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TRF

รายงานวิจัยฉบับสมบูรณ์

โครงการ " การศึกษากลุ่มไนตริไฟอิงแบคทีเรียในนากุ้งโดยใช้เทคนิคเชิงโมเลกุล"

โดย พูนสุข ประเสริฐสรรพ์
จรรยารัตน์ พ่วงพู

กันยายน 2545

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รายงานวิจัยฉบับสมบูรณ์

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

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ชื่อโครงการ: การศึกษากลุ่มไนตริไฟอิงแบคทีเรียในนากุ้งโดยใช้เทคนิคเชิงชีวโมเลกุล

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ระยะเวลาโครงการ: 2 ปี

บทคัดย่อ นำแบคทีเรียไนตริไฟอิงจากน้ำและตะกอนของนากุ้งและจากหัวเชื้อทางการค้ามาเพิ่มจำนวนในระบบเอสบีอาร์ (Sequencing Batch Reactor, SBR) คัดเลือกที่มีประสิทธิภาพในการกำจัดแอมโมเนียได้ดีที่สุดของแต่ละแหล่ง นำไปเลี้ยงใน SBR โดยป้อนน้ำเสียสังเคราะห์ที่มีความเค็ม 25 ส่วนในพันส่วน และความเข้มข้นแอมโมเนีย 100 มิลลิกรัม/ลิตร ตรวจสอบปริมาณของแบคทีเรียที่กำจัดแอมโมเนียและแบคทีเรียที่กำจัดไนไตรท์โดยวิธี Most Probable Number (MPN) พบว่าจำนวนของแบคทีเรียไนตริไฟอิงทั้ง 2 กลุ่มเพิ่มขึ้นตามระยะเวลาการเลี้ยงใน SBR ทั้ง 2 ถัง ซึ่งสอดคล้องกับการเปลี่ยนแปลงของปริมาณแอมโมเนีย ไนไตรท์ และไนเตรท เมื่อระบบเข้าสู่ภาวะคงที่ในการกำจัดแอมโมเนีย นำตัวอย่างตะกอนแบคทีเรียไปศึกษาโครงสร้างประชากรด้วยเทคนิคเชิงชีวโมเลกุล คือ fluorescence *in situ* hybridisation (FISH) และ phylogenetic analysis พบว่าจากการใช้เทคนิค FISH แบคทีเรียที่กำจัดแอมโมเนียของตัวอย่างจากนากุ้งและหัวเชื้อทางการค้า มีปริมาณ 44 ± 4% and 61 ± 4% เทียบกับแบคทีเรียทั้งหมด ตามลำดับ และตรวจไม่พบแบคทีเรียที่กำจัดไนไตรท์ด้วย FISH probe ที่มีอยู่ในปัจจุบัน ซึ่งคาดว่าอาจจะมีแบคทีเรียกำจัดไนไตรท์ชนิดใหม่จากเชื้อทั้ง 2 แหล่ง จึงศึกษาโครงสร้างประชากรของกลุ่มแบคทีเรียไนตริไฟอิงด้วยเทคนิคทาง phylogenetic analysis พบว่า *Cytophaga-Flavobacterium* group (CFB) และ *alphaproteobacteria* เป็นประชากรกลุ่มหลักในระบบ ผลสัมฤทธิ์จากการวิจัยนี้คือ การจำแนกกลุ่มเชื้อด้วยวิธี phylogenetic analysis และการออกแบบตัวติดตาม (probe) จำเพาะสำหรับเทคนิค FISH ของแบคทีเรียที่กำจัดแอมโมเนียในนากุ้ง

คำหลัก การกำจัดแอมโมเนีย, แบคทีเรียไนตริไฟอิง, fluorescence *in situ* hybridisation (FISH), ระบบเอสบีอาร์ (SBR), นากุ้ง

Abstract

Project Code: BGJ/31/2543

Project Title: Use of Molecular Biology Techniques in Studying the Nitrifying Bacteria Community from Shrimp Farming System

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Project Period: 2 years

Abstract Nitrifying bacteria from shrimp farm water and sediment and from commercial seed cultures were enriched in sequencing batch reactors (SBR). The microbial consortia from each source giving the best ammonia removal were used as inocula for two SBRs. Nitrifiers were cultivated in the SBRs containing artificial wastewater with 25 ppt salinity and 100 mg NH_4-N/l. The quantity of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) was determined using the most probable number (MPN) technique. Both AOB and NOB increased in number over the long-term operation of both SBRs. This agreed with the quantity changes of ammonia, nitrite and nitrate. When the system was steady state on ammonia removal, the microbial consortia samples was taken for community structure studies using the fluorescence *in situ* hybridisation (FISH) and phylogenetic analysis. According to FISH probing, AOB from the natural seed and commercial seed comprised of 44 ± 4% and 61 ± 4% of total bacteria, respectively. NOB could not be detected with currently-reported FISH probes, suggesting that novel NOB may be present in both sources. Therefore, the bacterial community structure of nitrifying bacteria was determined by phylogenetic analysis. *Cytophaga-Flavobacterium* group, CFB and *alphaproteobacteria* of bacterial phylum were the dominant group in the system. Phylogenetic identification and design of a specific probe for FISH (fluorescence *in situ* hybridization) of AOB in shrimp farming were achieved in this study.

