Table 5 Comparison on different major bacterial divisions in the Nitrifying Bacteria in Sequencing Batch Reactor (NFSBR) sludge by fluorescence *in situ* hybridisation, clone libraries and polymerase chain reaction (PCR)

Probe/Primer	Specificity	FISH(%)[a]	BLAST(%)[b]	PCR[c]
EUBMIX	Domain bacteria	any	-	-
NSO190	Ammonia oxidizing β proteobacteria	40	2 clones	Positive
NEU	Halophilic and halotolerant member of the genus *Nitrosomonas*	Not specific	na[d]	na[d]
NIT3	*Nitrobacter* spp.	Not specific	2 clones	Positive
NSR1156	Freshwater *Nitrospira* spp.	Not specific	Not specific	Negative
NSR662	whole genus Nitrospira, including the 4 sublineages	Not specific	Not specific	Positive
ALF1b	∝-subclass of proteobacteria	19 ± 2	31	na[d]
BET42a	β-subclass of proteobacteria	20 ± 2	3	na[d]
GAM42a	γ-subclass of proteobacteria	8 ± 1.3	11	na[d]
CF319a	some Cytophagales; also one group of e-Proteobacteria	25 ± 2.5	32	na[d]
HGC69a	Actinobacteria	1.3 ± 0.3	11	na[d]
LGC354c	part of Low G+C Gram positive division	Not specific	1	na[d]

[a] FISH – fluorescence *in situ* hybridisation. Percentages are of cells in the NFSBR sludge binding the EUB338 MIX probe for all Bacteria.

[b] The percentage of the total clones in the library determined by analysing sequence with BLAST.

[c] Polymerase Chan Reaction of DNA in the NFSBR sludge.

[d] na = not applicable.

Microbiology of ammonia oxidising bacteria in sequecing bathch reactor (AOSBR)

Amplified PCR product for AOB clone library was constructed using 2 primers; Nso190f; 5'-GGAGAAAAGCAGGGGATCG–3' (Mobarry *et al.* 1996) and 1492r; 3'-TTCAGCATTGTTCCATYGGCAT-5' (Lane, 1991). The cloning methods were described in methodology.

One hundred clones were selected from plates, then 50 clones were screened for full sized gene *inserted E. coli*. Only 26 clones were found inserted proper size of insert gene. After that *E coli* which having proper size insert gene were analysed by restriction fragment length polymorphism (RFLP) (Table 6). HinP1 I enzyme was used in this experiment.

Most clones belonged to OTU C and OTU A, thus, the representative clones were sequenced and compared to publicly available databases using the basic local alignment search tool (BLAST) to determine approximate phylogenetic affiliations (Table7).

Table 6 Thirteen different operational taxonomic units (OTUs) pattern and their frequency of occurrence during restriction enzyme analysis (REA) analysis within the ammonia oxidising bacteria in sequecing bathch reactor (AOSBR) clone library

OTU	Clone member	Total
A	1,30,46	3
B	3	1
C	4,7,13,11,25,16,32,36,39,41,42,44	12
D	12	1
E	14	1
F	19	1
G	22	1
H	23	1
I	28	1
J	21	1
K	33	1
L	37	1
M	43	1
Total		26

Table 7 The basic local alignment search tool (BLAST) identity according to partial 16S rDNA sequence

Accession No.	Length (bp)	Definition	%Identity
OTU A, AF272419	614	*Nitrosomonas* sp. NM 33, betaproteobacteria	96
OTU A, AF272419	339	*Nitrosomonas* sp. NM 33	98
OTU C, AJ298733	741	*Nitrosomonas* sp. Nm33, betaproteobacteria;	98
OTU C, AF272419	309	*Nitrosomonas* sp. NM 33 16S ribosomal RNA gene	95

Nitrosomonas sp. was dominant in AOSBR clone library which was the as the NFSBR clone library (using 27f and 1492r primers to amplify 16S rDNA). Thus, *Nitrosomonas sp* is responsible for AOB clone.

