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Characterization of Nitrifying Bacterial Community in a Mariculture Wastewater Treatment Using SBR System

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Abstract

Sequencing batch reactors (SBR) have been used in the biological treatment of aquaculture wastewater. In this study, we investigated the microbial community of a SBR that used diatomite earth (20 g/L) as the sludge carrier material. Marine wastewater in which ammonia content was 42.08 to 55.88 mg/L was supplied to the SBR every 12 h over a treatment period of 65 days. During the first 20 days, the concentration of NH_4-N decreased gradually, while nitrite (NO_2-N) became the major nitrogen compound, reminiscent of the development of an ammonia-oxidizing process. Over the next 20 days, the concentration of NH_4-N decreased further due to conversion to NO_3-N. More than 99% of the NH_4-N was converted to NO_3-N over a period of 40-65 days. Denaturing gradient gel electrophoresis (DGGE) assay showed that bacteria of the genus *Flavobacterium* were present during the entire treatment period, while *α-Proteobacteria*, *β-Proteobacteria*, *γ-Proteobacteria*, and *Sphingobacteriia* started to accumulate after the first 20 days. Fluorescence in situ hybridization (FISH) assay identified *Nitrobacter* and *Nitrosomonas* as the main bacteria involved in the conversion of NH_4-N to NO_3-N. Diatomite earth therefore acted as an efficient sludge carrier by shortening the settling time and facilitating bacterial colonization. This SBR was capable of rapid removal of NH_4-N. This warrants further investigation at the pilot-scale in an actual mariculture farm.

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Introduction

The coastline of China measures approximately 18,400 km (Xie et al. 2013). Many commercially important marine animals have been successfully cultured along its coast, making China one of the largest producers and consumers of mariculture products in the world. Rapid growth of intensive mariculture is extremely profitable, however there are major environment problems, such as eutrophication, algae bloom (Keesing et al. 2011), and nitrogenous compounds (NH_4-N, NO_2-N, and NO_3-N) which are considered major contaminants in aquaculture wastewater (Cao et al. 2007). NH_4-N is the principal nitrogenous waste produced from residual feed, urine, feces, and excreta, of aquatic animals. NO_2-N is the product of ammonia oxidation, i.e. intermediate product of the nitrification process. NO_3-N is the result of nitrite oxidation, i.e. the most oxidized form of nitrogen in the nitrification process and is relatively non-toxic to fish (Schreier et al. 2010).

Marine aquaculture is mainly carried out in the four sea regions of China, i.e. Bohai Sea, Yellow Sea, East China Sea, and South China Sea (Cao et al. 2007). About 43 billion tons of wastewater from aquaculture systems spill into the coastal waters every year (Xie et al. 2007), the equivalent of more than five tons of nitrogenous pollutants are thus discharged into the sea (Cui et al. 2005). The Bohai Bay in the north of China has reached a critical point and could become a 'dead' sea due to severe pollution (Cao et al. 2007). Thus, it is imperative to develop suitable mariculture wastewater treatment systems and promote their use to improve aquaculture sustainability in China.

In previous studies of aquaculture wastewater treatment, systems with biofilter (Gregory et al. 2012), biofilm (Seca et al. 2011), side-stream bioreactor (Smith et al. 2006), Aquamats (Voltolina et al., 2013), and active sludge biofloc technology (Avnimelech, 2009) have been used. However, in some cases the costly equipment and complex operations sometimes discourage extensive use of these systems. To attempt to solve these issues, a SBR system was introduced for shrimp wastewater treatment initially in Thailand (Paungfoo et al. 2007) and subsequently in South Carolina (Boopathy et al. 2009; Fontenot et al. 2007; Roy et al. 2010). The use of one tank with multiple reaction steps considerably lowered the running costs and complexity of the system. However, the functional nitrifying bacteria in the systems were not thoroughly studied, the sludge carrier material was either absent or carried with air stones, and the processing time was relatively long, i.e. about 3.5-8 d of hydraulic retention time (HRT) (Paungfoo et al. 2007; Boopathy et al. 2009 ; Fontenot et al. 2007; Roy et al. 2010). In this study, a four sequential step SBR system was developed to treat mariculture wastewater and the effects of local nitrifying bacteria was identified with molecular and microscopy techniques. Diatomite earth, which is mainly produced in China, was used as sludge carrier material because of its multiple-porosity (Xu et al. 2009).