Keywords ammonia removal; nitrifying bacteria; fluorescent *in situ* hybridization (FISH); sequencing batch reactor (SBR); shrimp farm

Introduction

Aquaculture is a major industry in the ASEAN region with Thailand being the world's major exporter of shrimp, having a capacity in excess of 250,000 tonnes per annum (FAO/NACA, 1995). Excessive feed given during the culture causes water and sediment in the pond to contain organic matter and nutrients in high concentration (Sansanayuth *et al.*, 1996). The nutrient load discharged from shrimp ponds can form a significant source of nutrients causing pollution and eutrophication in littoral areas. Nitrogen compounds, which accumulate in seawater aquaria, aquacultural ponds and disposal sites, also are toxic to aquatic animals at certain concentrations (Kawabata *et al.*, 1997).

A primary concern in shrimp farm culture systems is the toxic effect of ammonia on shrimp (Hovanec and DeLong, 1996). Chemolithoautotrophic nitrifying bacteria are responsible for ammonia oxidation and its removal from the ecosystem. Understanding nitrifier ecology is the first step towards determining how to remediate toxic ammonia. Molecular approaches to microbial community studies of wastewater treatment systems have given a better understanding than culture-dependent approaches (Amann *et al.*, 1996; Kämpfer *et al.*, 1996). Among these molecular methods are probe-based techniques that have allowed detailed quantitative studies of environmental microbial community structure (Bouchez *et al.*, 2001). Oligonucleotide probes, which target chemolithoautotrophic nitrifying bacteria, were used for examining nitrifying bacterial populations associated with shrimp ponds. The nitrifying capacities of the microbial consortia were also determined and compared. The studied populations were enrichments of nitrifiers from a natural source (a shrimp pond sediment and water) and a commercial seed. In this study, oligonucleotide probes specific for Ammonia Oxidising Bacteria (AOB) were designed using phylogenetic analysis. Phylogenetic analysis of aligned data sets is carried out by using ARB, freeware for sequence database and phylogeny software, available from the Department of Microbiology, Technical University, Munich. Phylogenetic trees were constructed by carrying out evolutionary distance analyses on the 16S rDNA alignments, using the appropriate tool in the ARB database.

Materials and Methods

Sample collection

Samples were taken from six shrimp farms and several commercial microbial products. The samples from shrimp farms consisted of the sediment and water. Shrimp pond water was determined for pH, dissolved oxygen (DO), salinity, conductivity, chemical oxygen demand (COD) and nitrogen components (NH_4-N, NO_2-N, NO_3-N).

Enrichment of nitrifying bacteria population

Ten ml of each collected sample was used in enrichments with Modified Alexander medium containing (g/l): $(NH_4)_2SO_4$, 2, KH_2PO_4, 0.5, $NaHCO_3$, 0.5, $MgSO_4.7H_2O$, 0.5, HEPES, 11.9, Sea salt, 25, trace element 1 ml (modified from Liu *et al.*, 2000). Primary enrichment was performed in a 250 ml flask containing 50 ml of Modified Alexander medium, incubated on a rotary shaker (200 rpm) at room temperature for 4 days. Further enrichment occurred by inoculating the total volume of specific primary enrichments to 3 l reactors containing 1 l of artificial wastewater containing (g/l): $(NH_4)_2SO_4$, 0.5, $MgSO_4.7H_2O$, 0.2, $Na_2HPO_4.2H_2O$, 9.0, KH_2PO_4, 1.5, $NaHCO_3$, 0.5, sea salt, 25, and 1 ml trace element (modified from Liu *et al.*, 2000). Mixing and aeration were achieved by bubbling air through the reactor which was operated at room temperature (28-30°C). From the numerous primary enrichments setup, six from each source (natural seed and commercial seed) were chosen for further enrichment, on the basis of ammonia removal efficiency. Those with the best ammonia removal were chosen and their biomass from the 3 l reactors were used as inocula for cultivation in sequencing batch reactors (SBRs). One enrichment from each source type (natural seed and commercial seed) was taken for further studies.

Cultivation in SBR

The 5 l operating volume SBR was employed with bubbling air for aeration and in a 24 h-cycle consisting of 10 min fill, 21 h aeration react, 2 h 40 min settle, and 10 min supernatant withdrawal. Support media made from perforated plastic were placed in the SBR for encouraging bacterial attachment. Modified artificial wastewater containing 100 mg NH_4-N/l was fed at 430 ml per cycle to achieve a 7 d HRT. Nitrification in the SBR was monitored daily by determining the NH_4-N, NO_2-N, and NO_3-N concentrations in SBR effluent using a Spectroquant NOVA 60, Merck Ltd., Co. The SBRs reached steady state and after 77 d of operation, a HRT of 3.5 days was applied with a 12-h cycle consisting of 10 min fill, 9 h aeration react, 2 h 40 min settle, and 10 min supernatant withdrawal.