Microbiology of nitrite oxidising bacteria in sequecing bathch reactor (NOSBR)

Amplified PCR product for NOB clone library was constructed using 2 primers; Ntspa662f ; 5'- AGAGGAGCGCGGAATTCC -3' (Daims *et al.*, 2000) and 1492r ; 3'- TTCAGCATTGTTCCATYGGCAT-5' (Lane, 1991). The cloning methods were described in methodology. The result of restriction enzyme analysis (REA) was given in Table 8.

Total of 24 clones comprised of OTUs A while the remaining 7 OTUs were comprised of individual clones (each OTU = 1 clone, except 2 clones in OTU C). The representative clones of each OTU were sequenced and compared to publicly available databases using the basic local alignment search tool (BLAST) to determine the approximate phylogenetic affiliations (Table 9).

Table 8 Nine different operational taxonomic units (OTUs) pattern and their frequency of occurrence during restriction enzyme analysis (REA) analysis within the nitrite oxidising bacteria in sequecing bathch reactor (NOSBR) clone library

OTUs	Clone member	Total
A	1,2,4,8,10,11,13,16,22,25,26,27,29,30,31,32,33,34,38,39,41,43,47,49	24
B	6	1
C	9,19	2
D	3,7,12,14,15,17,18,20,23,37,46,48,28,42	14
E	21	1
F	40	1
G	45	1
H	44	1
I	50	1
Total		46

Table 9 The basic local alignment search tool (BLAST) identity according to partial 16S rDNA sequence

Accession No.	Length (bp)	Definition	%Identity
OTU D, AF139997	640	*Xanthomonas* sp. ML-122 gamma-Proteobacteria	95
OTU C, AF139997	672	*Xanthomonas* sp. ML-122	95
OTU A, AF139997	557	*Xanthomonas* sp. ML-122	95
OTU A, AF494542	729	*Rhodanobacter* sp. gamma-Proteobacteria	94
OTU D, AF139997	421	*Xanthomonas* sp. ML-122	90
OTU A, AF139997	507	*Xanthomonas* sp. ML-122	95
OTU B, AF139997	504	*Xanthomonas* sp. ML-122	94
OTU G, AF290420	355	*Xanthomonas campestris*; gamma-Proteobacteria	97
OTU H, AF513086	451	uncultured bacterium Bacteria	91
OTU F, AY102317	598	uncultured bacterium Bacteria	96
OTU E, AF139997	737	*Xanthomonas* sp. ML-122	95

Most of the clones were *Xanthomonas* sp. ML-122. *Nitrospira* sp. was not found in clone library. Nevertheless, after checking sequence of Nstpa 662 primer, it was hybridized specifically at the 3'-OH of *Xanthomonas*. Thus, it was not surprising to find *Xanthomonas* in the clone library. Primer specific to NOB was not found at present, thus, the nitrite enrichment was needed for the following experiment to discover the NOB in the NFSBR.

Design specific oligonucleotide probe for nitrifying bacteria in SBR containing saline artificial wastewater

Specific probes were designed using the probe design tool in the ARB software package. The design parameters were as described by Hugenholtz *et al.* (2001). Based on comparative analysis of all sequences in the ARB database consisted of publicly-available sequences and our in-house clone sequences, the program selected specific regions within the selected sequences which allowed their discrimination from all other reference sequences.

Phylogenetic analysis was performed with the full sequences 16S rDNA of NFSBR clone (NFSBR0442_full, 1479), representative members of the *Nitrosomonas* phylum and the OTU group bacteria (*Nitrosospira briensis*) (Figure 5). The 50 specific probes for NFSBR clone were shown in Table 10.