Materials and Methods

Sequencing batch reactor.

The SBR system was made of polyethylene material molded into a cylindrical shape, diameter 120 mm, height 600 mm. It has a total volume of 6.8 L and a working volume of 5.0 L. The water inlet of the SBR system   was set at the bottom of the tank and the effluent outlet was fixed on the wall of the tank, 250 mm from the bottom. The outlet allowed approximately 50 % of the working volume (2.5 L) to be exchanged with each recycle. Four bubble diffusion devices were installed at the bottom of the SBR (Fig. 1). The aeration was performed by pumping air at 0.22 m^3/h through the bubble diffusion devices whenever necessary.
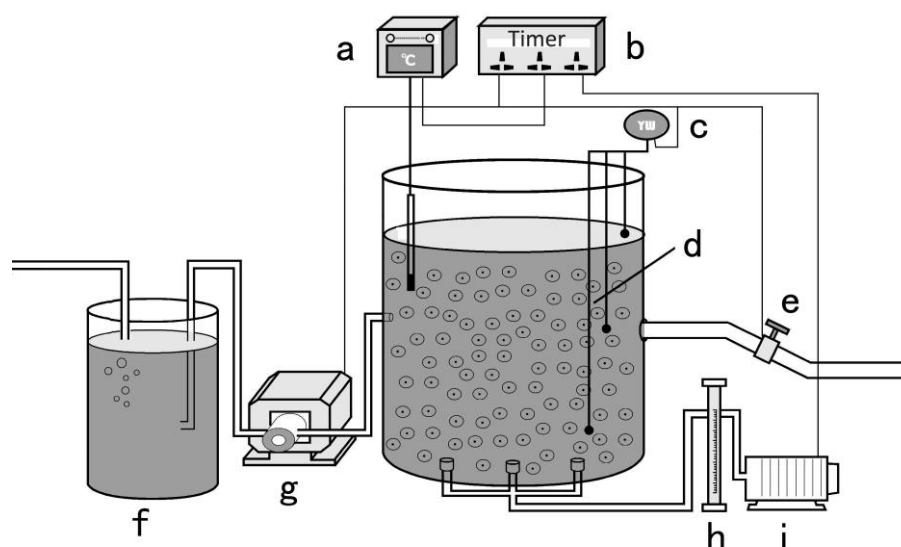
Figure 1. Schematic graph of SBR treatment system. a, Temperature controller; b, Microcomputer timer; c, Liquid level controller; d, Carrier; e, Electromagnetic valve; f, Wastewater tank; g, Pump; h, Gas-flow meter; i, Air compressor.

Sludge, diatomite, seawater and preparation of wastewater. The original sludge containing 2.0 g/L mixed liquor volatile suspended solids (MLVSS) and 2.5 g/L mixed liquor suspended solids (MLSS) was collected at the sewage outlet in Dalian Heshengfeng aquaculture farm, Liaoning, China. Seaweed and gravel were removed from the sludge after collection. The processed sludge was pooled and stored at 4 °C for later use in the SBR. Diatomite (oven-dried form) 80-110 μm diameter, SiO_2 content > 86.5 %, surface area of 68 m^2/g, and pH value of 7.0 was purchased from Qingshanyuan Crop. (Jilin, China). About 20 g diatomite was used per liter of wastewater. Wastewater was also collected from the marine aquaculture farm in Dalian bay, in the Yellow sea, salinity of 32-34‰, NH_4-N concentration of 42.08 to 55.88 mg/L, NO_2-N concentration of 1.02 to 1.37 mg/L and chemical oxygen demand (COD) of 15.96 to 18.09 mg/L.

