Temporal heterogeneity of prokaryotic micro-organism communities in sediment of traditional freshwater cultured fish ponds in Southwest China

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ABSTRACT
The temporal heterogeneity of prokaryotic microorganism communities in sediment of traditional freshwater cultured fish ponds in Southwest China were investigated by 16S rRNA clone libraries. The result indicated that a total of 47 archaea clones and 392 bacterial clones were recovered from three samples, among them, the bacteria Mitsuria chitosanitabida, Prolixibacter bellariivorans and Flavihumibacter petaseus and archaea Methanoregula formicica were dominant in the cultivation 1 year (Y1) sample, the bacteria Cetobacterium somerae, Allochromatium vinosum and Rhabdorhamatium marinum and archaea Methanolinoa tarda were dominant in the cultivation 2 years (Y2) sample, the bacteria Cetobacterium somerae and Bacteroides fragilis and archaea Methanolinoa tarda were dominant in the cultivation 4 years (Y4) sample. The Shannon–Wiener (H) diversity index and the Simpson dominancy index respectively varied from 2.23 to 2.37 and from 7.30 to 9.04 for three gene libraries. Maximum operational taxonomic unit (OTU) richness was respectively estimated with two different models SChao1 and SACE; and it revealed SChao1 15 and SACE 15 for Y1, SChao1 15.25 and SACE 15 for Y2, SChao1 12 and SACE 12 for Y4. However, the B. fragilis which was found in the Y4 sample was 99% similar to the human and animal pathogen B. fragilis. No pathogenic organisms related to humans or animals were found at any other sample. Mentionable, the more detailed information about the safety of fish pond cultivation environment should receive more attention and further studies.

Introduction
China has been the largest fishery producer in the world since 2002, and freshwater fish pond aquaculture produces 60%–70% of the total freshwater aquaculture production in China in 2004 [1]. These data suggest that aquaculture production in China depends heavily on the pond aquaculture. Especially in the south-western part of China, which has been known as ‘homeland for rice and fish’ owing to the munificent climate and water resources, pond fish farming serves as the traditional freshwater aquaculture and has always played an important role.

In the recent two decades, due to the increasing demand for aquaculture products and the need for new food supplies, aquaculture rapidly developed in scope and intensity, and intensive aquaculture leads to localized, high inputs of organic feed to pond [2]. However, Christensen et al. [3] demonstrated that only 12%–30% of total nitrogen and phosphorous from organic feed (feed pellets) was absorbed and utilized by fish. Thus, large amounts of organic materials such as uneaten fish feed and fish feces, which are known to contribute to eutrophication, are released into the environment and accumulate in the underlying sediments [4]. The impact of this organic enrichment has a serious negative effect on fish ponds. With the increasing cultivation years of fish in such conditions, the organic rich sediment below fish ponds tends to exhibit highly reduced conditions with the production of hydrogen sulhide and ammonia, and to cause deficiency of dissolved oxygen in bottom water [5]. Organic pollution of sediments or eutrophication also influences the biogeochemical process and changes the microbiology of the sediment [6]. Since microbial populations rapidly respond to environmental variations, the changes in sediment microorganisms is a useful indicator for assessing the pollution states of freshwater cultured fish ponds. So it is necessary to investigate the microbial diversity of fish pond sediment, which serves as an important part of the aquaculture ecosystem.

Nowadays, the advancement of molecular biology such as the analysis of nucleic acids from microorganisms has revolutionized the research in environmental...
microbiology. Nucleic acids can be analyzed either from isolates obtained from the sample by traditional culture-dependent microbiological methods, or from the whole environmental sample by direct extraction of nucleic acids, i.e. culture-independent methods. However, traditional culture-dependent methods introduce a strong bias because of the selective enrichment during cultivation resulting in failure to describe the whole microbial diversity [7]. Among various culture-independent tools, the direct amplification of the 16S rRNA genes from environmental samples using the polymerase chain reaction (PCR) is a commonly employed technique used to describe the composition of complex microbial communities and to gain a descriptive overview of possible differences among communities [8]. In particular, 16S rRNA clone library construction has previously been used to investigate the microbial diversity of sediment environments such as Antarctic continental shelf sediments [9], the Vlasa hot spring of Bulgaria [10], the northern south China sea [11] and a tropical upwelling system [12]. These studies have provided much information on the microbial communities that inhabit sediments and their large bacterial diversity. However, to date, there has been no investigation published about the temporal heterogeneity of prokaryotic microorganism communities and their yearly variations in sediments of the traditional freshwater culture fish ponds of Southwest China.