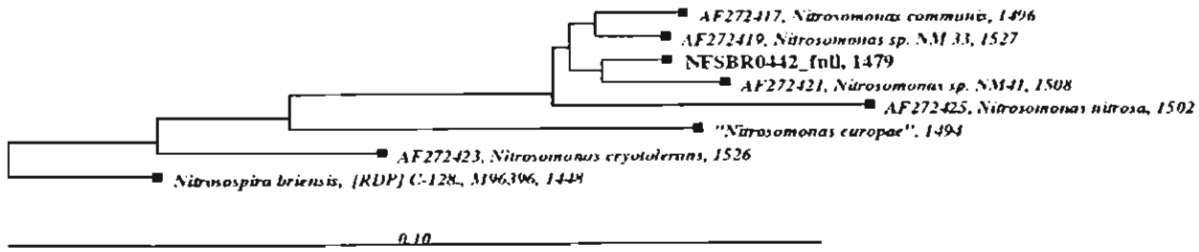
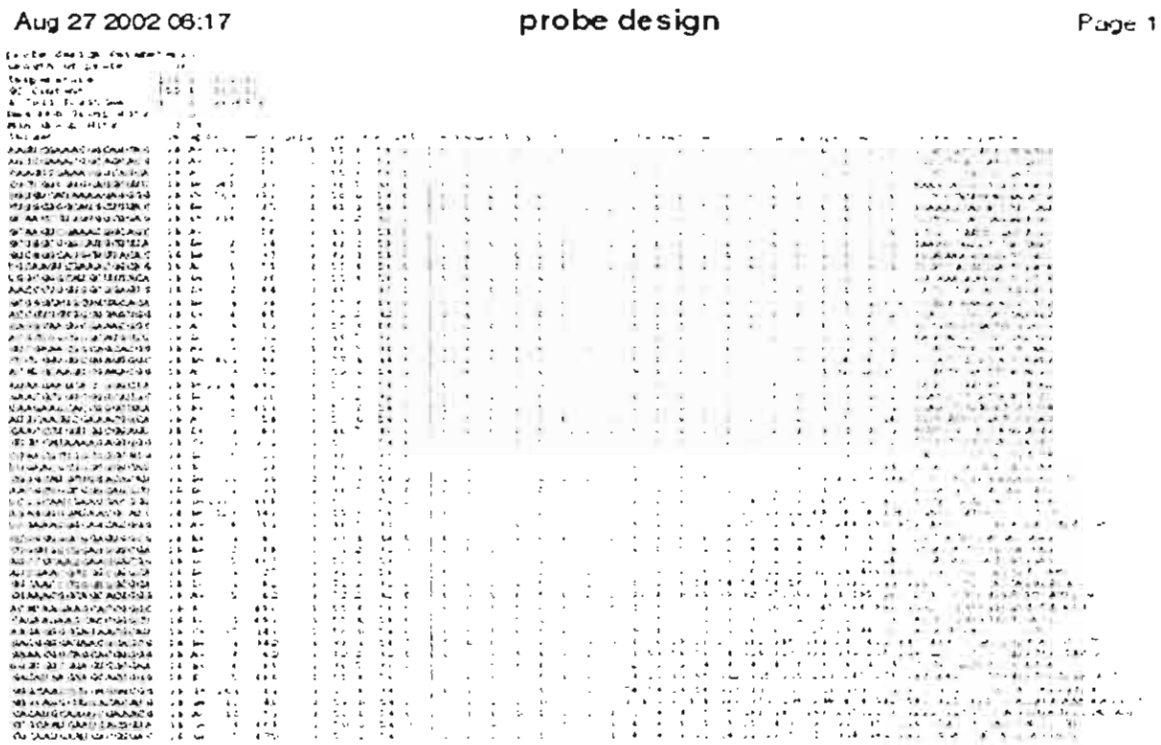


Figure 5 Evolution distance tree of NFSBR clone (NFSBR0442_full, 1479) and selected members of *the Nitrosomonas* phylum. The bar represents 0.1 estimated change per nucleotide

Table 10 The information of 50 probes specific to Nitrifying Bacteria in Sequencing Batch Reactor (NFSBR) clone according to the ARB freeware program



The most appreciated oligonucleotide probes for AOB in NFSBR were saline AOB058 and saline AOB033. Probes sequence, target site according to the *E. coli* numbering system, and melting temperature (Tm) of nearest neighbor were represented in Table 11.

Table 11 Probe sequence, melting temperature (Tm), and target position of the probes designed from this study

Probe	Tm	Probe sequence	Target site
salineAOB058	58	5'- CCGTGCTGCCGTTTCGACTT -3'	16S (58-78)
salineAOB033	59	5'- AAAGCATGCCCGCCAGCG -3'	16S (33-51)

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว. (คปก. และ วปก.)