Experimental design. Duplicate SBR systems were operated at room temperature (20-25 °C) for 65 days. On Day 1, 5.0 l of wastewater containing about 50 mg/L NH_4-N was pumped into the SBR system, followed by additions of 200 g of sludge and 50 g of diatomite with 24 h aeration. On Day 2, the water was allowed to settle for 1 h, and approximately 2.5 L was then discharged through the outlet, after which 2.5 L wastewater was pumped into the system through the inlet, and the system was aerated for 24 h. On Day 3, the aerated water was allowed to settle for 1 h and 2.5 L was again discharged from the system. Then 50 g of diatomite was added into the system. Three days later, residual seaweed and gravel debris were thoroughly removed, and the system was prepared for wastewater treatment. From Day 3 onwards, the system operated on a 12-h cycle, with 5 min of filling, 11 h of aeration, 30-50 min for settling, and 5 min of discharge of 3.0 L treated wastewater. The settling time was 50 min for Day 4-40 and 30 min for Day 41-65. In addition, an appropriate amount of $NaHCO_3$ was also added to adjust the pH value to 7.0-8.5. The effluent was sampled every 2-3 d for quality assessment. The sludge was sampled on Days 1, 10, 20, 30, 35, 40, 50 and 60, and stored at -80°C before molecular analyses. The sludge sample obtained on Day 60 was also subjected to FISH and SEM analyses.

Water quality assessment. The salinity, pH, temperature, and dissolved oxygen (DO) level of the wastewater in the SBR system were monitored every 2-3 d using a salinity meter (WZ-211, Wancheng Co., Beijing, China), a pH meter (Sartorius, Bradford, MA), and a thermoprobe & DO probe (JPB-607, Leici Co., Shanghai, China), respectively. NH_4-N, NO_2-N, and NO_3-N concentrations in the effluent were determined in triplicate using a standard chromatographic method for seawater analysis (GB17378.4 2007). The

differences in nitrogen concentrations at every two time points were analyzed using student's *t*-test, and differences were considered to be statistically significant at the *P* < 0.05 level.

DGGE assay. Genomic DNA was extracted from the active sludge samples collected on Day 1, 10, 20, 30, 35, 40, 50 and Day 60 using FastDNA® Spin Kit (Mpbio Corp., USA) according to the manufacturer's protocol. The extracted DNA was subjected to 16S rDNA PCR using the universal 16S rDNA primer set for GC-341F (5'-CGCCCGCC GCGCGCGGC GGGCGGGG CGGGGGC ACGGG GGGCCTACG GGAGGC AGCAG-3') and 758R (5'-CTACCA GGGTATCT A ATCC-3') (Rolleke et al. 1996). The PCR sample contained 2 µl of DNA, 0.2 µM of each primer and 20 µl of 1 × Premix Ex Taq™ master mix (Takara Co., Dalian, China) in total volume of 50 µl. Amplification was performed using a Dice® Thermocycler (Takara) with the following conditions: 95°C for 5 min; 35 cycles of 94°C for 30 s; 55°C for 30 s; 72°C for 30 s; and a final step of 72°C for 10 min. PCR products were analyzed by DGGE assay using the Dcode™ system (BioRad, Hercules, CA, USA). About 200 ng of PCR product was loaded onto 6 % (w/v) polyacrylamide gel prepared to contain a 45-70 % denaturing gradient and run at 80 V and 60°C for 16 h in 1 × TAE buffer (pH 7.4) (Tresse et al. 2007). After electrophoresis, the gel was stained with 1 × Genefinder (BayGene Co., Beijing, China) in 1 × TAE buffer for 30 min and documented with a Gel-Doc XR system (Bio-Rad). Clear linearized bands were cut from the gel, rinsed three times with double distilled H_2O (ddH_2O), and soaked in 100 µl ddH_2O at 4°C overnight. The water fraction was then amplified using the primers 341F (5'-CCTACGGGAGGC AGCAG-3') and 758R, followed by DNA sequencing performed by Beijing Genomics Institute (BGI, Shenzhen, China). The sequencing data were subjected to a BLAST search. Sequences having more than 95 % identity were considered to be of the same species.