Enumeration of nitrifying bacteria populations using most probably number (MPN) techniques

One 1 ml of sample was added to 9 ml of sterile artificial seawater and approximately 10 sterile glass beads (3 mm in diameter) and the mixture was vigorously shaken for 1 min. The artificial seawater contained (g/l): NaCl, 25; $MgCl_2$.7H2O, 5; $CaCl_2$, 1; KCl, 1 (Stephen *et al.*, 1996). For each of AOB and NOB, 0.15 ml of the in modified Watson's medium (Jones and Hood, 1980) and appropriate sterile medium (see below) was dispensed into each well of a 96-well microtiter plate, and eight replicate two-fold serial dilutions of 0.15 ml sample were performed. After incubation in the dark for 21 d at room temperature, the presence of AOB or NOB was determined (see below) and the numbers were calculated using MPN tables (Rowe *et al.*, 1977).

AOB were enumerated in modified Watson's medium (Jones and Hood, 1980) prepared in artificial seawater and the pH was adjusted to 7.5 using sterile 5% Na_2CO_3. Phenol red was added into the medium to indicate when the pH dropped below 7. Wells in the microtiter plate were recorded as positive for AOB by acid production and by detection of NO_2^- and NO_3^- through development of a blue colour after addition of 1 or 2 drops of 0.2% (w/v) diphenylamine in concentrated H_2SO_4 (McCaig *et al.*, 1999). NOB were enumerated in the medium of Alexander and Clark (1965) prepared in artificial seawater. Wells in the microtiter plate were recorded as positive for NOB when nitrite was absent, as indicated by the lack of colour change upon addition of Griess Ilosvay reagents 1 and 2.

Fluorescence *In situ* Hybridisation (FISH) Analysis

Samples from the SBRs were analysed by FISH using oligonucleotide probes detailed in Table 1 and methods described by Manz *et al.* (1992). The paraformaldehyde-fixed samples were spotted on gelatin-coated slide glasses, dried and dehydrated in an ethanol series (50%, 80% and 98%) each for 3 min. Hybridisation buffers were made from 0.9 M NaCl, 20 mM Tris/HCl, 0.01% sodium dodecyl sulfate (SDS) and formamide (see

Table 1), and the pH was adjusted to 7.2. Fluorescent-labeled oligonucleotide probes were dissolved with the hybridization buffer, and hybridized with the sample for 2 h at 46°C. After hybridization, washing was done in a wash buffer without formamide (20 mM Tris/HCl, 0.01% SDS and NaCl concentration depended on probe, pH 7.2) for 20 min at 48°C. Slides were rinsed with distilled water, air dried, mounted with Citifluor to avoid bleaching and observed using a confocal laser-scanning microscope (CLSM). Image Analysis (Crocetti *et al.* submitted) was used to quantify the proportion of bacteria that were AOB.

Implementation of modern molecular biology techniques in the study of microbial ecology of nitrogen removal system in shrimp farm

Complex dominant microbial communities are detected and analyzed using molecular ecological methods. The total bacterial community DNAs from the SBR sludge is extracted, the 16S rRNA genes (16S rDNA) were polymerase chain reaction (PCR) amplified with a set of bacteria specific primers (Lene, 1991) and cloned. Restriction enzyme analysis (REA) of clone inserts was used to analyze clones in clone library. Clones having the same REA bands pattern were grouped into operational taxonomic units (OTUs) by restriction fragment length polymorphism banding profiles (RFLP). Representatives of each OTU were sequence of the 16S rDNA. Then phylogenetic identification and design of a specific probe for FISH (fluorescence *in situ* hybridization) were constructed.

Table 1. Name, %FA, sequences, target positions and specificity of the probes used in this study

Probe	%FA	Probe sequence (5'-3')	Target site	Specificity	Reference
EUBMIX					
-EUB338	0-70	GCTGCCTCCCGTAGGAGT	16S (338-355)	most bacteria	Amann *et al.* (1990)
-EUB338-II	0-50	GCAGCCACCCGTAGGAGT	16S (338-355)	Bacterial domain (Planctomycetes)	Daims et al., (1999)
-EUB338-III	0-50	GCTGCCACCCGTAGGAGT	16S (338-355)	Bacterial domain (Verrucomicrobia)	Daims et al., (1999)
NSO190	55	CGATCCCCTGCTTTTCTCC	16S (190-208)	Ammonia oxidizing β proteobacteria	Mobarry *et al.* (1996)
NEU	40	CCCCTCTGCTGCACTCTA	16S (653-670)	Halophilic and halotolerant member of the genus *Nitrosomonas*	Wagner *et al.* (1995)
NIT3	40	CCTGTGCTCCATGCTCCG	16S (1035-1048)	*Nitrobacter* spp	Wagner *et al.* (1996)
NSR1156	30	CCCGTTCTCCTGGGCAGT	16S (1156-1173)	Freshwater *Nitrospira* spp	Schramm *et al.* (1998)
NSR662	35	GGAATTCCGCGCTCCTCT	16S (662-679)	Whole genus Nitrospira, including the 4 sublineages	Daims *et al.* (2001)
NSR827	20	GTAAMCCGCCGACACTTA	16S (827-847)	Nitrospira genus	adapted from Schramm (1998)
BET 42a	35	GCCTTCCCACTTCGTTT	23S (1027-1043)	β-subclass of *proteobacteria*	Manz *et al.* (1992)
GAM 42 a	35	GCCTTCCCACATCGTTT	23S (1027-1043)	γ-subclass of *proteobacteria*	Manz *et al.* (1992)
ALF 1b	25	CGTTCGCTCTGAGCCAG	16S (19-35)	α-subclass of *proteobacteria*	Manz *et al.* (1992)
CF319a	35	TGGTCCGTGTCTCAGTAC	16S (319-336)	some Cytophagales, also one group of δ-Proteobacteria	Amann (1995)
HGC69a	25	TATAGTTACCACCGCCGT	23S (1901-1918)	Actinobacteria	Amann (1995)
LGC354c	35	CCGAAGATTCCCTACTGC	16S (354-371)	part of Low G+C Gram positive division	Meier *et al.* (1999)