1. ผลงานตีพิมพ์ในวารสารนานาชาติ

Paungfoo C, Prasertsan P, Intrasungkha N, Blackall L.L. and Bhamidimarri R. (2002) Microbial Community Structure in Shrimp Farm Wastewater during Treatment in SBR System. (submitted for publication in Water Science and Technology)

2. การนำผลงานไปใช้ประโยชน์

- เชิงพาณิชย์

1) เมื่อทำการวิจัยจนถึงขั้นสุดท้ายแล้ว หากมีการทำเป็นระบบที่ใหญ่ขึ้นและพัฒนาเป็นหัวเชื้อเพื่อใช้ในการเพาะเลี้ยงสัตว์น้ำ ก็สามารถผลิตจำหน่ายในท้องตลาดต่อไป

2) การจดลิขสิทธิ์ Probe/Primer ที่ได้

- เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)

งานวิจัยนี้สามารถสร้างความร่วมมือทั้งภายในประเทศและต่างประเทศ โดยภายในประเทศเป็นการสร้างความร่วมมือและทำวิจัยร่วมกัน ระหว่างอาจารย์ของมหาวิทยาลัยสงขลานครินทร์และอาจารย์ของมหาวิทยาลัยทักษิณ (ดร. นุกูล อินทรสังขา) ส่วนความร่วมมือกับต่างประเทศ ได้ร่วมมือกับอาจารย์ของ University of Queensland (Assoc. Prof. Dr. Linda Louise Blackall) ประเทศออสเตรเลีย และอาจารย์ของ Massey University (Prof. Rao Bhamidimarri) ประเทศนิวซีแลนด์

- เชิงวิชาการ

งานวิจัยเรื่องนี้ได้พัฒนานักศึกษาระดับปริญญาเอกภายใต้ทุน คปก. ให้มีความรู้ความสามารถด้านเทคนิคเชิงชีวโมเลกุล โดยเฉพาะ Phylogenetic analysis เทคนิค Fluorescence *in situ* Hybridisation (FISH) ซึ่งปัจจุบันได้มีการนำมาประยุกต์ใช้ในด้านต่างๆมากมาย และสามารถพัฒนา Probe ที่ใช้ตรวจสอบ Nitrifying bacteria ในนากุ้งต่อไป นอกจากนี้ความรู้ที่ได้รับสามารถใช้ในการพัฒนาการเรียนการสอนในระดับบัณฑิตศึกษาของภาควิชาด้วย

3. อื่นๆ (เช่นผลงานตีพิมพ์ในวารสารวิชาการในต่างประเทศ การเสนอผลงานในที่ประชุม หนังสือ การจดสิทธิบัตร)

1) ผลงานในรูปแบบของ Poster ในงาน Conference of Science and Technology of Thailand ครั้งที่ 27 ระหว่างวันที่ 16-18 ตุลาคม 2544 ที่หาดใหญ่ ในหัวข้อ Ammonia Removal from Saline Wastewater by Nitrifying Bacteria in Sequencing Batch Reactor (SBR)

2) ผลงานในรูปแบบของ Poster ในหัวข้อ Comparison between Ammonia Removal Efficiencies of Natural and Commercial Nitrifying Bacteria in Saline Wastewater by Sequencing Batch Reactor (SBR) ในงาน BioThailand 2001 ระหว่างวันที่ 7-10 พฤศจิกายน 2544 ที่กรุงเทพฯ

3) นำเสนอผลงานแบบปากเปล่าใน Environmental Biotechnology Conference ที่ Massey University, Palmerston North, New Zealand ในหัวข้อ Microbial Community Structure in Shrimp Farm Wastewater during Treatment in SBR System ระหว่างวันที่ 15-17 เมษายน 2545

ภาคผนวก

ผลงานตีพิมพ์ในวารสารนานาชาติ

Paungfoo C, Prasertsan P, Intrasungkha N, Blackall L.L. and Bhamidimarri R. (2002) Microbial Community Structure in Shrimp Farm Wastewater during Treatment in SBR System. (submitted for publication in Water Science and Technology)

