Short communication

# The application of bioflocs technology in high-intensive, zero exchange farming systems of *Marsupenaeus japonicus*

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## ARTICLE INFO

## ABSTRACT

A 106-day experiment was conducted to investigate the effectiveness of bioflocs technology for maintaining good water quality, supplying feed nutrition and inhibiting potential pathogen in high-intensive, zero exchange farming systems of *Marsupenaeus japonicus*. The experiment was progressed with 2 groups differentiated by bioflocs treatment and relative control in 6 indoor concrete ponds. Sucrose was added to the water of bioflocs treatment ponds based on the amount of daily feed. The monitoring indicated that ammonia-N and nitrite-N concentrations of water in the bioflocs treatment group were significantly lower than the relative control group ($P<0.05$). Comparing with the relative control group, the bioflocs treatment resulted in a 41.3% higher shrimp yield, 12.0% higher protein efficiency ratio, and 7.22% lower feed conversion rate. The production of *M. japonicus* reached to 1.3 kg m$^{-2}$ at a body length of 11.33 cm after 106-day farming in the bioflocs treatment group. The predominant microbe analyzed with denaturing gradient gel electrophoresis (DGGE) was characterized by *Bacillus* sp. in the bioflocs treatment group, but by *Vibrio* sp. in the relative control group. It was concluded that bioflocs technology performed well in high-intensive, zero exchange farming systems of *M. japonicus*, and the performance may be associated with microbial diversity.

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## 1. Introduction

The Kuruma shrimp, *Marsupenaeus* (*Penaeus*) *japonicus* (Bate, 1888), is indigenous to Pacific rim countries, together with Mediterranean, East Africa and Persian Gulf (Peˊrez Farfante and Kensley, 1997). It is one of the most valuable aquaculture species in many Asian countries (Chen, 1990). Farming of the shrimp *M. japonicus* is generally conducted extensively in grow-out ponds, and has been developed in an indoor high-intensive farming system to meet growing world demand (Lin et al., 2001; Zhou, 2001). With the rapid expansion and intensification, however, there is also a growing concern about the ecological sustainability of shrimp farming (Naylor et al., 2000). The cultured shrimp retain only 20–30% of feed nutrient, therefore, 70–80% of high dietary protein is excreted and accumulated in water, which leads to water deterioration finally (Avnimelech and Ritvo, 2003). Moreover, worsening of water quality has resulted in disease outbreaks and heavy financial losses (Samocha et al., 2004). Such environmental issues have created a large demand for

productive, efficient and sustainable shrimp farming systems that have low impact on the environment and are more likely to be free of disease (Horowitz and Horowitz, 2001).

One of the potential management measures to improve production and nutrients retention in shrimp farming systems is the application of bioflocs technology according to manipulating the carbon/nitrogen ratio (C/N ratio) (Avnimelech, 1999). If carbon and nitrogen are well balanced according to either the use of lower protein diet or supplying additional carbon sources, e.g. glucose, sucrose, and starch to the pond, the inorganic nitrogen components (ammonia, nitrite, and nitrate) in pond will be converted into bacterial biomass (Avnimelech, 1999; Crab et al., 2007; Hargreaves, 2006). As such, nutrients from excretion and remnant feed are recycled into bacterial biomass and formed bioflocs which can be taken up as an additional feed for aquatic animals (Avnimelech, 2006). In addition to water quality control and *in situ* feed production, bioflocs technology has protected brine shrimp (*Artemia franciscana*) larvae from vibriosis. (Crab et al., 2010a).

Bioflocs technology have been applied and developed in high-intensive farming systems of several shrimp species, such as *Penaeus monodon*, *Litopenaeus vannamei*, and *Macrobrachium rosenbergii* farming (Burford et al., 2003; Crab et al., 2010b; Hari et al., 2006). However, due to the specific hiding sand behaviour of *M. japonicus*

by daylight, little is known about the practicability of biofloc technology for the intensive farming of the shrimp *M. japonicus*. Moreover, few investigations were shown about the microbial diversity in bioflocs technology ponds.

We conducted an intensive farming trial for *M. japonicus* through bioflocs technology. The characters of water quality and growth performance of intensive *M. japonicus* farming in bioflocs treatment groups were investigated. In addition, our study also evaluated the microbial diversity and obtained the predominant microbes from microcosmic view, which may help to explore the potential mechanism of bioflocs technology.

## 2. Materials and methods

### 2.1. Experimental design and facilities

An on-station trial was conducted with a $2 \times 3$ factorial design through sucrose addition. The group without sucrose addition was referred to as relative control, and the group with sucrose addition was referred to as bioflocs treatment. Both groups were executed in triplicate and assigned randomly.

The experiment was carried out in 6 square concrete ponds with 1 m depth and 30 m$^2$ area each, and shrimps were farmed in Baorong Aquaculture Station (Qingdao, China) from June 30th till October 13th, 2009. The ponds were built in a 60 m $\times$ 11 m dark workshop which was closed with 5 cm thick polystyrene foam boards under the plastic film roof with a closable skylight above each of the ponds. Eight airstones were arranged at the bottom of each pond and other 8 aerators inside of upward flow tube were installed circumjacent of each pond to keep fine particles in suspension. A layer of 8 cm thick sand was spread on the bottom of each pond to provide hiding place for *M. japonicus*. All of the ponds were filled with water from impounding reservoir.

### 2.2. Shrimp stocking and pond management

Postlarvae of *M. japonicus* ($0.09 \pm 0.005$) g ($X \pm SE$) purchased from Huida Aquaculture Station (Tangshan, China) were stocked in the ponds on June 30th at a density of about 175 postlarvae m$^{-2}$. Pellet feed (Tongyi, Taiwan) containing 42% protein with C/N ratio about 7.8 and fresh feed (*Aloidis laevis*, Qingdao) containing 78.8% protein (dry weight) with C/N ratio about 4.0 were applied. The daily feeding rate was 5% body weight at the start of experiment, and declined gradually to 3% body weight at the end of the farming period with assuming 80% survival of total stock in each pond. Feed was distributed evenly over the ponds every forth day at 4:00 am, 9:00 am, 5:00 pm, and 10:00 pm. Weights of 20 shrimps were measured individually in every month to adjust the feeding rate. Sucrose was used as carbohydrate source for manipulating the C/N ratio. In order to raise the C/N ratio, 0.5 kg (2 kg) sucrose was applied for each kg of pellet (fresh) feed in bioflocs treatment ponds (Avnimelech, 1999). The preweighted sucrose was mixed in a beaker with pond water and uniformly distributed over the ponds directly after the feed application. Probiotics containing *Bacillus subtilis*, *pseudomonad, nitrobacteria*, and *denitrifying bacterium* (Total bacterial account $= 10^9$ CFU g$^{-1}$, provided by South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences) were dosed into all the experimental ponds at the rate of 100 g m$^{-3}$ 7-day before stocking and 10 g m$^{-3}$ on 29th, 43rd, and 85th day post stocking. From 11:00 am to 3:00 pm, the skylights were opened to increase the illumination intensity to 9000–10 000 lx, during the remaining time of the day, the skylights were closed to decrease the illumination intensity below 5–10 lx. During the farming period, there was no water exchange among all the experimental ponds.

### 2.3. Determination of floc volume and morphostructure

Floc volume was determined by sampling 1000 mL pond water into a series of Imhoff cones (1000-0010, Nalgene) at 10:00 am every 7-day post stocking. The volume of the floc plug accumulating on the bottom of the cone was determined 15 min following sampling. Thereafter, floc plug was collected from the turn-knob at the bottom tip of the cone, and the floc morphostructure was observed by biologic microscope (DH-2, Olympus) and photoed with a microimaging system (LY-WN-HPCCD, Liyang).

### 2.4. Assessment of water quality parameters

Water samples were collected using a horizontal water sampler from each pond and pooled before analysis. Water quality parameters, such as temperature, dissolved oxygen, pH, and salinity were monitored twice daily with a multiparameter water quality instrument (556MPS, YSI) *in situ* at 6:00 am and 6:00 pm, separately. Water samples were collected at 10:00 am every 7-day. Before biochemical analysis, water samples were filtered through microfibre glass filterpaper (GF/C, Whatman), using a vacuum pressure air pump. Ammonia-N, nitrite-N and nitrate-N concentrations in the filtrate were measured by a spectrophotometer (V5000, Metash) using the method of Mudroch et al. (1996).