In the present study, the temporal heterogeneity of prokaryotic microorganism communities in sediments of the traditional freshwater cultured fish ponds of Southwest China were investigated by constructing 16S rRNA clone libraries. The information gathered may be useful to improve our understanding of the composition and the microbial populations in the sediment of the traditional freshwater cultured fish ponds of Southwest China, and may help us to better understand the long-term responses of microorganism communities to the aquaculture cultivation process.

**Materials and methods**

**Study sites and sediment sampling**

The experiments were carried out in an earthen fishpond (82 × 65 m, mean water depth 1.5 m) located in the southwest of China (Chengdu, Sichuan). Pond sediments were collected from 2011 to 2015 at the same site by means of a 8.5 cm deep × 17 cm wide Van Veen grab. The sediments accumulated for cultivation 1 year (Y1), 2 years (Y2) and 4 years (Y4) were respectively collected in sterile tubes and then stored frozen in liquid nitrogen until analysis.

**DNA extraction and purification**

The metagenomic DNA pool of homogenized samples from the sediment was directly extracted using UltraCleanTM Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, California, USA). Then, DNA purity and concentration was analyzed spectrophotometrically using the e-Spect ES-2 (Malcom, Japan). The extracted DNA was stored at −20 °C until use.

**Construction of 16S rRNA libraries**

The nearly full-length 16S rRNA gene in prokaryotes was amplified by PCR using 16S forward and reverse primers (Eu27F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1490R: 5′-GGTTACCTGTACGACTT-3′ for bacterial 16S rRNA, Ar28F: 5′-TGGTGTGATCCTGCGAGG-G-3′ and 1490R: 5′-GGTTACCTGTACGACTT-3′ for archaeal 16S rRNA) [13]. The thermal cycling conditions for the bacteria and the archaea 16S primers were performed as previously described by Xiang et al. [13]. The PCR products were purified using TaKaRa purification kits. Then, the amplified 16S rRNA fragments were ligated into the plasmid pGEM-T vector (Promega, Madison, WI) according to the manufacturer’s protocol. The recombined plasmids were then transformed into the chemically competent E. coli JM109 cells and transformants were identified using blue-white selection on Luria–Bertani agar plates containing 100 μg/mL ampicillin, 40 μg/mL X-gal and 24 μg/mL IPTG (isopropyl β-D-1-thiogalactopyranoside). The white clones were used to construct a library of clones, which corresponded to a prokaryotic rDNA amplicon.

**Sequencing and phylogenetic analysis of 16S rDNA**

Recombined plasmids with 16S rRNA segments were prepared from individual recombined colonies and used as templates for sequencing. Sequencing was performed by a DNA sequencer (Applied Biosystems, Foster City, CA, USA) with a DYEnergy ET Terminator Cycle Sequencing Kit (Amersham Bioscience, Piscataway, NJ, USA). Chimera Check software was used to exclude chimeras. The 16S rRNA sequences without chimeras were submitted to compare with available published sequences in the GenBank database and the percent similarity was then determined. Multiple alignments of 16S rRNA were run by using the Clustal X program. The phylogenetic trees based on the16S rRNA were constructed with MEGA program version 5.0 by the neighbour-joining method with the Kimura two-parameter model [14].
**Statistical analysis**

BioEdit software was employed to carry out the pairwise comparison of all 16S rRNA sequences in this study. Sequences with 3% dissimilarity levels to each other were grouped in one operational taxonomic unit (OTU) [15], while the others were grouped alone. A value of 97% similarity is commonly set as the threshold to encompass the sequence microheterogeneity that may occur between closely related taxa [16]. All prokaryotic 16S rRNA represent sequences from the OTU were respectively classified by program RDP Seqmatch and GenBank Database. The rarefaction analysis was performed by the Analytic Rarefaction 1.3 calculator displayed using Origin 8.0 Software based on the numbers of OTUs versus the numbers of clone. More indices including $S_{obs}$, $S_{chao1}$, $S_{ACE}$, Shannon diversity ($H$), Simpson and Coverage were calculated using Estimates Win 8.20 software for each library.

