way of these two phyla responding to the culture of tilapia. Although some species of Proteobacteria are also photoautotrophic (Bryant and Frigaard 2006), most members are heterotrophic, so less input of dissolved organic matter might be the cause that Proteobacteria showed the lowest abundance in pond 2. Taken together, the data about Cyanobacteria, Chlorobi and Proteobacteria suggested that the feed coefficient rather than the stocking densities might be the key factor affecting the microbial community in pond water.

Although there existed some abundance differences of Cyanobacteria, Chlorobi and Proteobacteria between the three ponds, the richness, diversity and abundance of the total bacterial community in pond water were only affected by season changes rather than stocking densities or feed coefficient. The results were also supported by the similarity analysis and redundancy analysis (RDA) of the water samples on the basis of bacterial compositions. In addition, the study on bacterioplankton in surface water of Igoumenitsa Gulf (Meziti et al. 2015) showed the similar results of the bacterialplankton community being more pronounced by temporal differences than by spatial ones. And the results that the water temperature and the sunlight made the temporal differences happen could also explain our results. The water temperature and the sunlight were the driving forces of making the bacterial communities in ponds different between months except that in May when no feed were used and the water depth were lower than that in other months. On the basis of that, some water parameters between different months also appeared significantly different. In May, the water depth was low, and the dissolved organic matter was still high after the operation of adding water, which released the organic matter in sediment into the water. That might be the reason that resulted in the different bacterial community in May. In July, the specification of the fish was still small, the plankton in water was also important source of food for them, and so in this period of time, the feed coefficient might be lower to

make the concentrations of total nitrogen and total phosphorus higher. However, the water temperature was still not so high that too much nutrient input could not make the microorganisms in water respond correspondingly. In September and October, too much bait and feces, high water temperature, together with some specific bacterial community, made the concentrations of dissolved inorganic nitrogen (ammonia nitrogen and nitrite nitrogen) higher.

The abundance of some members of bacterial community was specific in specific months or specific ponds, which might mean the seasonal or breeding characteristics in that season or that pond. For example, in July the phylum of Actinobacteria was specific. We have previously stated that Actinobacteria was sensitive to the conditions leading to Cyanobacterial blooms. Thus for the reasons that we stated previously, in this period of time, the environmental conditions were better, although the level of total nitrogen and total phosphorous were high. Meanwhile, the phylum of Fusobacteria that was commonly present in animal gastrointestinal tract (Gupta and Sethi 2014) was one of the specific members in September. This result suggested that in September the fish experienced high speed of food intake and excretion, which made the fast communications of bacterial communities happen between the gastrointestinal tract of fish and water. Meanwhile, some specific members of bacterial community in specific ponds were also notable. In pond 1 where the feed coefficient was the highest, the family of *Saprospiraceae*, with many members related to organic matter degradation (Schauer et al. 2006), appeared in higher abundances, suggesting the abundance of *Saprospiraceae* might be affected by the high feed coefficient. In pond 2 where the feed coefficient was lowest, the class of Deltaproteobacteria appeared in higher abundance than other two ponds, at the same time the class of Betaproteobacteria showed the lowest abundance. Generally, the class of Betaproteobacteria was thought to be one of the typical members in freshwater (Hahn 2006), and it was also one of the major groups contributed to bacterial biomass production in freshwater (Cottrell and Kirchman 2003). However, most groups of the class Deltaproteobacteria were anaerobic, they were proven to be the predominant microbe in such anaerobic conditions as deep layer of horizontal subsurface flow constructed wetland (Bouali et al. 2014), sediment of deep sea (Wang et al. 2010) and seagrass-dominated estuary (Guevara et al.2014), and so on. In this study, the orders of *Myxococcales* and *Desulfuromonadales* were two dominant groups among the class Deltaproteobacteria, that were also reported to be two dominant orders in oil-polluted subtidal sediments (Acosta-Gonzalez et al. 2013). So the group of Deltaproteobacteria in water might be from the pond sediment after the digging of tilapia, and it is possible that its relative abundance in water might be related to that in sediment. Overall, the reduction of Betaproteobacteria abundance together with the increase of Deltaproteobacteria abundance might have been the response to reduction of the feed coefficient.

In summary, we studied the bacterioplankton community in tilapia ponds under the effects of season changes and stocking densities. Our results showed that season changes had more effects on the bacterioplankton community than the stocking densities did. And even some differences between different ponds were more likely to be caused by feed coefficient than by stocking densities.

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