### 2.5. Shrimp harvesting and estimation of yield parameters

Shrimps were harvested on October 13th after 106-day experiment by draining the ponds. Individual length and weight were measured by vernier caliper (500-197-20, Mitutoyo) and electronic balance (YP/JY, Yueping) respectively. Specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and net yields were calculated as follows:

$$SGR = [\ln(finalweight) - \ln(initialweight)] \times 100/(daysofexperiment)$$

$$FCR = (feedconsumed, dry)/(liveweightgain, wet)$$

$$PER = (liveweightgain)/(proteinconsumed)$$

### 2.6. Statistical analysis of apparent parameters

Growth and yield parameters (growth, yield, FCR, SGR, and survival) were analyzed by a one-way ANOVA (analysis of variance) using carbon addition as factor. Water quality parameters (temperature, dissolved oxygen, pH, salinity, ammonia-N, nitrite-N, and nitrate-N concentrations) were compared by repeated measures ANOVA using carbon addition as factor. The assumptions of normal distributions and homogeneity of variances were checked before analysis. The percentage and ratio data were analyzed using arcsine-transformed data. All ANOVA were tested at 5% level of significance using SPSS (Statistical Package for Social Science) version 14.

### 2.7. Sample preparation and denaturing gradient gel electrophoresis (DGGE)

Water samples for DNA extraction were taken from all experimental ponds on the 105th day post stocking and sent to the laboratory in Yellow Sea Fisheries Research Institute. Water samples were taken from 50 mL of pond water at 10:00 am, and biomass was collected by centrifugation (10 min, 8000 rpm). Total DNA was extracted from centrifugation using a soil DNA extraction kit (E.Z.N.A.[R], Omega). To obtain DNA for further analysis of the total bacterial community by DGGE, a single round PCR was performed with Premix Ex Taq (DRR001A, TaKaRa) which contained 2$\times$ Ex Taq buffer with 4 mmol L$^{-1}$ MgCl$_2$,
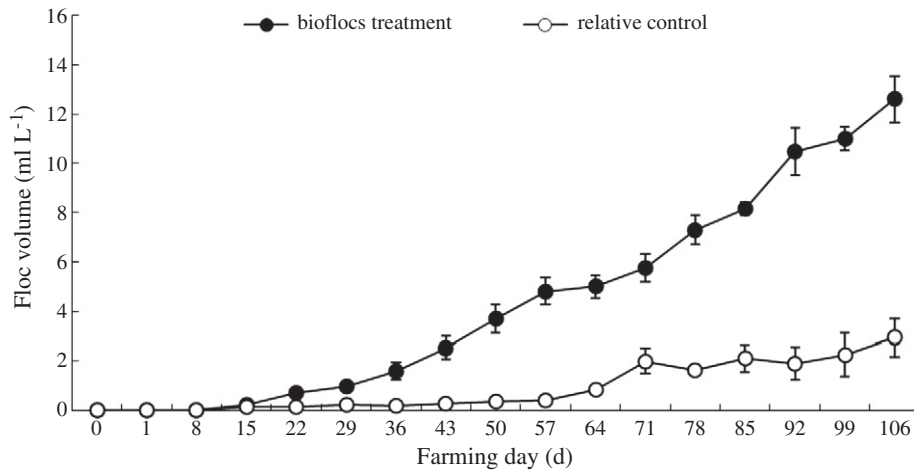
**Fig. 1.** Dynamic changes of floc volume in different groups throughout the experimental period. Values are means (± standard deviation) of three replications in each sampling date respectively for the bioflocs treatment and relative control groups.

0.4 mmol L$^{-1}$ each of deoxynucleoside triphosphate, 0.05 U/μL$^{-1}$ of Ex Taq DNA polymerase. The primers V338 and V534 (Muyzer et al., 1993) were used in this study for amplifying of the 16S rDNA genetic amplicon of all bacteria. A 1 μL DNA template was added to 24 μL master mixture

(12.5 μL Premix Ex Taq, 10 pmol primer V338 and V534 each) followed by the PCR with the program: an initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min; and a final extension at 72 °C for 10 min. After the PCR, the size of the amplicon was verified by electrophoresis on a 1.5% agarose gel with low range DNA Marker (D501A, TaKaRa).

Denaturing gradient gel electrophoresis (DGGE) based on the protocol of Muyzer et al. (1993) was performed using the Bio-Rad Dcode system (Decode™, Bio-Rad). PCR fragments were loaded onto an 8% (w/v, prokaryotic) polyacrylamide gel in 1× TAE (20 mmol L$^{-1}$ Tris, 10 mmol L$^{-1}$ acetate, 0.5 mmol L$^{-1}$ EDTA, pH 7.4). To separate the amplified DNA fragments, the polyacrylamide gel was made with denaturing gradients ranging from 30% to 60% (100% denaturing contains 7 mol L$^{-1}$ urea and 40% formamide). The electrophoresis was run for 16 h at 60 °C and 100 V. Staining of the gel was performed as described previously (Boon et al., 2000). DGGE banding patterns were assessed by cluster analysis with a percent agreement coefficient, and the similarities among the profiles of AMF communities were depicted as a dendrogram constructed by the unweighted pair group with mathematical average method (UPGMA) using the software Quantity One.
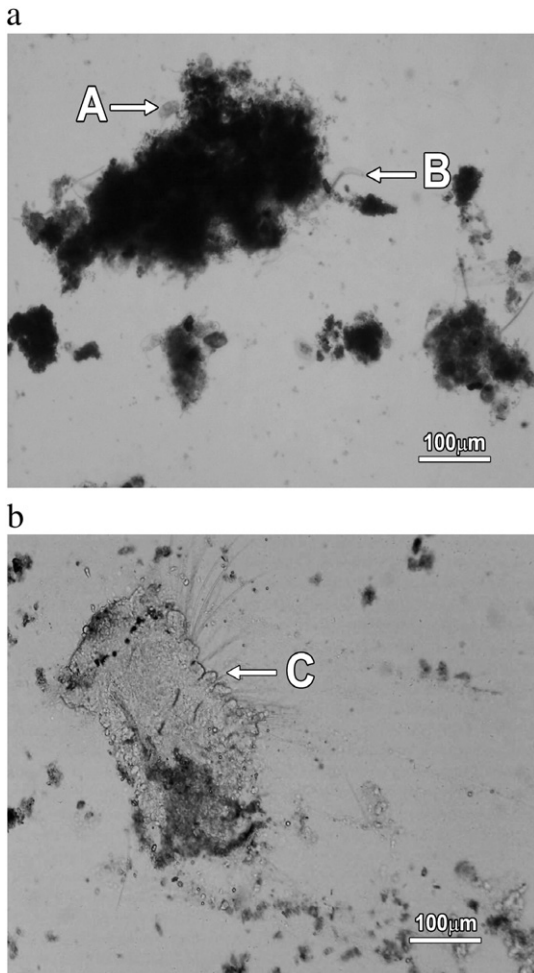


**Fig. 2.** Morphology of floc under microscope in different groups. (a) bioflocs treatment; (b) relative control. A) *Eucalanus subcrassu*; B) *Limnodrilus* sp.; C) partial sloughs.