Enrichment of Nitrifying Microbial Communities from Shrimp Farms and Commercial Inocula

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Abstract Nitrifying bacteria were selected from shrimp farm water and sediment ("natural" seed) in Thailand and from commercial seed cultures. The microbial consortia from each source giving the best ammonia removal during batch culture pre-enrichments were used as inocula for two sequencing batch reactors (SBRs). Nitrifiers were cultivated in the SBRs with 100 mg NH_4-N/l and artificial wastewater containing 25 ppt salinity. The two SBRs were operated at a 7 d hydraulic retention time (HRT) for 77 d after which the HRT was reduced to 3.5 d. The amounts of ammonia removed from the influent by microorganisms sourced from the natural seed were 85% and 92% for the 7 d HRT and the 3.5 d HRT, respectively. The ammonia removals of microbial consortia from the commercial seed were 71% and 83% for these HRTs respectively. The quantity of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) was determined in the SBRs using the most probable number (MPN) technique. Both AOB and NOB increased in number over the long-term operation of both SBRs. According to quantitative fluorescence *in situ* hybridisation (FISH) probing, AOB from the natural seed and commercial seed comprised 44 ± 4% and 61 ± 4%, respectively of all bacteria. NOB could not be detected with currently-reported FISH probes, suggesting that novel NOB were enriched from both sources. Taken collectively, the results from this study provide an indication that the nitrifiers from shrimp farm sources are more effective at ammonia removal than those from commercial seed cultures.

Keywords ammonia removal; effective microorganisms (EM); fluorescent *in situ* hybridization (FISH); nitrifying bacteria; 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 in this study 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

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 assessed 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 collected samples 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 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, trace element 1 ml (modified fromLiu *et al.*, 2000). Mixing and aeration was achieved by bubling air through the reactor. The reactor was operated at room temperature (28-30°C). From the numerous primary enrichments setup, five from each source (natural seed and commerical seed) were chosen for further enrichment, on the basis of ammonia removal capacity. Those with the best ammonia removal were chosen. Biomass from the 3 l reactors, demonstrating the best ammonia removal capacities were used as inocula for cultivation in sequencing batch reactors (SBRs). One enrichment from each source type (natural seed and commercial seed) was taken forward.

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

A volume of 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. Artifical seawater contained (g/l): NaCl, 25; MgCl2.7H2O, 5; CaCl2, 1; KCl, 1 (Stephen *et al.*, 1996). For each of AOB and NOB, 0.15 ml of the 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 of 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 (see above) 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.

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

Table 1. Information relevant to FISH oligonucleotides used in this study

Probe	%FA	Probe sequence (5'-3')	*E. coli* 16S rRNA position	Specificity	Reference
EUBMIX	20		338-355	Domain bacteria	
-EUB338		GCTGCCTCCCGTAGGAGT			(Amann *et al.*, 1990)
-EUB338-II		GCAGCCACCCGTAGGAGT			(Daims *et al.*, 1999)
-EUB338-III		GCTGCCACCCGTAGGAGT			(Daims *et al.*, 1999)
NSO190	55	CGATCCCCTGCTTTTCTCC	190-208	Ammonia oxidizing *betaproteobacteria*	(Mobarry *et al.*, 1996)
NEU	40	CCCCTCTGCTGCACTCTA	653-670	Halophilic and halotolerant members of *Nitrosomonas*	(Wagner *et al.*, 1995)
NIT3	40	CCTGTGCTCCATGCTCCG	1035-1048	*Nitrobacter* spp.	(Wagner *et al.*, 1996)
NSR1156	30	CCCGTTCTCCTGGGCAGT	1156-1173	Freshwater *Nitrospira* spp.	(Schramm *et al.*, 1998)
NSR662	35	GGAATTCCGCGCTCCTCT	662-679	All *Nitrospira* spp.	(Daims *et al.*, 2001)