**Nucleotide sequence accession numbers**

The 16S rRNA sequences of every OTU in this study were deposited in the GenBank databases at the National Center for Biotechnology Information (NCBI). The prokaryotic 16S rRNA sequences were assigned the following GenBank IDs: KJ461494–KJ461513.

**Results and discussion**

**16S rRNA libraries of fish ponds sediment**

In this study, 16S rRNA analysis was used to characterize the temporal heterogeneity of prokaryotic micro-organism communities in fish pond sediment of Southwest China. For each sample, a 16S rRNA clone library was produced. A total of 47 archaea clones and 392 bacterial clones were recovered from three samples; among them, 20 archaea clones and 142 bacterial clones from the Y1 sample, 16 archaea clones and 130 bacterial clones from the Y2 sample, and 11 archaea clones and 120 bacterial clones from the Y4 sample. All clones were selected for sequencing and then the obtained sequences without chimeras were aligned and sorted into OTUs. It was determined that there were 4 archaea OTUs and 11 bacterial OTUs in Y1; 3 archaea OTUs and 12 bacterial OTUs in Y2; and 2 archaea OTUs and 10 bacterial OTUs in Y4. The results showed that most OTUs contained multiple clones, while only a few OTUs were unique and represented by only a single clone.

The molecular biology method based on PCR technology can objectively evaluate the microbial diversity of samples. Differences in microbial community structure represent a potential useful tool for investigating environmental perturbations due to the rapid response to changes in nutrient loading [17]. Compared to the three sediment samples, the lower species richness that was observed in the Y4 sample could be attributed to the higher accumulation of organic matter in the Y4 sample.

**Estimated complexity of gene libraries**

The rarefaction curves based on the 16S rRNA sequences in three samples were respectively obtained by plotting the number of OTUs observed against the number of clones (Figure 1). Some curves not reaching clear saturation indicated that some microorganisms might have escaped detection. However, the obvious decrease in the rate of OTUs detection indicated that the major part of the diversity of prokayotic micorbes in the present study had been detected (Figure 1). This conclusion was further supported by calculating the coverage of three libraries, which was from 99.2% to 100% (Table 1). The biodiversity for the prokaryotic populations was evaluated using the Shannon–Wiener ($H$) diversity index and Simpson dominancy index. The $H$ index and the Simpson index respectively varied from 2.23 to 2.37 and from 7.30 to 9.04 for the three gene libraries (Table 1). Maximum OTU richness was estimated with two different models $S_{Chao1}$ and $S_{ACE}$, and it revealed $S_{Chao1}$ 15 and $S_{ACE}$ 15 for Y1, $S_{Chao1}$ 15.25 and $S_{ACE}$ 15 for Y2, $S_{Chao1}$ 12 and $S_{ACE}$ 12 for Y4.

![Figure 1. Rarefaction analysis of prokaryotic microorganisms in Y1, Y2 and Y4 samples based on the 16S rRNA libraries, displaying the number of OTUs detected versus the number of sequences analysed.](image-url)

**Table 1.** Prokaryotic microorganism richness and diversity estimations of OTUs derived from Y1, Y2 and Y4 samples.

| Sample | Individual | $S_{obs}$ | $S_{ACE}$ | $S_{chao1}$ | H     | Simpson | Coverage |
|--------|------------|-----------|-----------|-------------|-------|---------|----------|
| Y1     | 162        | 15        | 15        | 15          | 2.24  | 7.30    | 100%     |
| Y2     | 146        | 15        | 15        | 15.25       | 2.37  | 9.04    | 99.2%    |
| Y4     | 131        | 12        | 12        | 12          | 2.23  | 8.08    | 100%     |

*Percentage of coverage: $S_{obs}$/mean ($S_{chao1}$, $S_{ACE}$) × 100.
for Y4 (Table 1). Based on these estimations, 99.2%–100% of the prokaryotic diversity was covered by the applied sampling survey.