**Table 1**
Effects of sucrose addition on growth and yield parameters of shrimps based on one-way ANOVA. Values are means (± standard deviation) of three replications respectively in the bioflocs treatment and relative control groups. The superscript letters following each value indicate significant difference at 0.05. If the effects were significant, ANOVA was followed by Turkey test. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; NS: not significant.

| Growth and yield parameters | Bioflocs treatment | Relative control | ANOVA significance |
|---|---|---|---|
| Individual stocking weight (g) | 0.09 ± 0.005 | 0.09 ± 0.005 | NS |
| Individual harvesting weight (g) | 11.33 ± 0.02[a] | 9.98 ± 0.05[b] | ** |
| Individual weight gain (g) | 11.24 ± 0.2[a] | 9.89 ± 0.12[b] | ** |
| Specific growth rate (%bw d$^{-1}$) | 4.56 ± 0.2[a] | 4.42 ± 0.33[b] | * |
| Feed conversion rate (FCR) | 1.67 ± 0.11[b] | 1.8 ± 0.17[a] | * |
| Protein efficiency ratio (PER) | 1.42 ± 0.13[a] | 1.25 ± 0.1[b] | ** |
| Survival (%) | 65.7 ± 4.6[a] | 52.3 ± 6.1[b] | *** |
| Net yield (kg m$^{-2}$ · 106 d$^{-1}$) | 1.3 ± 0.2[a] | 0.92 ± 0.1[b] | *** |

**Table 2**
Statistics of daily water quality data in different groups throughout the experimental period. Values are means (±standard deviation) of three replications in each sampling time everyday respectively in the bioflocs treatment and relative control groups.

| RW | Temperature (°C) | Salinity (ppt) | DO (mg·L$^{-1}$) | pH |
|---|---|---|---|---|
| *6:00 am* | | | | |
| Bioflocs treatment | 26.2 ± 1.3 | 22.7 ± 0.8 | 5.6 ± 0.5 | 7.8 ± 0.3 |
| Relative control | 26.4 ± 1.6 | 22.9 ± 1.2 | 6.2 ± 1.2 | 8.3 ± 0.2 |
| | | | | |
| *6:00 pm* | | | | |
| Bioflocs treatment | 32.7 ± 1.5 | 23.2 ± 0.6 | 4.0 ± 0.8 | 7.8 ± 0.2 |
| Relative control | 33.1 ± 1.2 | 23.4 ± 1.1 | 4.9 ± 1.3 | 8.4 ± 0.1 |

### 2.8. Sequencing and phylogenetic analysis

Stable single bands in DGGE gel, verified by DGGE three times to ensure a single band at the same location, were labeled with S1–S20 (bioflocs treatment) and C1–C18 (relative control). The bands were excised from gel and eluted in 30 μL of TE buffer (10 mmol L$^{-1}$ Tris and 1 mmol L$^{-1}$ EDTA, pH8.0). The supernatant after centrifugation (12 000 rpm, 5 min, 4 °C) was used as a template for 16S rDNA-V3 sequence amplification using the same primer pairs without GC-clamp. The templates in 2 μL supernatants were amplified in 50 μL reaction mixtures with same PCR protocol mentioned in 2.7. PCR products were analyzed by electrophoresis in 1.5% agarose gels and DGGE gel.

The amplified 16S rDNA-V3 segment was cloned into pMD18-T Vector (D101A, TaKaRa) after being purified with a PCR purification kit (DV807A, TaKaRa). The positive recombinants were screened on X-Gal (5-bromo-4-chloro-3-indolyl-h-d-galactopyranoside)-IPTG (iso-propyl-h-d-thiogalactopyranoside)-ampicillin–tetracycline indicator plates by color-based recombinant selection. Positive clones were identified by PCR amplification with pMD18-T Vector primer pairs, using the same program as 16S rDNA-V3 amplification. PCR products were affirmed by electrophoresis in 1.5% agarose gels to confirm its amplicons of 16S rDNA-V3 and pMD18-T Vector. Positive recombinants were then submitted for sequencing using a DNA Sequencer (3730, ABI) with T7 primer in Sunny Biotechnology Company, China. Sequences were compared to those in the GenBank database by BLAST algorithm to identify sequences with a high degree of similarity.

### 2.9. Intensity analysis of DGGE profile

Each lane of the DGGE profile was digitized to intensity (8 bit gray) − mobility profile with SigmaScan software. The gray value of the intensity in each lane was converted to optical density by the formula: OD = −lg(gray/256). The OD − mobility profile of each lane

was disassembled into Gaussian narrow band peaks and wide background peaks with the PeakFit 4.0 software. The intensity percentage of a specific band was calculated with the percentage of the specific band peak area within the sum of all band peaks area in the lane.

## 3. Results

### 3.1. Shrimp performance and floc investigation

In the beginning of the farming period (1–35 d), shrimp were fed with pellet feed according to the feeding scale described in Section 2.2. There were always many remanent feed that settled to the bottom of the pond after feeding and only a few shrimps were observed to eat in relative control group. However, few remanent feed settled to the bottom of the pond after feeding and many shrimps were observed to eat in the bioflocs treatment group. Except for feeding time, shrimps generally hid in sand by daylight and infrequently moved at night. In the middle of the farming period (36–71 d), fresh feed was substituted for pellet feed. Few remanent feed settled to the bottom of the pond and many shrimps were observed to eat in both groups. Except for feeding time, shrimps in both groups moved frequently at night and shrimps in the bioflocs treatment group even moved by daylight. In the late of the farming period (72–106 d), shrimps were fed with fresh feed continuously and moved more frequently. However, on the 80th day post stocking, many shrimps were found died in the drain of the ponds, therefore, pollution discharged intensively in both groups.

Water transparency of the bioflocs treatment group began to reduce on the 10th day post stocking, then the transparency reduced gradually to 5 cm on the 105th day post stocking and the color was identified to be tan. However, water transparency of the relative control group kept at a high level of about 40 cm during the beginning and middle of the farming period, then the transparency reduced gradually to 20 cm on the 105th day post stocking and the color was identified to be pale yellow. The average volume of floc that settled from the water samples of the 3 replicated ponds in the Imhoff cones as a function of farming days was shown in Fig. 1. The floc volume in the bioflocs treatment group increased gradually to the top (11.6 ml L$^{-1}$) along with sucrose addition. On the contrary, the value in the relative control group kept at low levels (0–1.9 ml L$^{-1}$). The structure of floc was observed under a microscope with 10× objective. In the bioflocs treatment group, floc was shown in anomalous flocculation with *Eucalanus subcrassus* and *Limnodrilus* sp. shuttling in it (Fig. 2a). However, in the relative control group, there were a few of detritus and sloughs in the sediment of the Imhoff cone (Fig. 2b).
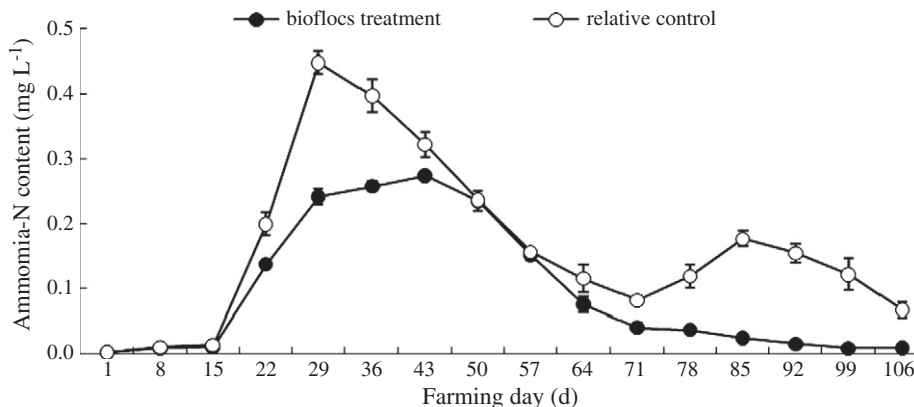


**Fig. 3.** Dynamic changes of ammonia-N concentrations in different groups throughout the experimental period. Values are means (±standard deviation) of three replications in each sampling date respectively for the bioflocs treatment and relative control groups.