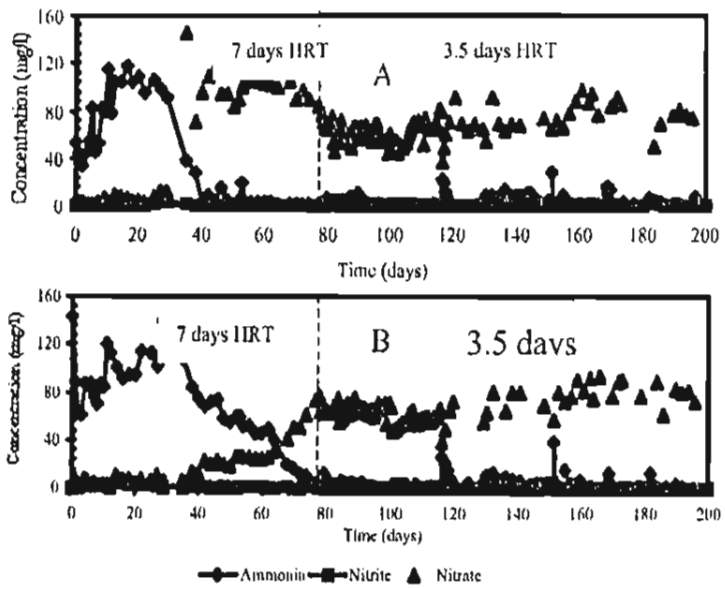

Figure 1. Data for effluent values of ammonia, nitrite and nitrate determined during the long-term operation of the SBRs at 7 d HRT and 3.5 d HRT. The source for data given in A was a shrimp farm and for the data given in B, the source was from commercial seed

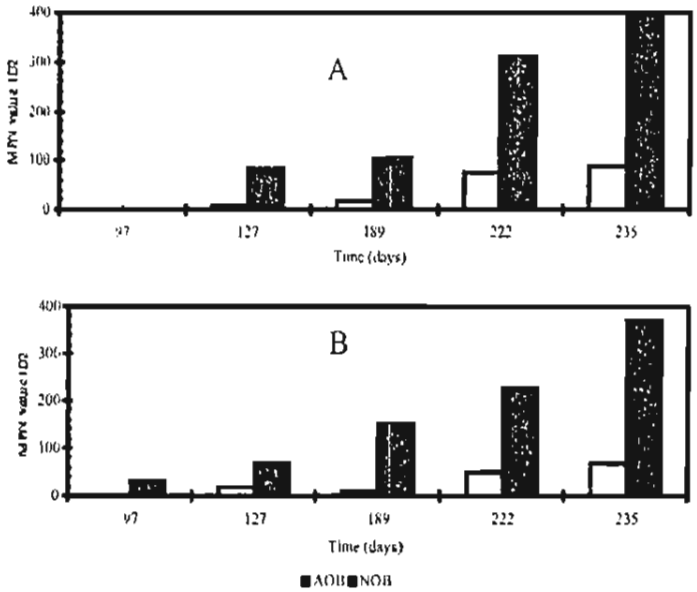

Figure 2. MPN data for AOB and NOB in the SBRs. The source for data given in A was a shrimp farm and for the data given in B, the source was from commercial seed.

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.

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 microorganisms in either 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 will now be done with the enrichments containing NOB.

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.

Conclusions

1. SBRs operating with artificial wastewater containing 100 mg NH_4-N/l and 25 ppt salinity and enriched AOB and NOB from different seed sources.
2. Both high (3.5 d HRT) and low nutrient loading (7 d HRT) were evaluated in the SBRs and nitrification was recorded for both.
3. The ammonia removed from the influent in the SBR with natural (shrimp farm sediment and water) seed was 85% and 92% for the 7 d HRT and 3.5 d HRT, respectively. The SBR with commercial seed gave 71% and 83% ammonia removed, for 7 d HRT and 3.5 d HRT, respectively.
4. The shrimp farm wastewater seed might contain highly efficacious AOB as concluded by their capacity to remove more ammonia by fewer AOB compared to those sourced from a commercial seed. This could have significant practical ramifications.

Acknowledgement

This research was financially supported by the Thailand Research Fund (TRF) and Prince of Songkla University Graduate School Fund.