Clone library preparation and analysis

DNA extraction

The biomass (500 ml) is centrifuged at 12,000 xg for 5 minutes. The supernatant is discarded, and the pellet is resuspended in 500 µl of saline-EDTA (150 mM NaCl, 100 mM EDTA [pH 8.0]). A volume of 100 µl of freshly prepared 100-mg/ml lysozyme is added to the mixture and incubated at 37°C for 1.5 hours. The mixture was then subjected to four cycles of freezing and thawing at –20°C and 65°C, respectively. Following this, 100 µl of 25% (w/v) sodium dodecyl sulfate (SDS) and 50 µl of 2% (w/v) protenase K are added to the mixture and the mixture is incubated at 60°C for 1.5 hours. The DNA is recovered from the tube by phenol-chloroform extraction. The nucleic acids from the 0.5 ml aqueous phase are precipitated by adding 0.12 ml of sterile 3 M sodium acetate and 1 ml ice- cold 100% ethanol and incubating for 1 hour at –70°C. The DNA pellet is recovered by centrifuging the solution at 12,000 x g for 20 minutes at 4 °C. The pellet is washed by adding 500 µl of 70% ice-cold ethanol then centrifugation at 12,000 *x* g for 10 minutes at 4°C. The pellet is then air dried, and the nucleic acids are dissolved in 100 µl of sterile milliQ-purified (mQ) water. Residual RNA is removed from the nucleic acid solution by adding 3 µl of 10-mg/ml RNase and incubating at 37°C for 1 hour. The DNA is visualized by agarose gel electrophoresis (Burrell and Blackall, 1998).

Polymerase chain reaction of the 16S rDNA (16S rRNA genes)

Amplification of the near complete 16S rRNA genes from the extracted DNA was done by employing the conserved bacterial primers (Lane, 1991). The PCR reagents were composed of 10x *Tth* Plus reactions buffer (Biotech International, Australia), 1.5 mM $MgCl_2$, 200 µM (each) deoxynucleotide phosphate (dNTPs), 0.3 U of *Tth* Plus DNA Polymerase (Biotech International, Australia), 1 µl of 200 ng/µl of each primer and 10-100 ng of DNA template. The sterile MQ water was added to made up a final volume of 100 µl.

Amplification reactions placed in a thermocycler (Perkin-Elmer DNA Thermal Cycler) with the cycling program of 28 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 2 min. This was followed by an extension cycle of 48°C for 2 min and 72°C for 5 min. The PCR products were detected by 1% agarose gel electrophoresis, stained with Ethidium Bromide and viewed on a UV trans-illuminator.

Cloning of the 16S rDNAs

Amplicons were cloned immediately in a ligation reaction mixture using the TA Cloning kit (Invitrogen, CA, USA). Ligation reaction was composed of 1 µl of T_4 DNA ligase (1 U/ µl), 5 µl of 10 x ligation buffer, 1 µl of pGEM-T vector (50 ng), 3 µl of amplicons (24 ng). Ligation occurred at 4 ^{o}C overnight. Ultracompetent Epicurian coli XL2-Blue MRF' cells are thawed on ice in preparation for the transformation step. A volume of 50 µl of thawed cells is gently placed in a chilled 50-ml Falcon tube, and 1 µl of β-mercaptoethanol is added. The mixture is incubated on ice for 10 minutes with gentle swirling every 2 minutes. Then, 5 µl of the ligation mixture is added to the cells, and the cells are incubated on ice for 30 minutes. A heat shock step is done by immersing the Falcon tube in a 42 ^{o}C water bath for exactly 30 seconds. Cells are then returned to ice for 2 minutes. A volume of 450 µl of preheated (42 ^{o}C) sterile NZY^{+} broth is added to each tube of transformed cells. These were then shaken at 37^{o}C for 1 hour.

A volume of 50-100 µl of transformed cells is spread inoculated onto Luria-Bertani (LB) agar plates containing ampicillin, X-Gal (5-Bromo-4-Chloro-3-indolyl-β-D-galactopyranoside, and IPTG (isopropyl-β-D-thiogalacto pyranoside (LB Ampicillin/ X-Gal/ IPTG), which are incubated at 37 ^{o}C for 12 to 16 hours and then at 4 ^{o}C for 1 hours. Positive clones (those containing 16S rDNA PCR inserts) appeared white and negative clones (no inserts) were blue. Positive clones are picked and patched onto LB Ampicillin/ X-Gal/ IPTG agar plates to ensure that the first screening is correct. Positive clones were picked, homogenized into 100 µl of sterile 20% glycerol, and stored at –70^{o}C until required. These clones constituted the clone libraries.