FISH assay. The active sludge sample on Day 60 was immobilized and hybridized according to a standard FISH protocol (Ito et al. 2002). The published FISH ammonia-oxidizing bacteria (AOB) oligonucleotide probe, Nso1225 (5'-Cy5-CGCCATTGTATTACGTGTGA-3'; red), theoretically targets most of the recognized ammonia-oxidizers of the *β-Proteobacteria* class with the exception of *Nitrosococcus mobilis*. The nitrite-oxidizing bacteria (NOB) oligonucleotide probe, NIT3 (5'-Cy3-CCT GTG CTC CAT GCT CCG-3'; green) was used to target nitrite-oxidizing bacteria of the genus *Nitrobacter* (Mobarry et al. 1996). Fluorescent signals were visualized using a laser scanning spectral confocal microscope (Leica TCS-SP2, Germany) and quantitatively analyzed using the software of Image-Pro Plus 6.0.

SEM assay. The active sludge from Day 60 was freshly sampled and prepared for SEM within 4 h. Samples were ultrasonically processed for 5 min and fixed in 0.1 M sodium cacodylate buffer (SCB, pH 7.2) supplemented with 50 mg/L glutaraldehyde for 2 h (Eighmy et al. 1983). Excess glutaraldehyde was removed by five successive 20 min washes in SCB. The samples were re-suspended in SCB at 4°C, and after dehydration via a graded ethanol series, the samples were critical point-dried under carbon dioxide, affixed to aluminum stubs with silver paste and then sputter-coated with gold. The specimens were observed using a Hitachi S4800 scanning electron microscope (Tokyo, Japan).

Results

Water quality. Throughout the 65 days of treatment, the water in the SBR had the following characteristics: pH of 6.0-8.1; temperature of 18-25°C; DO at 4.5-5.5 mg/L; and salinity at 32-34‰ (data not shown). From day 2-20, the NH_4-N concentration gradually decreased, accompanied by peak production of NO_2-N, indicating the development of an ammonia-oxidizing process. From Day 20-40, transient increases in NH_4-N level occurred, but this rapidly decreased to a level lower than the lowest level observed during between day 2 and 20 (Fig. 2). The reduction in NH_4-N was accompanied by low production of NO_2-N, and at the same time a significant amount of NO_3-N was produced. From Day 40-65, more than 99% of the initial NH_4-N in the water was converted to NO_3-N, indicating that ammonia was almost completely removed within 12 h of the treatment.
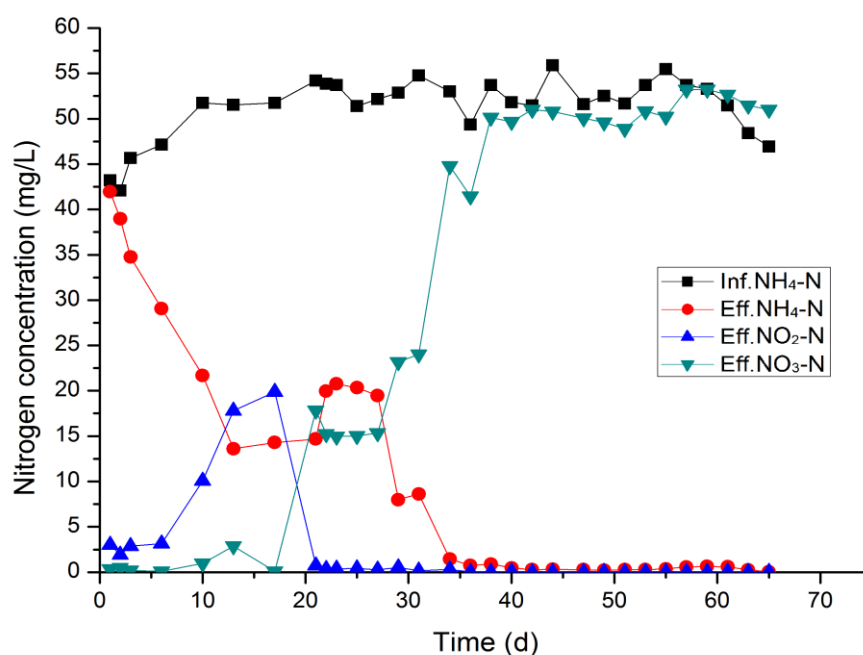
Figure 2. The concentration of NH_4-N, NO_2-N, and NO_3-N in the influent (Inf) and effluent (Eff) liquid of SBR throughout 65 d of treatment. Data represent average of duplicate reactors.