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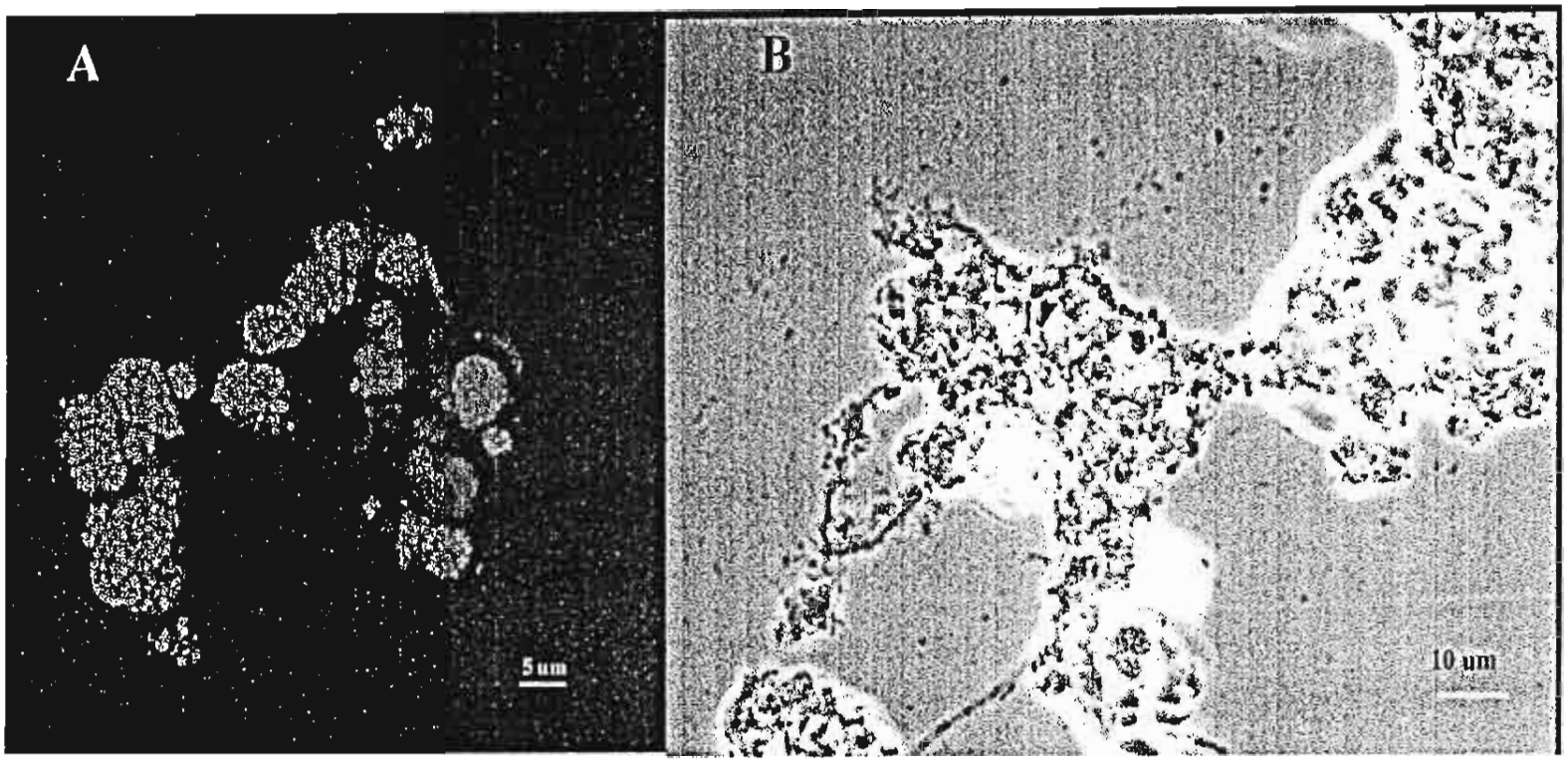

Figure 3. Micrographs of cultures from the 3.5 d HRT SBR inoculated with shrimp farm water and sediment. (A) Confocal laser scanning micrograph of culture dual hybridised with EUBMIX (blue) for all bacteria and NSO190 (red) for *betaproteobacteria* AOB. Magenta coloured cells have bound both probes and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

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