**Fig. 4.** Dynamic changes of nitrite-N concentrations in different groups throughout the experimental period. Values are means (± standard deviation) of three replications in each sampling date respectively for the bioflocs treatment and relative control groups.
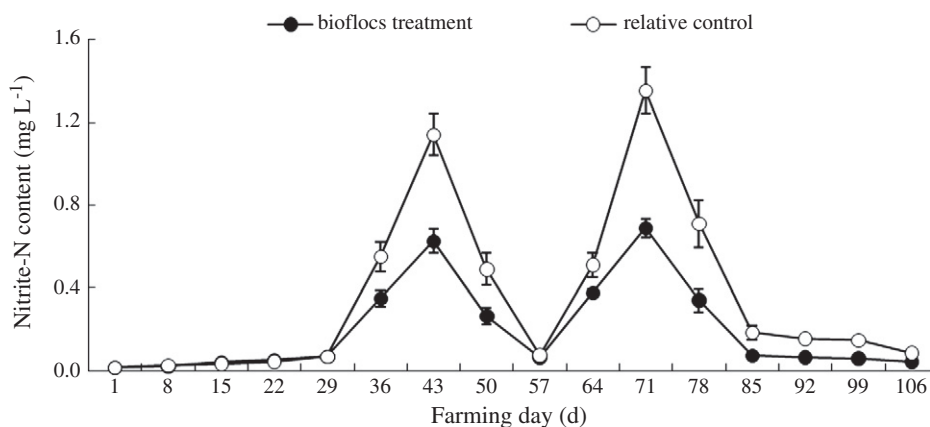
### 3.2. Shrimp growth and yield parameters

After the 106-d experiment, the shrimp growth and yield parameters in both groups were summarized by a one-way ANOVA (analysis of variance) using carbon addition as factor (Table 1). The ANOVA results showed that bioflocs technology significantly increased the individual shrimp weight at harvest ($P<0.01$). The SGR value of the bioflocs treatment group showed higher than that of the relative control group ($P<0.05$). Bioflocs technology had a significant effect on the protein efficiency ratio ($P<0.01$). The FCR value of the bioflocs treatment group was lower than that of the relative control group ($P<0.05$). Comparing with the relative control group, the bioflocs treatment group resulted in a 41.3% higher shrimp yield, 12.0% higher protein efficiency ratio, and 7.22% lower feed conversion rate. The ANOVA result showed that bioflocs technology significantly increased the shrimp survival from 52.3 to 65.7% ($P<0.001$) and the net yield from 0.92 to 1.3 kg m$^{-2}$ ($P<0.001$).

### 3.3. Daily changes of the water quality parameters

Daily changes of water quality parameters were presented in Table 2. There were no significant differences in temperature (26.2–33.1 °C) and salinity (22.7–23.4 ppt) among the different groups both in the morning and evening ($P>0.05$). Dissolved oxygen (DO) varied either from the different groups ($P<0.05$) or from the detection time ($P<0.01$). Sucrose addition significantly reduced pH from 8.4 in the relative control group to 7.8 in the bioflocs treatment group ($P<0.05$).

### 3.4. Inorganic nitrogen dynamics

Temporal variations in ammonia-N, nitrite-N, nitrate-N and total nitrogen concentration among the 106-d farming period in both groups were shown in Figs. 3–6. The ammonia-N concentration was significantly reduced through bioflocs technology ($P<0.05$). On the 29th day post stocking, the ammonia-N concentration peaked at 0.449 mg L$^{-1}$ in the relative control group, but only 0.242 mg L$^{-1}$ in the bioflocs treatment group (Fig. 3). Concurrent with the decrease of the ammonia-N concentration, the nitrite-N concentration showed two definite peaks in both groups during the 29th to 71th farming period, the fluctuation of the nitrite-N concentration in the bioflocs treatment group was significantly reduced by sucrose addition ($P<0.05$), but both treatments peaked at the same farming day (Fig. 4). There were no significant differences in the nitrate-N concentration during the whole farming period in both groups ($P>0.05$). The mean concentration of Nitrate-N in both treatments fluctuated between 1.44 and 7.52 mg L$^{-1}$ (Fig. 5). The fluctuation of the total nitrogen concentration was similar as the nitrate-N concentration ($P>0.05$), therefore, the total nitrogen concentration was mostly influenced by the nitrate-N concentration but not the ammonia-N or nitrite-N concentration (Figs. 3–6).

During the 29th to 43rd farming period, the ammonia-N concentration was linearly decreased in the relative control group while the nitrite-N concentration was linearly increased, which may indicate nitrification of ammonia to nitrite by probiotics addition on the 29th day post stocking, although the nitrite-N concentration was
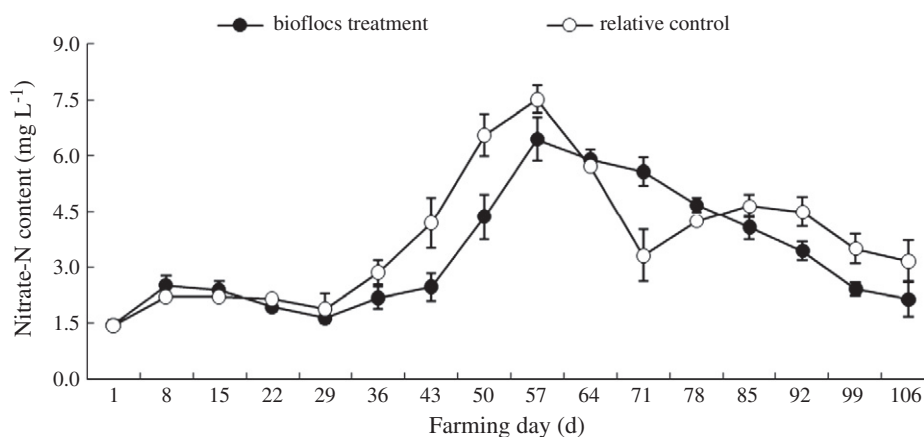


**Fig. 5.** Dynamic changes of nitrate-N concentrations in different groups throughout the experimental period. Values are means (± standard deviation) of three replications in each sampling date respectively for the bioflocs treatment and relative control groups.

**Fig. 6.** Dynamic changes of total nitrogen concentrations in different groups throughout the experimental period. Values are means (± standard deviation) of three replications in each sampling date respectively for the bioflocs treatment and relative control groups.
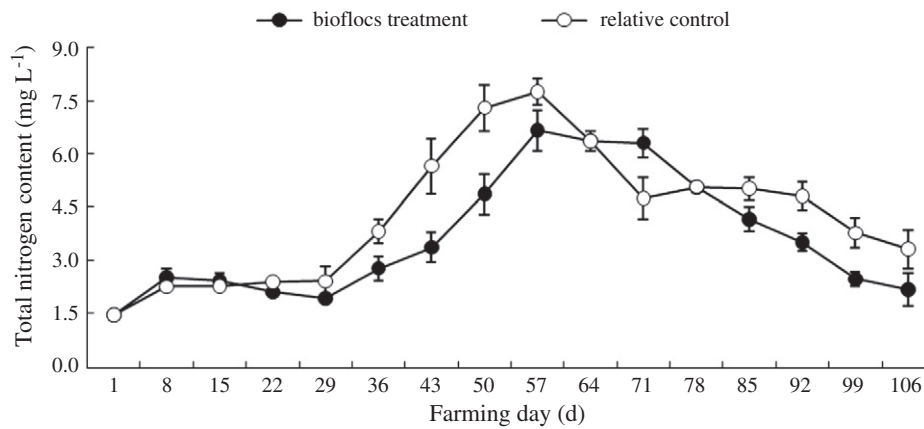
linearly increased in the bioflocs treatment group, the ammonia-N concentration was tardily increased, which may indicate partial bioflocs in the bioflocs treatment group were fading and releasing ammonia (Figs. 3 and 4). During the 43rd to 57th farming period, the nitrite-N concentration linearly decreased in both groups while the nitrate-N concentration linearly increased, which may indicate the oxidation of nitrite to nitrate through probiotics addition (Figs. 4 and 5). During the 57th to 71st farming period, the ammonia-N concentration continuously decreased, and the nitrite-N concentration repeatedly peaked on the 71st day post stocking, which may indicate the second nitrification of ammonia to nitrite (Fig. 3 and 4). During the 71st to 85th farming period, the ammonia-N concentration increased gradually in the relative control group but decreased continuously in the bioflocs treatment group most probably due to the dead shrimp in the relative control group, concurrent with the decreased nitrite-N concentration in both groups, the nitrate-N concentration was increased in the relative control group but continuously nitrification in the bioflocs treatment group, which may indicate the second oxidation of nitrite to nitrate in relative control but continuously nitrification in bioflocs treatment (Figs. 3–5). After intensively pollution discharge and probiotics addition, the ammonia-N, nitrite-N and nitrate-N concentrations all decreased gradually during the 85th to 106th farming period (Figs. 3–5).