Bacterial community indicated by DGGE assay. Changes in bacterial communities in the SBR over the 65 d treatment period were profiled using DGGE gel. About 26 different electrophoretic bands were assigned as shown in Fig. 3.

Figure 3. 16S rDNA DGGE profiles of bacterial community in the active sludge from different times of the 65-d treatment. Arrows and numbers represent the DNA bands that were excised and sequenced.

On Day 1 and 10, the DGGE profiles showed almost identical patterns in that the DNA bands were not clear and widely distributed, with no major bands being recognized. DGGE profiles for the water samples taken on Days 20, 3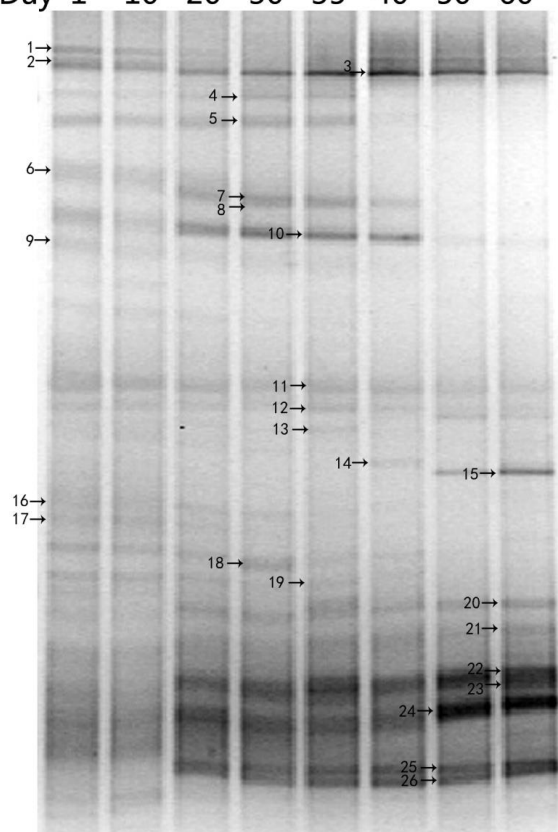0, 35 and 40 also showed similar patterns, but with a few DNA bands becoming more intense (bands 3, 10, 22-26 in Fig. 3). Sequencing and BLAST analysis revealed the presence of four major groups of bacteria in the SBR (Table 1); these were *Sphingobacteriia*, *α-Proteobacteria*, *β-Proteobacteria* and *γ-Proteobacteria,* which corresponded to bands 24, 22-23, 26 and 25, respectively, of the DGGE patters*.* The analysis also identified *Flavobacteriia* as another major group of bacteria in the SBR (bands 1-10 in Table 1). The distribution of the bacteria within the SBR became stable on Days 50 and 60, yielding patterns that were similar to those of Day 20-40. These bacteria appeared to accumulate to the point when their number became stable, and then aggregated into larger masses of cells. On Days 50 and 60, the nitrification process was fully completed, yielding a NH_4-N to NO_3-N conversion rate of 99%. Thus *α-Proteobacteria*, *β-Proteobacteria*, *γ-Proteobacteria*, and *Sphingobacteria* were the major classes of nitrifying bacteria, whereas *Flavobacteria* was the minor one but persisted during whole treatment period.

Table 1. Sequencing and BLAST results of DNA bands selected from the DGGE gel. [1]

Band No.	Accession No.	Phylogenetically related organism	Phylogenetic group	Identity (%)
1	NR104531	*Olleya aquimaris*	*Flavobacteriia*	99
2	JX412959	*Bizionia* sp.	*Flavobacteriia*	99
3	JX854357	Flavobacteriaceae bacterium	*Flavobacteriia*	99
4	GU949542	Marine bacterium	NA	98
5	JQ723603	Uncultured *Lutibacter* sp.	*Flavobacteriia*	96
6	EU328155	*Formosa crassostrea*	*Flavobacteriia*	99
7, 8	JX530396	Uncultured *Lutibacter* sp.	*Flavobacteriia*	98
10	FJ387163	*Marinitalea sucinacia*	*Flavobacteriia*	98
14	JF947798	Uncultured alpha proteobacterium	*α-Proteobacteria*	96
15	HQ601863	Uncultured bacterium	NA	97
19	EF215752	Uncultured gamma proteobacterium	*γ-Proteobacteria*	96
22	HM003639	Uncultured *Rhodobacter* sp.	*α-Proteobacteria*	98
23	KF618620	*Nitrobacter* sp.	*α-Proteobacteria*	99
24	EU328037	Uncultured Cytophagales bacterium	*Sphingobacteriia*	97
25	NR042618	*Marinobacter guineae*	*γ-Proteobacteria*	99
26	AF272418	*Nitrosomonas marina*	*β-Proteobacteria*	99