It is important to note that Chao-1 richness estimates are sensitive to the sampling effort and a greater sampling effort at the Y1 sample may also account for some of the additional richness at this sample. It needs to be considered that pyrosequencing errors can lead to an overestimation of the 16S rRNA diversity if not stringent quality filtering and clustering thresholds that are not greater than 97% identity are applied [18]. The negative relationship between organic matter accumulation and richness (Chao1) and bacterial diversity (H) observed in the present study supports the postulation of Levin et al. [19] that species richness is lower in nutrient-rich areas as the enhanced resources support larger populations where the dominance of a few species occurs, often leading to a decrease in habitat heterogeneity. In addition, one of the most likely explanations for the decrease in diversity with increasing the cultivation time is that the high organic loading leads to hypoxia. This is reasonable, since not only the total resource available, but also the balance of resources play critical roles in maintaining the community’s diversity.

**Distribution of the clones at the genus level**

The prokaryotic 16S rRNA without chimeras were accurately classified to the species level by the program RDP MultiClassifier. All prokaryotic sequences obtained in this study fell into the genera of *Cetobacterium*, *Mitsuaria*, *Desulfomicrobium*, *Flavihumibacter*, *Sulfuritalea*, *Thiohalocapsa*, *Sterolibacterium*, *Ignavibacterium*, *Allochromatium*, *Thiohalomonas*, *Bacteroides*, *Rhabdoclammatium*, *Prolixibacter*, *Thiocapsa*, *Longilinea* and *Bellilinea*, a lineage of the Bacteria domain, and Archaea domain *Methanosaeta*, *Methanolinea*, *Methanospirillum* and *Methanoregula* genera (Figure 2). Among these genera, the bacteria *Mitsuaria chitosanitabida* (34/142, number of bacterial clones/total number of bacterial clones), *Prolixibacter bellariivorans* (28/142) and *Flavihumibacter petaseus* (37/142) and archaea *Methanoregula formicica* (10/20, number of archaea clones/total number of archaea clones) were dominant in the Y1 sample; the bacteria *Cetobacterium somerae* (27/130), *Allochromatium vinosum* (26/130) and *Rhabdoclammatium marinum* (24/130) and archaea *Methanolinea tarda* (12/16) were dominant in Y2 sample, the bacteria *Cetobacterium somerae* (26/120) and *Bacteroides fragilis* (30/120) and archaea *Methanolinea tarda* (8/11) were dominant in Y4 sample.

In the present study, the prokaryotic microorganism communities were shown to be different from each other during the investigated period. Since the fish pond was mainly formed by digging, at the beginning of the cultivation process, the fish pond sediment originated from the compacted subsurface soil which contains some prokaryotic microorganisms. In the study, some microorganisms which belong to bacteria *Mitsuaria chitosanitabida*, *Prolixibacter bellariivorans* and *Flavihumibacter petaseus* and archaea *Methanoregula formicica* were dominant in the Y1 sample. Zhang et al. [20] report that *Flavihumibacter petaseus* was isolated from the soil of a subtropical rainforest and Holmes et al. [21] report that *Prolixibacter bellariivorans* was isolated from marine sediment. *Mitsuaria chitosanitabida* is a chitosan-degrading species that produces chitosanases similar to ChoA [22].

The microbial community structure in the Y2 and Y4 samples, however, was different. The bacteria *Cetobacterium somerae*, *Allochromatium vinosum* and *Rhabdoclammatium marinum* and archaea *Methanolinea tarda* were abundant in the Y2 sample. Among them, the bacteria

*Figure 2. Distribution of prokaryotic microorganisms at the genus level in Y1, Y2 and Y4 samples from fish pond sediment.*
Cetobacterium somerae and Methanolinea tarda were also abundant in the Y4 sample. Finegold et al. [23] report that Cetobacterium somerae was consistently more abundant in human feces, and thus could serve as indicator of sewer and fecal pollution, respectively. Further, the bacterium was successfully used to track sewer and fecal contamination in Lake Michigan [24]. Fish farm sediments generally have high organic matter content which can be strongly anoxic and rich in sulphides [25]. Allochromatium vinosum and Rhodobacterium marinus possess the ability to carry out different types of metabolism using a variety of alternate electron donors (e.g. elemental sulphur, sulphide) and acceptors (e.g. sulphite, nitrate) and they play an important role in sulphur cycles [26]. The concentration of organic-rich sediment increases the microbial activity and decreases the dissolved oxygen in the ponds or tanks [27].