Sequencing DNA fragments

16S rDNA inserted gene from individual clones were amplified and grouped according to restriction fragment length polymorphism (RFLP) analysis using restriction enzyme analysis (REA). Clones having the same REA bands pattern were grouped into operational taxonomic units (OTUs). Selected clones for sequence determinations are representatives of each OUT. Sequencing reactions are comprised of 4 µl of version 3 BDT (Big Dye Terminator, ready reaction mix), X µl of SP6-T7 PCR product (50-100 ng) and 1 µl of 530f primer (25ng/l). Made up to a final volume of 10 µl with sterile MQ water. The sequencing cycle on the thermocycler consisted of 1 cycle at 96 ^{o}C for 2 minutes and 24 cycles of 50 ^{o}C for 15 second, 60^{o}C for 4 minutes and 94 ^{o}C for 30 seconds.

Analysis of sequence data

The partial 16S rDNA sequences were compiled using the software package SeqEd (Applied Biosystems, Australia). Each of the compiled sequences was compared to publicly available databases using the basic local alignment search tool (BLAST) to determine approximate phylogenetic affiliations. The sequences are also manually aligned, considering secondary structural constraints, with sequences from members of the domain *Bacteria*. Phylogenetic analysis of aligned data sets is carried out by using ARB software package and database (http://www.arb-home.de/). Phylogenetic trees were constructed by carrying out evolutionary distance analyses on the 16S rDNA alignments, using the appropriate tool in the ARB database.

Results and Discussion

Data on the composition of shrimp pond water is presented in Table 2.

Table 2. The composition of shrimp farm water from six ponds in Songkhla Province and Nakhon Si Thammarat Province

Parameter	Pond					
	1	2	3	4	5	6
NH_4^+-N (mg/l)	0.26	0.12	0.68	0.20	0.04	0.20
NO_2^- (mg/l)	0.24	0.07	0.04	0.06	0.00	0.32
COD (mg/l)	75.24	47.52	75.24	71.28	99.00	nd
Salinity (ppt)	19.40	7.20	37.00	24.60	16.50	18.30
DO (mg/l)	7.57	7.38	12.00	7.46	7.46	7.39
Conductivity (µs/cm)	nd	12.54	64.00	43.30	30.10	nd
pH	8.01	8.34	7.94	8.29	8.45	8.09

nd = not determined

COD = Chemical Oxygen Demand, DO = Dissolved Oxygen.

Note

Pond number 1, water was taken from Marine Development Center, Songkhla Province.

Pond number 2, water was taken from Singhanakorn District, Songkhla Province.

Pond number 3-5, water were taken from Ranod District, Songkhla Province.

Pond number 6, water was taken from Hoasai District, Nakhon Si Thammarat Province.

Standard values for coastal water quality are less than 0.4 mg/l ammonia and less than 0.004 mg/l nitrite. Therefore, Ranod District (0.68 mg/l ammonia) and all ponds except pond No.5 (> 0.004 mg/l nitrite) had water qualities exceeding coastal standard values. These data strongly support the notion that the shrimp farm wastewater should be treated before discharging to the environment.

Cultivation in SBR

The operation of SBRs was for the enrichment of AOB and NOB from two different source inocula - shrimp pond water and sediment (natural seed) and commercial seed cultures. The SBRs were evaluated in terms of their nitrification performance and microbial community composition. Two operating HRTs for each inoculum source were evaluated. The first was a 7 d HRT for 77 d and the second was 3.5 d HRT for 123 days. Results for ammonia removal capacity were calculated from the raw data presented in Figure 1. The natural seed was capable of ammonia removals of 85% (7 d HRT) and 92% (3.5 d HRT). The commercial seed gave 71% (7 d HRT) and 83% (3.5 d HRT) ammonia removal values.

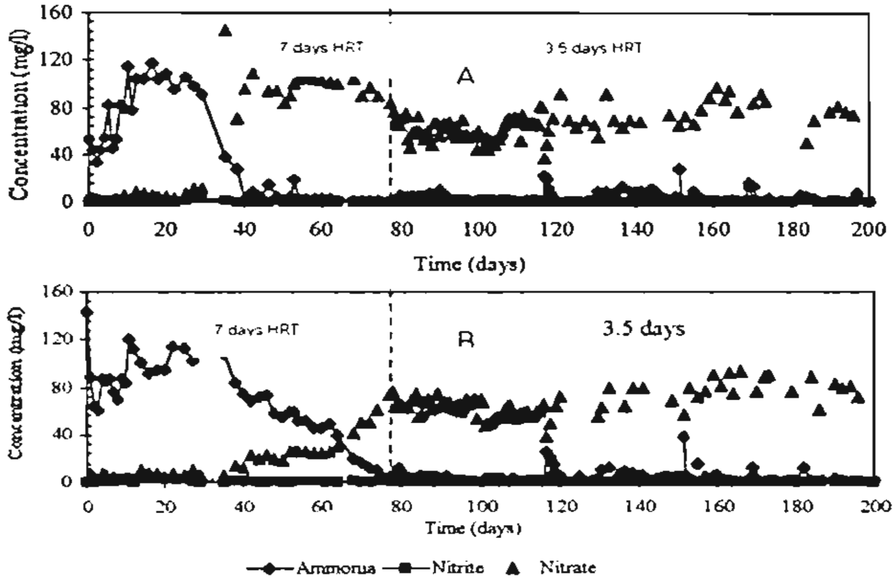

Figure 1. Data for effluent values of ammonia, nitrite and nitrate determined during the long-term operation of the SBRs at 7 d HRT and 3.5 d HRT. The source for data given in A was a shrimp farm and for the data given in B, the source was from commercial seed