[1] Bands 12 and 18 could not to be sequenced. Bands 9, 11, 13, 16, 17, 20, and 21 retrieved no hit from Genebank with identity higher than 95 %. Bands 7 and 8 have the same sequences.

Bacterial communities indicated by FISH assay
Whole-cell hybridization revealed that the nitrifying bacteria were uniformly distributed within the sludge by the end of the 60-day period (Fig. 4A-B). *Nitrobacter* (band 23 in Fig. 3) cells formed small clusters that were in contact with *Nitrosomonas* (band 26 in Fig. 3) cell clusters (Fig. 4C). Quantitative analysis by Image-Pro Plus 6.0 showed that the ratio of *Nitrosomonas* spp. to *Nitrobacter* spp. was 1.3: 1. This spatial distribution of *Nitrosomonas* spp. and *Nitrobacter* spp. may reflect a syntrophic association between the two bacteria.

Bacteria morphology visualized by SEM
The sludge on Day 60 consisted mainly of diatomite earth, bacterial aggregates, and many micropores (Fig. 5). Fig. 5A shows the multiple-porosity structure of diatomite earth, the magnified view of which is shown in Fig. 5B-D. The pores within the diatomite earth have different sizes, with average pore sizes larger than 2 μm. The aggregated bacterial cells could be widely seen (indicated by black arrows in Figs. 4B-C), and some of these were embedded between the diatomite earth and sludge granules.
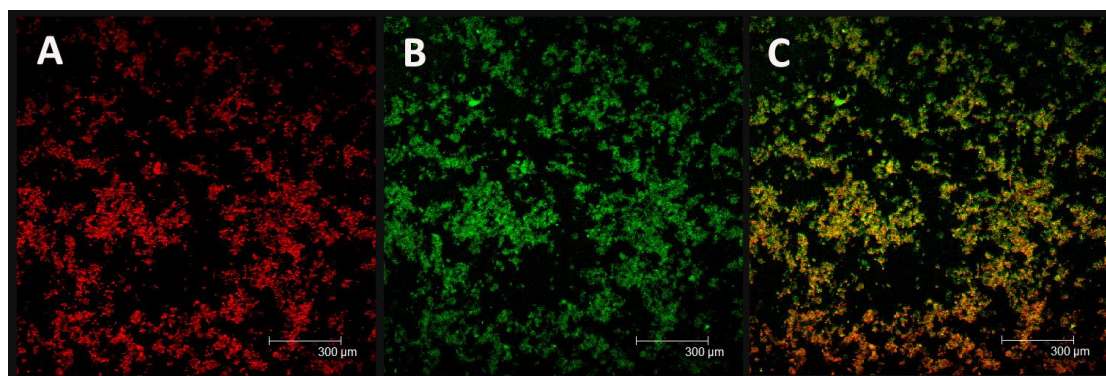
Figure 4. FISH assay for the sludge sample collected on Day 60. Identical microscopic fields were viewed. A, Cy5-labeled Nso1225 probe (red). B, Cy3-labeled NIT3 probe (green). C, Merging of Nso1225 and NIT3 probes. Yellow represents the regions represented by both probes.
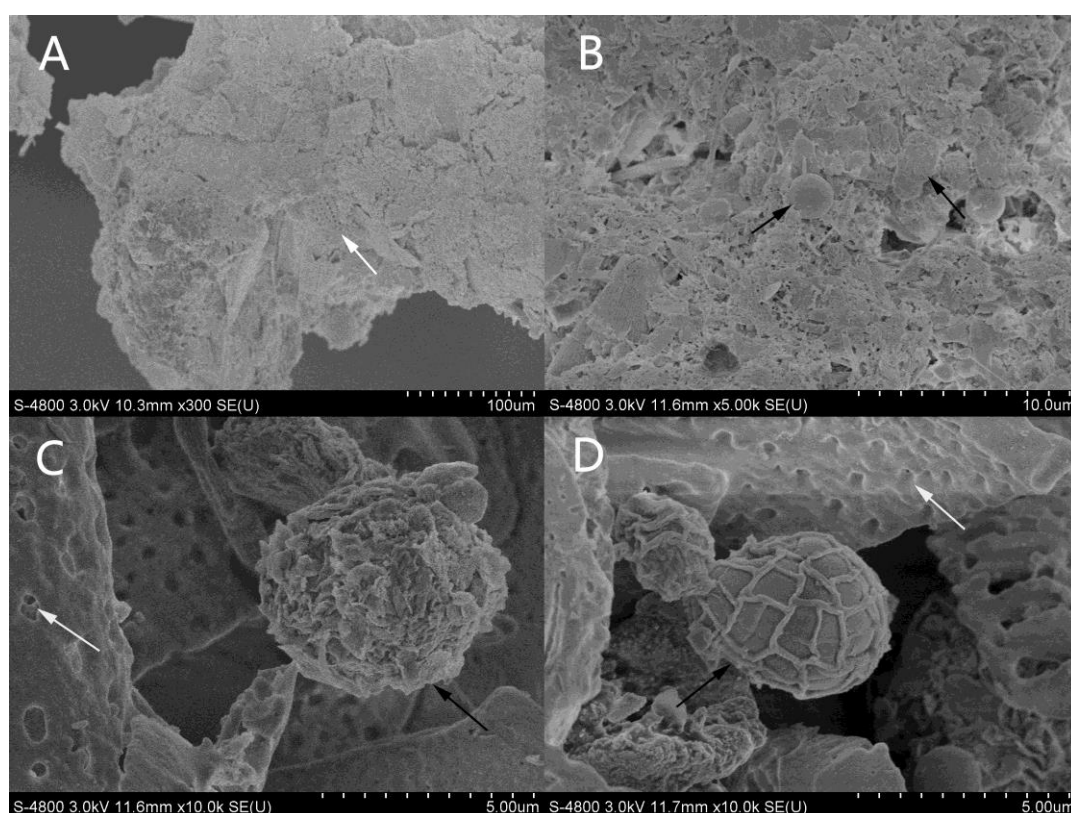