Microorganism communities and the sediment of fish pond aquaculture are an interacting complex system which has a natural hydrographical self-purification capability. Their diversity plays an important role in maintaining the biosphere, and provides large amounts of resources through photosynthesis to release oxygen, and to change the O₂ content in the sediment.

Figure 3. Phylogenetic tree based on the complete 16S rRNA sequences of representative clones from prokaryotic OTUs by using the neighbour-joining method (A) and the representative clone distributions in Y1, Y2 and Y4 samples from fish pond sediment (B).
**16S rRNA phylogenetic analysis**

Molecular phylogeny increasingly supports our understanding of the microbial relationships in the environment and provides the basis for the conventional identification techniques [21]. Comparative sequence analysis based on 16S rRNA is currently the most widely used approach for the reconstruction of microbial phylogeny [10].

In the present study, all representative 16S rRNA sequences from each OUT group were submitted to the NCBI public database to infer a possible phylogenetic classification. The BLAST searches revealed that most representative sequences shared more than 98% similarity with the strains in the public database at the 16S rRNA level. Eichler et al. [28] demonstrated that the microorganisms with a similarity higher than 98% of 16S rRNA sequences could be clustered into the same species. The bacteria represented by clone YT1-12 and YT2-68 showed 97% similarity with type strains *Met. formicica* SMSP and *Sul. hydrogenivorans*, respectively. To disclose the microbial taxonomic position and relationships, a phylogenetic tree based on the 16S rRNA of the representative sequences from OTUs was constructed by the neighbour-joining method (Figure 3). The tree revealed the phylogenetic affiliation of microorganisms inhabiting the sediment of the studied traditional freshwater culture fish pond of Southwest China.

In intensive aquaculture, fish or other aquatic animals are under high-density stress, and fishponds with a lot of humus (remnants of feeds and feces) are causing the fish or shrimp to be susceptible to diseases [27]. *Bacteroides fragilis* has been studied extensively and it is known to have been the cause of many intestinal inflammations and it is also the main cause of acute and chronic diarrheal diseases in humans and animals [29]. Unfortunately, the 16S rRNA of the dominant species *B. fragilis* which was found in the Y4 sample was 99% similar to the human and animal pathogen *B. fragilis*. No pathogenic organisms related to humans or animals were found in any other sample. High-density fish farming provides conditions where contagious illnesses spread rapidly through the farm. Therefore, it is typical to observe an enrichment of fish pathogenic organisms with the increase of cultivation time. High-density fish farming provides conditions where contagious illnesses spread rapidly through the farm. Baffone et al. [30] and Reith et al. [31] report that the fish pathogen *A. saolonicida* and the human pathogen *V. parahaemolyticus* were found in fish farming sediment. Fortunately, they were not detected or not prevalent in three samples. Noteworthy, the safety of the fish pond cultivation environment should receive more attention in further studies.

**Conclusions**

In the present work, the prokaryotic microorganism communities in the sediment of the traditional freshwater cultured fish ponds of Southwest China were investigated using 16S rRNA clone libraries. Our study provides qualitative and quantitative information on the bacterial diversity present in the sediment of freshwater cultured fish ponds at different periods. The knowledge of bacterial species in the sediment of fish pond might partially be useful for the scientific guidance of high-densities aquaculture. Noteworthy, the detection of pathogenic organisms should receive more attention, and more detailed information about the safety of the fish pond cultivation environment should be the subject of further studies.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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**References**

1. Hu BT. Cage culture development and its role in aquaculture in China. Aquaculture Res. 2008;25:305–310.
2. Edwards P. Aquaculture environment interactions: past, present and likely future trends. Aquaculture. 2015;447:2–14.
3. Christensen PB, Rysgaard S, Sloth NP, et al. Sediment mineralization, nutrient fluxes, denitrification and dissimilatory nitrate reduction to ammonium in an estuarine fjord with sea cage trout farms. Aquat Microb Ecol. 2000;21:73–84.
4. Kawahara N, Shigematsu K, Miyadai T, et al. Comparison of bacterial communities in fish farm sediments along an organic enrichment gradient. Aquaculture. 2009;287:107–113.
5. Kunihiro T, Miyazaki T, Uramoto Y, et al. The succession of microbial community in the organic rich fish-farm sediment during bioremediation by introducing artificially mass-cultured colonies of a small polychaete, Capitella sp. I. Mar Pollut Bull. 2008;57:68–77.
6. Asami H, Aida M, Watanabe K. Accelerated sulfur cycle in coastal marine sediment beneath areas of intensive shellfish aquaculture. Appl Environ Microbiol. 2005;71:2925–2933.
7. Shaﬁ S, Kamili AN, Shah MA, et al. Aquatic bacterial diversity: magnitude, dynamics, and controlling factors. Microb Pathog. 2017;104:39–47.
8. Yakimov MM, Denaro R, Genovese M, et al. Natural microbial diversity in supercritical sediments of Milazzo Harbor (Sicily) and community successions during microcosm
[9] Bowman JP, McCammon SA, Gibson JAE, et al. Prokaryotic metabolic activity and community structure in Antarctic continental shelf sediments. Appl Environ Microbiol. 2005;69:2448–2462.