### 3.5. DGGE profile and clustering analysis

The prokaryotic microbial communities in both bioflocs treatment and relative control groups were analyzed with DGGE of the PCR amplified 16S rDNA-V3 fragments (Fig. 7a). There were about 20 different main bands in the bioflocs treatment group and 18 main bands in the bioflocs treatment group and 18 main bands in the relative control group. The DGGE profiles of the bioflocs treatment group were characterized by a group of bands with slower mobility. Clustering analysis based on the values of Dice coefficients was visualized in an UPGMA dendrogram to study general patterns of community similarity among the six samples (Fig. 7b). Both the bioflocs treatment and relative control groups were clustered in two groups at a genetic similarity of 0.40. In the bioflocs treatment group, SII and SIII were clustered at a similarity of 0.58 and they both have the similarity of 0.47 with SI. In the relative control, CII and CIII were clustered at a similarity of 0.64 and they both have the similarity of 0.59 with CI.

### 3.6. BLAST and microbe identification

The DGGE bands were re-amplified and cloned into pMD-18T vector for sequencing. BLAST was used to analyze the sequence similarity with the GenBank for identification of the possible species of prokaryotic microbes originating the specific DGGE bands (Table 3). In the bioflocs treatment group, the bands 3, 4, 5, 13, and 19 had the highest homology to *Bacillus* sp. with 95 to 100% similarity; meanwhile, the bands 6, 7, 8, 9, 10, 14, 15, 16, 17, and 18 had the highest homology to Proteobacterium with 94 to 100% similarity. Besides, *Roseobacter* sp. and *Cytophaga* sp. represented bands 1 and 20 separately were detected in bioflocs treatment. In the relative control group, the bands 7, 9, 10, 11, 12, and 13 displayed the highest sequence homology to *Vibrio* sp. with 92 to 100% similarity, meanwhile, the bands 15, 17, and 18 showed the highest sequence homology to *Pseudoalteromonas* sp. with 93 to 95% similarity. All of the bacterial isolates (2, 3, 4 and 6) in the relative control group showed sequence similarity to Proteobacterium which were also represented in bioflocs treatment. Besides, *Cytophaga* sp. *Photobacterium* sp., *Paracoccus homiensis* and *Halomonas* sp. were observed in the relative control group.

### 3.7. Predominant analysis

The intensities of bands in the DGGE profiles (Fig. 7) reflected the template concentrations of the 16S rDNA-V3 fragments which directly related to the amount of specific bacterial cells. The intensity percentage of each band was calculated within each lane from the band peak area. The predominance of a specific bacterial group was summed with the intensity percentage of the band with same identity by the BLAST results (Table 3). The predominant rates of the 3 lanes of bioflocs treatment group and the 3 lanes of relative control group were statistically averaged respectively (Fig. 8). Both groups shared a large portion of Proteobacterium analogues, which were close to 5 Proteobacterium members, including Proteobacterium M3-2, α-Proteobacterium CLCM, α-Proteobacterium RS.Sph.017, α-Proteobacterium clone SHBC432, and ε-Proteobacterium clone F-a11. The predominant order of Proteobacterium analogues in the bioflocs treatment group was α-Proteobacterium clone SHBC432 (16.88% ± 11.24%), Proteobacterium M3-2 (13.24% ± 1.39%), ε-Proteobacterium clone F-a11 (10.15% ± 8.01%), α-Proteobacterium RS.Sph.017 (2.74% ± 0.73%), and α-Proteobacterium CLCM (0.35% ± 0.61%); while there were only 2 Proteobacterium analogues in the relative control group, which were Proteobacterium M3-2 (23.36% ± 3.14%) and α-Proteobacterium clone SHBC432 (5.80% ± 1.83%). Proteobacterium M3-2 was the most stable predominant analogue in both of the bioflocs treatment (13.24% ± 1.39%) and the relative control (23.36% ± 3.14%) groups. As a result of bioflocs treatment measure with sucrose addition, together with probiotics addition aperiodically, *Bacillus* sp. became the predominant bacteria in the bioflocs treatment group (27.71% ± 2.83%), while this bacterium was not
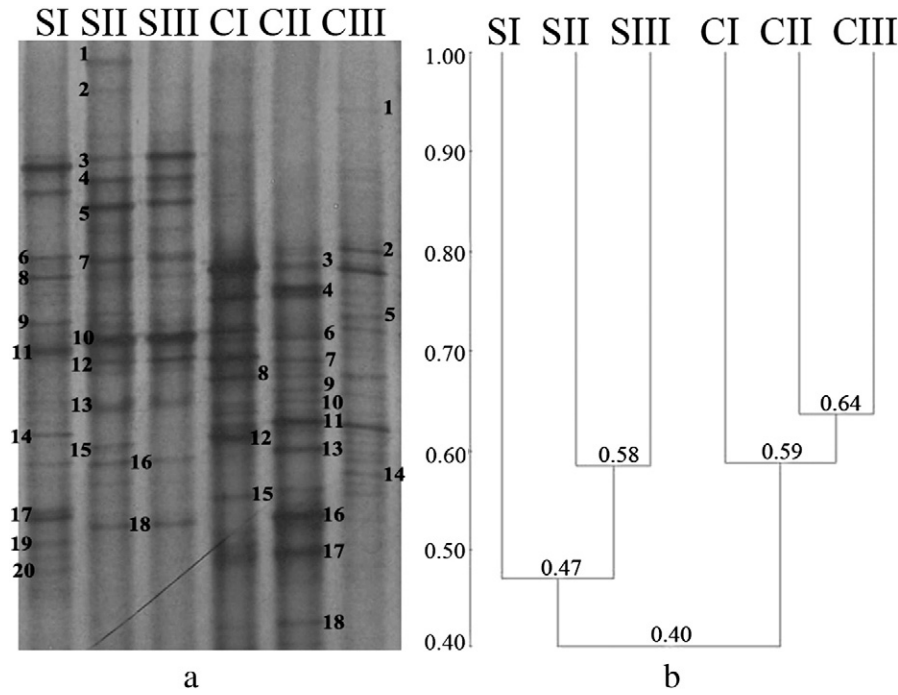
**Fig. 7.** DGGE profile and its clustering analysis of amplified 16S rRNA-V3 gene fragments of bacterial communities. (a) DGGE profile; (b) clustering analysis of DGGE profile; Lane SI, SII, and SIII, bioflocs treatment ponds; Lane CI, CII, and CIII, relative control ponds.

detectable in the relative control group. Instead, *Vibrio* sp. was the predominant bacteria in the relative control group ($22.65\% \pm 4.49\%$). Actinobacterium BGR 105 analogue was detected in the bioflocs treatment group ($8.16\% \pm 4.02\%$), while the alternative species in the relative control group was *Pseudoalteromonas* sp. ($11.02\% \pm 6.22\%$). There were some other minor bacteria species, including *Paracoccus homiensis* analogue ($5.45\% \pm 2.57\%$), *Photobacterium ganghwense* analogue ($1.53\% \pm 2.64\%$), and *Halomonas* sp. ($1.45\% \pm 2.51\%$) were detected in the relative control group; *Roseobacter* sp. ($1.53\% \pm 2.66\%$) in the bioflocs treatment group; and *Cytophaga* sp. in both bioflocs treatment ($0.97\% \pm 3.90\%$) and relative control ($1.67\% \pm 5.64\%$) groups.