Figure 5. Representative SEM image of the sludge sample collected on Day 60. A, low magnification of magnified sludge surface; B, fracture in the sludge surface; C and D, high-magnification of microcolonies. The white arrows indicate the multiple-porosity structure of the diatomite carrier, and the black arrows indicate the bacterial aggregate. Magnification factor and the scale are shown at the bottom of each image.

Discussion

Compared to other studies on SBR, our SBR system appeared to have a higher processing speed and probably different communities of nitrifying bacteria. The SBR used in the shrimp farm in Thailand can convert up to 92% of the NH_4-N in the system to NO_3-N in 3.5 days of HRT following the inoculation of shrimp farm sediment (Paungfoo et al. 2007), compared with 99% conversion in our SBR, which was inoculated with sea cucumber farm sediment from the North China Yellow Sea. The operating temperature in Thailand was 28-30°C and in our study was 20-25°C; the original sludge material in Thailand was shrimp farm sediment and in our study was sea cucumber farm sediment which could be the main reason for the differences in the bacterial communities. We believe that this difference in bacterial communities could be the factor affecting settling

time and the rate of NH_4-N conversion. As for the SBR used for re-circulating the water in the shrimp aquaculture in South Carolina, 99% of all nitrogen species, i.e. NH_4-N, NO_2-N and NO_3-N, were removed from the wastewater in 8-9 d of operation after molasses were added as an additional carbon source to raise the C:N ratio to 10:1. The present SBR provided a fast NH_4-N removal system within 12 h.