[10] Holmes DE, Nevin KP, Woodard TL, et al. Prolinibacter belTRANisors gen. nov., sp. nov., a sugar-fermenting, psychrotolerant anaerobe of the phylum Bacteroidetes, isolated from a marine-sediment fuel cell. Int J Syst Evol Microbiol. 2007;57:701–707.

[11] Xia XM, Guo W, Liu HB. Dynamics of the bacterial and archaeal diversity in Vlasa hot spring, Bulgaria, by using 16S rRNA and glycoside hydrolase family 4 genes. Biotechnol Biotechnol Equip. 2010;24:1979–1985.

[12] Newton RJ, Bootsma MJ, Morrison HG, et al. A microbial signature approach to identify fecal pollution in the waters off an urbanized coast of Lake Michigan. Microb Ecol. 2013;65:1011–1023.

[13] Brooks KM, Stierns AR, Mahnken CVW, et al. Chemical and biological remediation of the benthos near Atlantic salmon farms. Aquaculture. 2003;219:355–377.

[14] Takai K, Inagaki F, Nakagawa S, et al. Isolation and phylogenetic diversity of members of previously uncultivated e-Proteobacteria in deep-sea hydrothermal fields. FEMS Microbiol Lett. 2003;218:167–174.

[15] Zhou QL, Li KM, Xie J, et al. Role and functions of beneficial bacteria and archaea in the human gut. Biology (Basel). 2017 Jun 25;6:427. DOI: 10.3390/biology60300427

[16] Brooks KM, Stierns AR, Mahnken CVW, et al. Chemical and biological remediation of the benthos near Atlantic salmon farms. Aquaculture. 2003;219:355–377.

[17] Takai K, Inagaki F, Nakagawa S, et al. Isolation and phylogenetic diversity of members of previously uncultivated e-Proteobacteria in deep-sea hydrothermal fields. FEMS Microbiol Lett. 2003;218:167–174.

[18] Zhou QL, Li KM, Xie J, et al. Role and functions of beneficial microorganisms in sustainable aquaculture. Bioresource Technol. 2009;100:3780–3786.

[19] Eichler S, Christen R, Höltje C, et al. Composition and dynamics of bacterial communities of a drinking water supply system as assessed by RNA- and DNA-Based 16S rRNA gene fingerprinting. Appl Environ Microbiol. 2006;72:1858–1872.

[20] Pathela P, Hasan KZ, Roy E, et al. Enterotoxigenic bacteria isolated from healthy patients with diarrhea in rural Bangladesh. J Infect Dis. 2005;191:1245–1252.

[21] McCaig AE, Glover LA, Prosser JI. Molecular analysis of bacterial community characterization and biogeochemistry of sediments from a tropical upwelling system (Cabo Frio, Southeastern Brazil). Cont Shelf Res. 2016;130:1–13.

[22] Brooks KM, Stierns AR, Mahnken CVW, et al. Chemical and biological remediation of the benthos near Atlantic salmon farms. Aquaculture. 2003;219:355–377.

[23] Takai K, Inagaki F, Nakagawa S, et al. Isolation and phylogenetic diversity of members of previously uncultivated e-Proteobacteria in deep-sea hydrothermal fields. FEMS Microbiol Lett. 2003;218:167–174.

[24] Zhou QL, Li KM, Xie J, et al. Role and functions of beneficial microorganisms in sustainable aquaculture. Bioresource Technol. 2009;100:3780–3786.