## 4. Discussion

Farming of the shrimp *M. japonicus* is generally conducted extensively in grow-out ponds, shrimp yield was mostly of 20–30 kg mu$^{-1}$ (0.030–0.045 kg m$^{-2}$) and occasionally of 50 kg mu$^{-1}$ (0.075 kg m$^{-2}$) (Mu et al., 2008). Zhou, et al. (2008) farmed the shrimp *M. japonicus* (initial body length: 0.8 cm) in 300 m$^2$ vinyl tunnel with the stocking density of 120 PL m$^{-2}$, and the shrimp production reached to 0.55 kg m$^{-2}$ after 132-d farming period. Before this research, Lin, et al. (2001) had farmed the shrimp *M. japonicus* (initial body length: 1.6 cm) in 21 m$^2$ idle shrimp breeding pond with the stocking density of 294 PL m$^{-2}$, and the shrimp production reached to 0.62–1.0 kg m$^{-2}$ after 87-d farming period, however, the experiment made great efforts on water exchange and disinfect method such as antibacterials and probiotics, which ran up cost immensely. In our study, shrimp *M. japonicus* was farmed in 30 m$^2$ industrialized concrete ponds with the stocking density of 175 PL m$^{-2}$ by no water exchange, and the shrimp production reached to 1.3 kg m$^{-2}$ after 106-d farming period through bioflocs technology. The benefits of bioflocs technology were discussed following including water quality control, feed nutrition substitute, and potential pathogen inhibition.

### 4.1. Water quality control

Concentrations of dissolved inorganic nitrogen in the water of intensive shrimp ponds were all considerably higher than those in conventional shrimp ponds, characterized by lower stocking densities and periodic water exchange (Avnimelech et al., 2008). Li and Lovell (1992) reported that the ammonia-N concentration increased with increasing dietary protein concentration and protein feeding rate. In our study, shrimp *M. japonicus* was farmed with higher protein diet and higher feeding rate in high-intensive systems by no water exchange, so the ammonia-N concentration in water was shown obviously higher in the relative control group. Probiotics can help improve the water quality in aquaculture ponds (Moriarty, 1997). This is due to the ability of the probiotic bacteria to participate in the absorption of organic nutrients in the ponds (Weber et al., 1994). In our study, probiotics were added aperiodically in both groups post stocking, and the nitrification process was advanced to 29 days, which is slightly shorter than 31 days needed to establish the nitrification process in *P. monodon* concrete culture tanks by Hari et al. (2006). However, the ammonia-N and nitrite-N concentration was significantly reduced by bioflocs technology, which corresponded to the findings of Avnimelech et al. (1989, 1994) and Avnimelech (1999). Similar to the report from Hari et al. (2006), no significant difference in nitrate-N concentration was shown in both groups, although the nitrate-N concentration was significantly higher than the ammonia-N and nitrite-N concentration, the mean nitrate-N concentration of 1.44 and 7.52 mg L$^{-1}$ had no effect on shrimp performance, which was reported being tolerated in concentrations of several thousand mg per litre (Vinatea et al., 2010).

### 4.2. Feed nutrition substitute

The reduction in nitrogenous compound through carbon addition could lead to an increased microbial flocs, which immobilized nitrogen for microbial synthesis (Avnimelech, 1999; Hari et al., 2004). Microbial flocs performed in bioflocs technology ponds are demonstrated to be an effective potential food source for tilapia through the monitoring of floc volume (Avnimelech, 2007). In our study, floc volume was monitored at lower levels in the beginning of the farming period but higher levels in the middle and late farming period along with sucrose addition, which may indicate the feed nutrition substitute of microbial flocs in the beginning of the farming period.

**Table 3**
16S rDNA-V3 sequence similarities to the closest relatives of DNA recovered from the respective bands in DGGE gels.

| Band | Accession no. | Closest relative and its accession number | Similarity |
|---|---|---|---|
| *Bioflocs treatment* | | | |
| 1 | DQ993342.1 | *Roseobacter* sp. SPO804 16S rRNA gene, partial sequence | 100% |
| 2 | GU817016.1 | α-Proteobacterium CLCM 16S rRNA gene, partial sequence | 98% |
| 3 | HQ423381.1 | *Bacillus subtilis* strain P6 16S rRNA gene, partial sequence | 100% |
| 4 | HQ423381.1 | *Bacillus subtilis* strain P6 16S rRNA gene, partial sequence | 96% |
| 5 | HQ423381.1 | *Bacillus subtilis* strain P6 16S rRNA gene, partial sequence | 97% |
| 6 | AY880307.1 | Proteobacterium M3-2 16S rRNA gene, partial sequence | 93% |
| 7 | AY880307.1 | Proteobacterium M3-2 16S rRNA gene, partial sequence | 95% |
| 8 | AY880307.1 | Proteobacterium M3-2 16S rRNA gene, partial sequence | 99% |
| 9 | DQ097291.1 | α-Proteobacterium RS.Sph.017 16S rRNA gene, partial sequence | 94% |
| 10 | GQ350256.1 | Uncultured α-Proteobacterium clone SHBC432 16S rRNA gene, partial sequence | 100% |
| 11 | GU168008.1 | Actinobacterium BGR 105 16S rRNA gene, partial sequence | 99% |
| 12 | GU168008.1 | Actinobacterium BGR 105 16S rRNA gene, partial sequence | 92% |
| 13 | HM006908.1 | *Bacillus licheniformis* strain Pb-WC09009 16S rRNA gene, partial sequence | 98% |
| 14 | GQ350256.1 | Uncultured α-Proteobacterium clone SHBC432 16S rRNA gene, partial sequence | 99% |
| 15 | EF466030.1 | Uncultured ε-Proteobacterium clone F-a11 16S rRNA gene, partial sequence | 94% |
| 16 | EF466030.1 | Uncultured ε-Proteobacterium clone F-a11 16S rRNA gene, partial sequence | 96% |
| 17 | EF466030.1 | Uncultured ε-Proteobacterium clone F-a11 16S rRNA gene, partial sequence | 97% |
| 18 | GQ350256.1 | Uncultured α-Proteobacterium clone SHBC432 16S rRNA gene, partial sequence | 94% |
| 19 | HQ122449.1 | *Bacillus pumilus* strain AU MB 16S rRNA gene, partial sequence | 95% |
| 20 | AB015266.1 | Uncultured *Cytophaga* sp. gene for 16S rRNA, partial sequence, clone:JTB220 | 98% |
| *Relative control* | | | |
| 1 | AB305227.1 | *Halomonas* sp.Sa13-1I gene for 16S rRNA, partial sequence | 94% |
| 2 | AY880307.1 | Proteobacterium M3-2 16S rRNA gene, partial sequence | 95% |
| 3 | AY880307.1 | Proteobacterium M3-2 16S rRNA gene, partial sequence | 99% |
| 4 | AY880307.1 | Proteobacterium M3-2 16S rRNA gene, partial sequence | 95% |
| 5 | AY960847.1 | *Photobacterium ganghwense* strain FR1311 16S rRNA gene, partial sequence | 97% |
| 6 | GQ350256.1 | Uncultured α-Proteobacterium clone SHBC432 16S rRNA gene, partial sequence | 100% |
| 7 | AF500207.1 | *Vibrio* sp.CJ11052 16S rRNA gene, partial sequence | 95% |
| 8 | DQ342239.1 | *Paracoccus homiensis* strain DD-R11 16S rRNA gene, partial sequence | 93% |
| 9 | GU223593.1 | *Vibrio* sp.A975 16S rRNA gene, partial sequence | 92% |
| 10 | AF500207.1 | *Vibrio* sp.CJ11052 16S rRNA gene, partial sequence | 100% |
| 11 | GU223600.1 | *Vibrio* sp. K323 16S rRNA gene, partial sequence | 97% |
| 12 | FJ227113 | *Vibrio harveyi* isolate EHP7 16S rRNA gene, partial sequence | 95% |
| 13 | AY174868.1 | *Vibrio* sp. QY102 16S rRNA gene, partial sequence | 92% |
| 14 | GQ408900.1 | *Halomonas* sp. EM490 16S rRNA gene, partial sequence | 90% |
| 15 | AB491746.1 | *Pseudoalteromonas* sp. MIW01 gene for 16S rRNA, partial sequence | 93% |
| 16 | AB015266.1 | Uncultured *Cytophaga* sp. gene for 16S rRNA, partial sequence, clone:JTB220 | 93% |
| 17 | AB491746.1 | *Pseudoalteromonas* sp. MIW01 gene for 16S rRNA, partial sequence | 95% |
| 18 | AF227238.1 | *Pseudoalteromonas* sp. A28 16S rRNA gene, partial sequence | 93% |

Avnimelech et al. (1994) estimated that feed utilization is higher in bioflocs technology ponds, while tilapia in such ponds is fed a ration 20% less than conventional one. The feed requirement of shrimp growing in bioflocs technology ponds was studied recently by Panjaitan (2004). It was found that lowering feed application by up to 30% of conventional feeding ration, did not lower shrimp growth, probably due to the partial replacement of feed by the microbial flocs. In our study, the bioflocs treatment group together with higher protein diet (42% protein pellet feed and 78.8% protein fresh feed) resulted in a 3.1% higher specific growth rate, 12.0% higher protein efficiency ratio and 7.22% lower feed conversion rate compared with the relative control group, which probably indicated the higher feed utilization through bioflocs technology.