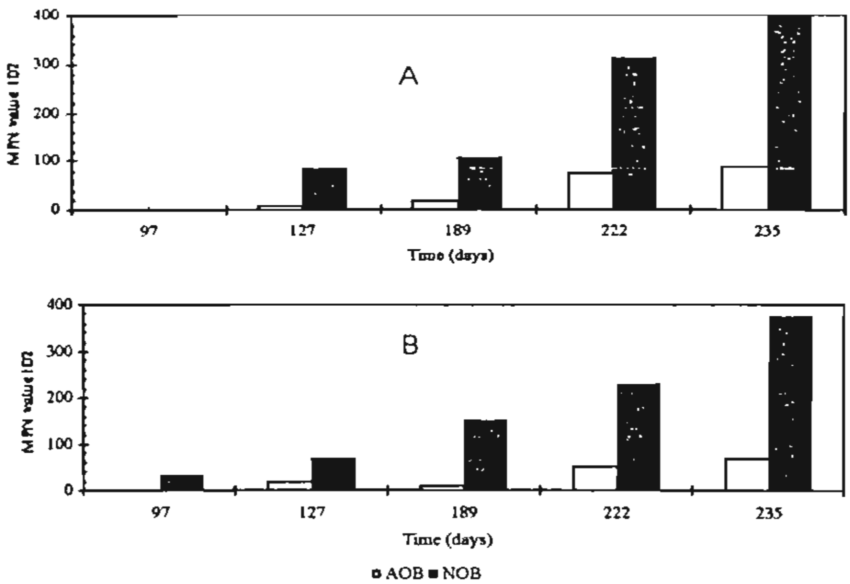

Figure 2. MPN data for AOB and NOB in the SBRs. The source for data given in A was a shrimp farm and for the data given in B, the source was from commercial seed.

MPN determination

The numbers of AOB and NOB in the SBRs using MPN are presented in Figure 2. It was clearly demonstrated that both of these populations increased in number over the operational time of the SBRs.

FISH analysis

Some bacteria enriched from the natural seed and the commercial seed hybridised with the *betaproteobacteria* AOB probe NSO190 (an example is shown in Figure 3). No microorganisms hybridised with NEU, which targets halophilic and halotolerant members of *Nitrosomonas*, including *N. mobilis*. No microorganism in either natural seed and the commercial seed enrichment hybridised with NIT3, NSR1156 and NSR662. This suite of probes should detect NOB from diverse habitats. According to the chemical analyses (Figure 1) and MPN results (Figure 2), it was concluded that NOB should be present. Clearly, the currently-available FISH probes for NOB are not appropriate to detect NOB in these enrichments suggesting that hitherto unknown NOB are present and responsible for the nitrite oxidation. Access to 16S rDNA sequence data for designing and evaluating FISH probes for NOB identification, can be obtained from 16S rDNA clone libraries from nitrifying enrichments. This have been done with the enrichments containing NOB in this study.

According to Image Analysis from FISH images, AOB from the SBR operated with the natural seed comprised 44 ± 4% of all bacteria. The AOBs comprised 61 ± 4% of all bacteria in the enrichment generated in the SBR with the commercial seed. The natural seed inoculated SBR was able to remove more ammonia with fewer AOB than the SBR inoculated with commercial seed. This result of a higher ammonia oxidation mediated by fewer AOB sourced from shrimp farms compared with commercial seed AOB should be further explored due to its practical ramifications since aquaculturists commonly use commercial nitrifier seeds in their process.