There are numerous published reports on high-throughput genome sequencing to examine microbial diversity. Here, DGGE was used to analyze the distribution of bacteria in SBR sludge because of its affordable price and the less accurate demands of this experiment. Similar results have previously been obtained by other investigators who also studied the functions of these nitrifying bacteria (Zhang et al. 2009). Although *Sphingobacteria* appeared to be the most dominant genus of bacteria in our SBR, only one species was identified for this genus (band 24) (Fig. 3). This bacterium has previously been identified in a freshwater nitrification drum filter study (Schneider et al. 2007). A number of other species were identified within the *a-Proteobacteria* class. Band 22, 23, 25 and 26 were identified as *Rhodobacter* spp., *Nitrobacter* spp., *Marinobacter* genus and *Nitrosomonas marina*, respectively; these have been detected in some other marine nitrification filter, nitrification and denitrification, and wastewater treatment studies (Michaud et al. 2009; Cytryn et al. 2005; Mobarry et al. 2007; Mota et al. 2005; Bellucci et al. 2011; Zheng et al. 2012; Cytryn et al. 2005; Leonard et al. 2000; Schreier et al. 2010; Zeng et al. 2013; Dang et al. 2010). However, there were also nitrifying bacteria, e.g., *Bacteroides*, and *Planctomycetales* classes, which have been found in other marine recirculating aquaculture systems (Schreier et al. 2010; Tal et al. 2006) but were absent in our system. We also noted several DGGE bands that could not be assigned to any known bacterial species by BLAST analysis of their sequences. This highlighted the complexity of the nitrifying bacterial community and showed that not all the species of nitrifying bacteria were revealed in the present study.

We speculated that the toxicity exerted by nitrite on the ammonia oxidizers and the low energy yield of nitrite oxidation might have promoted the close physical association between *Nitrosomonas* spp. and *Nitrobacter* spp. by providing these bacteria with a mutual advantage*.* In addition, it has been suggested that ammonia-oxidizing bacteria would first grow as microcolonies, and the nitrite that they produce would then allow the nitrite-consuming *Nitrobacter* to aggregate*.*

To our knowledge, this is the first study to report the use of diatomite as the sludge carrier material in marine aquaculture wastewater treatment. Our results suggested several advantages for using diatomite in wastewater treatment. First of all, using diatomite facilitated the settling step after aeration. In the SBR used in South Carolina (Fontenot et al. 2007) and Thailand (Paungfoo et al. 2007), the settling times were 1 d and 2.5 h, respectively, which are much longer than the settling times in our SBR (30-50 min). The multiple-porosity structure of diatomite earth in the SBR system offers high surface area to volume ratio that enhances bacterial attachment and accelerates the development of dominant bacterial aggregates, which may further facilitate the nitrification process. On the other hand, compared to the traditional heavy carrier materials, e.g. gravels, clay beads, and plastic particles (Gregory et al. 2012; Tal et al. 2006), diatomite earth is lighter and therefore can be more readily circulated in the reactor by aeration. The multiple-porosity structure of the diatomite earth provides a protective environment for the nitrifier to grow, since the active nitrifying bacteria can be embedded and oxygen transfer can be enhanced by aeration. Thus diatomite earth with high porosity, durability, and low cost when used as carrier material in marine aquaculture wastewater treatment is highly recommended.

There are many wastewater-treatment systems designed to reduce the nutrient discharge from aquaculture activities. However, many systems, such as those based on biofilter (Gregory et al. 2012), biofilm (Seca et al. 2011) and side-stream bioreactor systems (Smith et al. 2006), are relatively costly and/or involve complex operation, hindering their application in aquaculture farming in China. In this study, the conversion occurred within 12 h once the operation of the SBR was initiated. However, the system

needs improvement before such events can take place, in particular the problem posed by excessive NO_3-N. Although NO_3-N is relatively non-toxic to fish, the presence of excessive NO_3-N in the water together with other nutrients may cause eutrophication and algal blooms (Cao et al. 2007). Thus, further improvement of the system would involve the removal of excessive NO_3-N (via denitrification and nitrogen fixation processes for example which is necessary before the installation of a pilot scale of the SBR can be contemplated.

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