### 4.3. Potential pathogen inhibition

Manipulating the C/N ratios through carbon addition could result in a shift from an autotrophic to a heterotrophic system (Avnimelech, 1999; Avnimelech et al., 1994; Browdy et al., 2001). Further study showed heterotrophic bacteria was suspected to have a controlling effect on pathogen (Defoirdt et al., 2007; Michaud et al., 2006). In our study, the potential pathogen inhibition of microbial flocs was determined by microbial diversity analysis.

Clustering analysis from DGGE profile showed the clustering appeared to be influenced through sucrose addition, which was similar to the report from Crab et al. (2009). From the analysis of band intensity, we found the predominant microbes in the bioflocs treatment group were *Bacillus* sp. and Proteobacterium M3-2 analogue, and the predominant microbes in the relative control group were *Vibrio* sp. and Proteobacterium M3-2 analogue. In fact, band intensity from DGGE profile could not exactly show the predominance because of the differential amplication of 16S rDNA (Ihalin and Asikainen, 2006). In our experiment, the concentration of PCR product was determined and diluted to a same level before DGGE loading, which relatively avoided the inaccuracy. Majority of Proteobacterium detected in both groups were always considered to be symbiotic bacteria in aquaculture (Sakami et al., 2008). *Bacillus* sp. detected in bioflocs treatment was mostly considered to be the main probiotics in aquaculture (Li et al., 2006), however, *Bacillus* sp. needed to be added on a more continuous basis because of the minority of natural occurring (Moriarty, 1998). *Vibrio* sp. detected in the relative control group was reported to be the most numerous of the reported bacterial agents of penaeid shrimp (Mohney et al., 1994), however, some of vibrio species were the potential causative agents for diseases in aquaculture systems (Immanuel et al., 2004). Balcazar (2003) demonstrated that the administration of a mixture of bacterial strains (*Bacillus* and *Vibrio* sp.) positively influenced the growth and survival of juveniles of white shrimp and presented a protective effect against the pathogen *Vibrio harveyi*. In our study, sucrose addition together with probiotics addition resulted in *Bacillus* sp. predominant in the bioflocs treatment group, no sucrose addition together with probiotics addition resulted in *Vibrio* sp. predominant in the relative control group, which probably indicated the durability of *Bacillus* sp. and inhibition of *Vibrio* sp. through bioflocs technology. Finally, the bioflocs treatment group
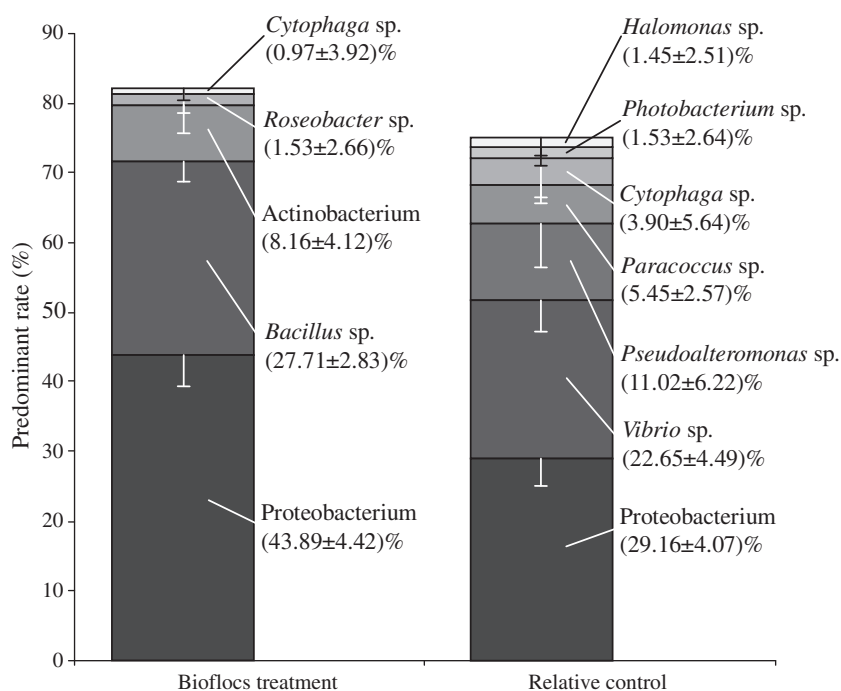
**Fig. 8.** Comparison of predominant bacterial communities among the bioflocs treatment and relative control groups based on the band intensity percentage of 16S rDNA-V3.

resulted in a 41.3% higher shrimp yield compared with the relative control group.

## 5. Conclusion

The application of bioflocs technology in high-intensive *M. japonicus* farming systems performed equally well as observed in other shrimp species. Comparing with the relative control group, the ammonium and nitrite concentration was significantly reduced in the bioflocs treatment group through sucrose addition. Concurrent with the reduction of inorganic nitrogen, the bioflocs treatment group resulted in a 13.4% higher shrimp yield, 12.0% higher protein efficiency ratio, and 7.22% lower feed conversion rate. Further analysis of microbial diversity showed the predominant microbe was characterized by *Bacillus* sp. in the bioflocs treatment group, but by *Vibrio* sp. in the relative control group.

Bioflocs technology offers the possibility to simultaneously maintain a good water quality within aquaculture systems and produce additional food for shrimp. The potential importance of the feed nutrition substitue fully justifies further research and widening of the knowledge base to help utilize the natural feed recycling potential of bioflocs technology. Microbes detected in the bioflocs treatment group could be useful in resisting disease in high-intensive shrimp farming systems. There exists scope for further improvement in bioflocs technology by inoculating different functional microbes. Radio or stable isotope studies are also needed to trace microbial dynamics of bioflocs among all the farming period. In addition, methods used to exactly evaluate the predominant microbes in water should be improved and refined.

## References

Avnimelech, Y., 1999. C/N ratio as a control element in aquaculture systems. Aquaculture 176, 227–235.

Avnimelech, Y., 2006. Bio-filters: the need for a new comprehensive approach. Aquacultural Engineering 34, 172–178.

Avnimelech, Y., 2007. Feeding with microbial flocs by tilapia in minimal discharge bioflocs technology ponds. Aquaculture 264, 140–147.

Avnimelech, Y., Ritvo, G., 2003. Shrimp and fish pond soils: processes and management. Aquaculture 220, 549–567.

Avnimelech, Y., Mokady, S., Schoroder, G.L., 1989. Circulated ponds as efficient bioreactors for single cell protein production. Israeli Journal of Aquaculture – Bamidgeh 41, 58–66.

Avnimelech, Y., Kochva, M., Diab, S., 1994. Development of controlled intensive aquaculture systems with a limited water exchange and adjusted C to N ratio. Israeli Journal of Aquaculture — Bamidgeh 46, 119–131.

Avnimelech, Y., Verdegem, M.C.J., Kurup, M., Keshavanath, P., 2008. Sustainable land-based aquaculture: rational utilization of water, land and feed resources. Mediterranean Aquaculture Journal 1, 45–55.

Balcazar, J.L., 2003. Evaluation of probiotic bacterial strains in Litopenaeus vannamei. Final Report, National Center for Marine and Aquaculture Research, Guayaquil, Ecuador.

Boon, N., Goris, J., De Vos, P., Verstraete, W., Top, E.M., 2000. Bioaugmentation of activated sludge by an indigenous 3-chloroaniline-degrading comamonas testosteroni strain, I2gfp. Applied and Environment Microbiology 66, 2906–2913.