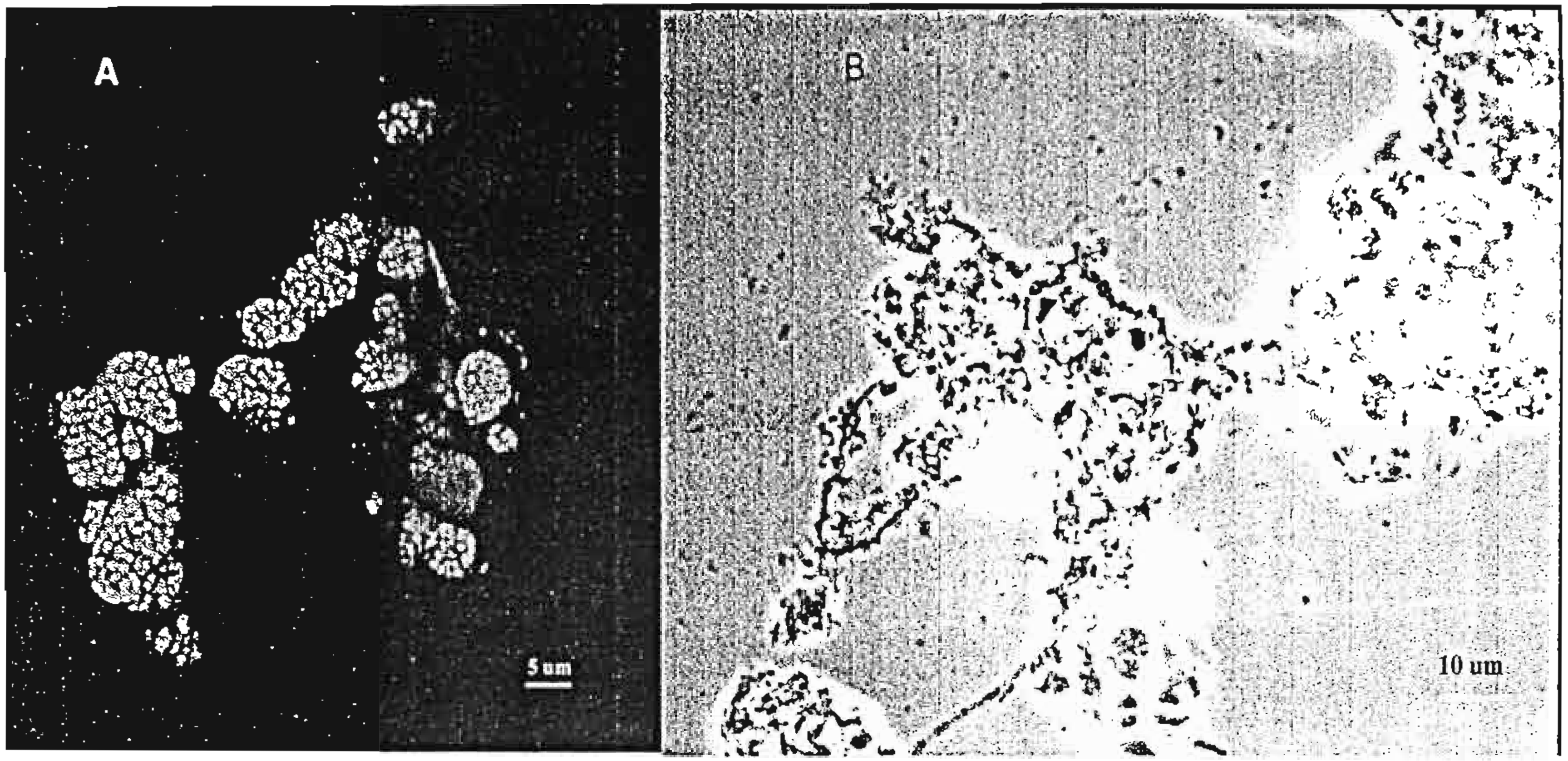

Figure 3 Micrographs of cultures from the 3.5 d HRT SBR inoculated with shrimp farm water and sediment. (A) Confocal laser scanning micrograph of culture dual hybridised with EUBMIX (blue) for all bacteria and NSO190 (red) for *betaproteobacteria* AOB. Magenta coloured cells have bound both probes and are the AOB. (B) Phase-contrast micrograph also of biomass from the 3.5 d HRT SBR inoculated with shrimp farm water and sediment demonstrating the floccular nature of the developed biomass

Clone library preparation and analysis

Restriction Enzyme Analysis (REA) has been used to analyse the nitrifying bacteria in sequencing batch reactor (NFSBR) clone library and it was found that there were 27 different OTUs from 100 selected clones (Figure 4). The information of the 27 OTUs were shown in Table 3. The 27 OTUs could be grouped into 7 bacterial division as given in Table 4. Most of the clones belong to *Cytophaga-Flavobacterium* (CFB) group, (32%) and *alphaproteobacteria* of bacterial phylum (31%). AOB is belonged to *betaproteobacteria* which accouted for 3% in clone library. In contrast, they were found abundant in sludge by FISH techniques. *Nitrobacter* were found 2 clones but none of them was found by FISH technique. It is possible that *Nitrobacter* gene from dead cell had been amplified by PCR. Thus, *Nitrobacter* clones were found in the NFSBR clone library. FISH technique and PCR were used to identify the microbial community in order to confirm the basic local alignment search tool (BLAST) result, (Table 5).

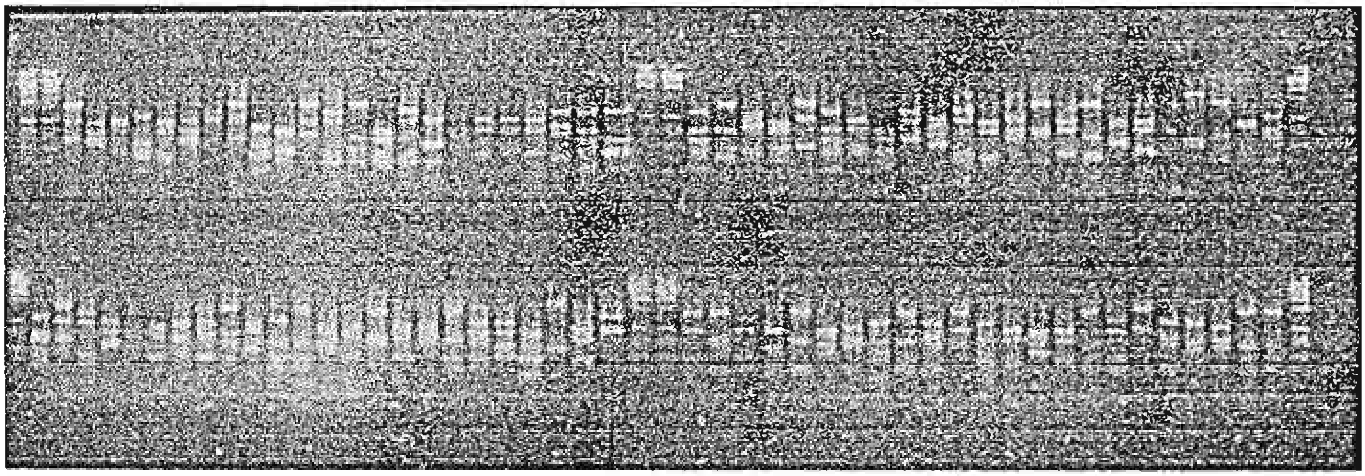

Figure 4 Digestion profiles of the operational taxonomic units (OTUs) in the Nitrifying Bacteria in Sequencing Batch Reactor (NFSBR) clone library

Table 3 Identities of the 27 different operational taxonomic units (OTUs), their frequency of occurrence during restriction enzyme analysis (REA) analysis within the Nitrifying Bacteria in Sequencing Batch Reactor (NFSBR) clone library and the BLAST identity according to partial 16S rDNA sequence

OTU	Clone member	Total	Sequence Identity
A	1.1, 11.1, 49.1, 34.2, 16.3, 3.3, 23.2	7	Gamma-Proteobacteria: *Xanthomonas*
B	2.1, 6.1, 17.1, 37.1, 1.2	5	Gamma-Proteobacteria: *Marinobacter* sp.
C	7.1	1	Gamma-Proteobacteria: *Pseudomonas* sp.
D	30.2	1	Gamma-Proteobacteria: environmental sample
E	8.2, 4.1, 23.1, 35.2, 9.1, 22.1, 24.1, 30.1, 11.3, 41.2	10	Alpha-Proteobacteria: Antarctica sediment
F	21.1, 48.1, 10.3, 16.2, 5.3, 9.3	6	Alpha-Proteobacteria: *Rhodobacter*
G	26.1, 15.2, 21.2, 17.3, 46.1, 3.2	6	Alpha-Proteobacteria: unspecified
H	27.1, 32.2	2	Alpha-Proteobacteria: unknown marine
I	32.1	1	Alpha-Proteobacteria: *Sphingomomas*
J	27.2	1	Alpha-Proteobacteria: *Rhizobiaceae; Mesorhizobium*
K	28.2	1	Alpha-Proteobacteria: *Rhodopseudomonas*
L	2.3, 6.3, 14.3	3	Alpha-Proteobacteria: *Hyphomonas* group
M	19.3, 18.3	2	Alpha-Proteobacteria: *Rhizobiaceae: Nitrobacter*
N	47.1, 44.2	2	Beta-Proteobacteria: *Nitrosomonas* sp.
O	14.3	1	Beta-Proteobacteria: *Rhodocyclus*
P	3.1, 4.2	2	Chlorobi: environmental sample
Q	18.1, 19.1, 20.1, 31.1, 36.1, 42.1, 5.2, 8.3, 48.2, 11.2, 13.2, 20.2, 36.2, 38.2, 39.1	15	CFB: *Cytophaga* sp.
R	12.1, 15.1, 35.1, 50.1, 40.2, 26.2, 50.2, 16.3, 4.3	9	CFB: environmental sample
S	29.1, 24.2, 5.1	3	CFB: *Flavobacteria*
T	13.1, 45.2, 46.2, 45.1, 39.2	5	CFB: *Flexibacter*
U	31.2	1	Fibrobacter/ Acidobacter group; *Acedobacter*
V	14.2	1	Actidobacteria: Environmental sample
W	16.1	1	Actidobacteria: *Rhodococcus* and *Nocardia*
X	41.1, 1.3, 10.2, 13.3	4	Uncultured Microbium; *Actinobacteria*
Y	37.2, 7.2, 34.1, 47.2, 13.3	5	*Actinobacteria*; unspecified
Z	33.1	1	Firmicutes: *Bacillus/ Clostridium* group
AA	10.1, 18.2	4	OP10
Total		100	

The result from FISH and clone library of AOB and NOB was different. NSO 190 hybridized specifically 14 ± 2% with sludge from NFSBR, but only 3% of them were found in clone library. Moreover, none of bacteria were hybridized with NOB probes (NIT3, NSR1156, and NSR662), while 2 clones of *Nitrobacter* were found in clone library. Thus 2 new clone libraries for AOB and NOB need to be constructed using their specific primer.

Table 4 Grouping of the 27 operational taxonomic units (OTUs) of Nitrifying Bacteria in Sequencing Batch Reactor (NFSBR) clone library into 7 groups of bacteria division

Bacterial Division	Total of Clone	Percentage of Clone
1. Proteobacteria		
- alpha	31	31
- beta	3	3
- gamma	11	11
2. Chlorobi	2	2
3. CFB	32	32
4. Firmicutes	1	1
5. Actinobacteria	11	11
6. OP10	4	4
7. Fibrobacter	1	1
Total	100	100