Browdy, C.L., Bratvold, D., Stokes, A.D., McIntosh, R.P., 2001. Perspectives on the application of closed shrimp culture systems. In: Browdy, C.L., Jory, D.E. (Eds.), The New Wave, Proceedings of the Special Session on Sustainable Shrimp Culture, Aquaculture 2001. The World Aquaculture Society, Baton Rouge, USA, pp. 20–34.

Burford, M.A., Thompson, P.J., McIntosh, R.P., Bauman, R.H., Pearson, D.C., 2003. Nutrient and microbial dynamics in high intensity, zero exchange shrimp ponds in Belize. Aquaculture 219, 393–411.

Chen, L.C., 1990. Aquaculture in Taiwan. Fishing News London, Great Britain, p. 273.

Crab, R., Avnimelech, Y., Defoirdt, T., Bossier, P., Verstraete, W., 2007. Nitrogen removal techniques in aquaculture for a sustainable production. Aquaculture 270, 1–14.

Crab, R., Kochva, M., Verstraete, W., Avnimelech, Y., 2009. Bio-flocs technology application in over-wintering of tilapia. Aquacultural Engineering 40, 105–112.

Crab, R., Lambert, A., Defoirdt, T., Bossier, P., Verstraete, W., 2010a. The application of bioflocs technology to protect brine shrimp (*Artemia franciscana*) from pathogenic *Vibrio harveyi*. Journal of Applied Microbiology 109, 1643–1649.

Crab, R., Chielens, B., Wille, M., Bossier, P., Verstraete, W., 2010b. The effect of different carbon sources on the nutritional value of bioflocs, a feed for *Macrobrachium rosenbergii* postlarvae. Aquaculture Research 41, 559–567.

Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., Bossier, P., 2007. Alternatives to antibiotics to control bacterial infections: luminescent vibriosis in aquaculture as an example. Trends in Biotechnology 25, 472–479.

Hargreaves, J.A., 2006. Photosynthetic suspended-growth systems in aquaculture. Aquacultural Engineering 34, 344–363.

Hari, B., Kurup, B.M., Varghese, J.T., Schrama, J.W., Verdegem, M.C.J., 2004. Effects of carbohydrate addition on production in extensive shrimp culture systems. Aquaculture 241, 179–194.

Hari, B., Kurup, B.M., Varghese, J.T., Schrama, J.W., Verdegem, M.C.J., 2006. The effect of carbohydrate addition on water quality and the nitrogen budget in extensive shrimp culture systems. Aquaculture 252, 248–263.

Horowitz, A., Horowitz, S., 2001. Disease control in shrimp aquaculture from a microbial ecology perspective. In: Browdy, C.L., Jory, D.E. (Eds.), The NewWave, Proceedings of the Special Session on Sustainable Shrimp Culture, Aquaculture 2001. The World Aquaculture Society, Baton Rouge, Baton Rouge, Louisiana, United States, pp. 199–218.

Ihalin, R., Asikainen, S., 2006. 16S rDNA PCR-denaturing gradient gel electrophoresis in determining proportions of coexisting *Actinobacillus actinomycetemcomitans* strains. Journal of Microbiological Methods 65, 417–424.

Immanuel, G., Vincybai, V.C., Sivaram, V., Palavesam, A., Marian, M.P., 2004. Effect of butanolic extracts from terrestrial herbs and seaweeds on the survival, growth and pathogen (*Vibrio parahaemolyticus*) load on shrimp *Penaeus indicus* juveniles. Aquaculture 236, 1–4.

Li, M., Lovell, R.T., 1992. Effect of dietary protein concentration on nitrogenous waste in intensively fed catfish ponds. Journal of the World Aquaculture Society 23, 122–127.

Li, Z.J., Guo, Z.X., Feng, J., Zhang, H.H., Yang, Y.Y., 2006. Primary study on mediating micro-ecological environment with *Bacillus* sp. in shrimp pond. Marine Science 30, 28–31.

Lin, Q.W., Shan, B.D., Liu, L.D., Huang, J.Q., 2001. Studies on high density culture of *Penaeus japonicus* in shrimp hatchery in autumn in north China. Journal of Oceanography in Taiwan Strait 20, 510–514.

Michaud, L., Blancheton, J.P., Bruni, V., Piedrahita, R., 2006. Effect of particulate organic carbon on heterotrophic bacterial populations and nitrification efficiency in biological filters. Aquacultural Engineering 34, 224–233.

Mohney, L.L., Lightner, D.V., Bell, T.A., 1994. An epizootic of vibriosis in Ecuadorian pond-reared *Penaeus vannamei* Bonne (Crustacea: Decapoda). Journal of the World Aquaculture Society 25, 116–125.

Moriarty, D., 1997. The role of microorganisms in aquaculture ponds. Aquaculture 151, 333–349.

Moriarty, D.J.W., 1998. Control of luminous *Vibrio* species in penaeid aquaculture ponds. Aquaculture 164, 351–358.

Mu, N.H., Han, J.M., Mu, G.Y., Jiao, W., Hou, Y.H., 2008. New techniques in effective aquaculture of *Marsupenaeus japonica*. Scientific Fish Farming 11, 38–39.

Mudroch, A., Azcue, J.M., Mudroch, P., 1996. Manual of Physico-chemical Analysis of Aquatic Sediments. Lewis Publishers. 286 pp.

Muyzer, G., Waal, E.C., Uitterlinden, A., 1993. Profiling of complex microbial populations using denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environment Microbiology 59, 695–700.

Naylor, R.L., Goldburg, R.J., Primavera, J.H., Kautsky, N., Beveridge, M.C.M., Clay, J., Folke, C., Lubchenco, J., Mooney, H., Troell, M., 2000. Effect of aquaculture on world fish supplies. Nature 405, 1017–1024.

Panjaitan, P., 2004. Field and laboratory study of Penaeus monodon culture with zero water exchange and limited water exchange model using molasses as a carbon source. Ph.D. Thesis, Charles Darwin Univ., Darwin, NT, Australia.

Pe´rez Farfante, I., Kensley, B.F., 1997. Penaeoid and sergestoid shrimps and prawns of the world. Key and diagnoses for the families and genera. Mémoires du Muséum National d'Histoire Naturelle Paris 175, 1–233.

Sakami, T., Fujioka, Y., Shimoda, T., 2008. Comparison of microbial community structures in intensive and extensive shrimp culture ponds and a mangrove area in Thailand. Fisheries Science 74, 889–898.

Samocha, T.M., Lawrence, A.L., Collins, C.A., Castille, F.L., Bray, W.A., Davies, C.J., Lee, P.G., Wood, G.F., 2004. Production of the Pacific white shrimp, *Litopenaeus vannamei*, in high-density greenhouse-enclosed raceways using low salinity groundwater. Journal of Applied Aquaculture 15, 1–19.

Vinatea, L., Ga'lvez, A.O., Browdy, C.L., Stokes, A., Venero, J., Haveman, J., Lewis, B.L., Lawson, A., Shuler, A., Leffler, J.W., 2010. Photosynthesis, water respiration and growth performance of *Litopenaeus vannamei* in a super-intensive raceway culture with zero water exchange: interaction of water quality variables. Aquacultural Engineering 42, 17–24.

Weber, J.T., Mintz, E.D., Canizares, R., Semiglia, A., Gomez, I., Sempertegui, R., Davila, A., Greene, K.D., Puhr, N.D., Cameron, D.N., Tenover, F.C., Barrett, T.J., Bean, N.H., Ivey, C., Tauxe, R.V., Blake, P.A., 1994. Epidemia cholera in Ecuador: multi-drug resistance and transmission by water and seafood. Epidemiology and Infection 112, 1–11.

Zhou, H.L., 2001. Growth of intensive farmed kuruma shrimp (*Penaeus japonicus* Bate) in earthen ponds. Journal of Guangxi Academy of Sciences 17, 91–95.

Zhou, C.G., Chen, J.Y., Huang, X.K., 2008. Preliminary study of high productive aquaculture of *Marsupenaeus japonica*. Shandong Fisheries 25, 27-27.