Chapter 3 Results and Discussion

Part 1: Enrichment of Nitrifying Microbial Communities from Shrimp Farms and Commercial Inocula

3.1.1. Introduction

A primary concern in shrimp farm culture systems is the toxic effect of ammonia on shrimp (Hovanec and DeLong, 1996). Nitrification takes place by two groups of autotrophic bacteria, both of which comprise slow-growing species. Both groups exist naturally in aquaculture water, but are readily washed out by frequent water exchange during the production cycle thereby impairing their potential beneficial effect on water quality (Shan and Obbard, 2001). Bioaugmentation using commercial bacterial inocula has been widely accepted (Shariff *et al.*, 2001; Devaraja *et al.*, 2002) because of strict legislation designed to prevent water pollution. In order to understand nitrifier ecology, 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.

3.1.2. Objectives

The first aim of this study was to investigate the general characteristics of water and sediment from several shrimp farms in Songkhla and Nakorn Sri Thammarat province, Southern Thailand. An additional aim was to compare the autotrophic nitrifying bacterial community from a shrimp farming system with that from a commercial seed by molecular techniques. The ammonia removal efficiencies of these two nitrifying bacterial consortia were compared.

3.1.3. Materials and Methods

3.1.3.1. Shrimp farm water composition

Biomass samples were taken from six shrimp farms (from water and sediment from Songkhla and Nakorn Sri Thammarat province, Southern Thailand) and eleven commercial microbial products (All Bactzyme, Batapure-XLSW, Bactapure-N3000, Bacillus 1070, Biobactcyme, Bigbac, Nature-Bacte, Starbact, TIM_2 Guard, TIM_3, and Treat Sludge). The shrimp farm samples were taken during the later stage of shrimp growth (week 12, 17 and 19) because this was when the highest total ammoniacal nitrogen (TAN) was produced in the ponds (Dierberge and Kiattisimkul, 1996). The samples were refrigerated (0-4 °C) until used (Takeno *et al.*, 1999). Shrimp pond water was assessed for pH (pH meter, WTW Germany), dissolved oxygen (DO meter, WTW, Germany), salinity (salinometer, WTW, Germany), conductivity (WTW, Germany), chemical oxygen demand (APHA, AWWA and WPCF, 1985), and NH_4 -N, NO_2 -N and NO_3 -N (Spectrometer NOVA 60, Merck, Ltd., Germany).

3.1.3.2. Enrichment of nitrifying bacteria population in SBR

Ten ml of collected samples was used in enrichments with Modified Alexander medium (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 primary enrichments which demonstrated nitrification to 3 L reactors containing 1 L of artificial wastewater (modified from Liu *et al.*, 2000). Mixing and aeration was achieved by bubbling air through the reactor. One enrichment from each source type ("natural seed" from shrimp pond samples and "commercial seed" from commercial microbial products), demonstrating the best ammonia removal capacities were then used as inocula for cultivation in two separate 3 L sequencing batch reactors (SBRs) which were constructed and operated similarly.

Air bubbling facilitated aeration and a 24 h-cycle consisted of 10 min filling, 21 h aeration reaction, 2 h 40 min settling, and 10 min supernatant

withdrawal (Figure 9). Support media made from perforated plastic were placed in the SBR for encouraging bacterial attachment. A volume of 430 ml of modified artificial wastewater containing 100 mg NH_4-N/L was fed to the SBRsand 430 ml removed during the withdrawal period 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. pH and dissolved oxygen were also monitored. Biomass was observed in unstained preparations and by Gram staining. When the SBRs reached steady state after 77 d of operation, a HRT of 3.5 days was applied in a 4 step 12-h cycle. The aeration reaction step was reduced from 21 h to 9 h.

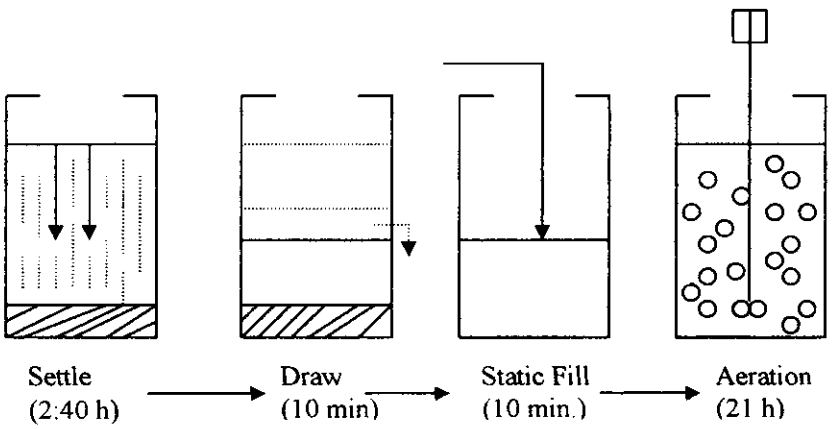

Figure 9. The operation of one SBR cycle in four discrete time periods of settle, draw, static fill and aeration.

3.1.3.3. Enumeration of AOB and NOB using most probable number (MPN) techniques

Sample from both SBRs at different periods of cultivation were obtained for enumeration of AOB and NOB using the MPN technique as detailed in Chapter 2 (2.2.1).

3.1.3.4. Enumeration of microbial community by fluorescence *in situ* hybridization (FISH) analysis

The microbial community and the proportion of nitrifying bacteria in the SBRs were analyzed by FISH (Chapter 2- 2.2.2) using oligonucleotide probes detailed in Table 7.

3.1.4. Results and Discussion

3.1.4.1. Shrimp farm water composition

Shrimp farm wastewaters are generally characterized by high values of BOD, salinity, total suspended solids, chlorophyll (a), orthophosphate, nitrite, nitrate and ammonia-nitrogen (Chaiyakam and Songsangjinda, 1992). The salinity (5-38 ppt) and NH_3-N are the most important components of shrimp pond waters likely to impact natural water bodies and shrimp survival. Standard values for coastal water quality are < 0.4 mg/l ammonia and < 0.004 mg/l nitrite (Standard coastal water quality group, 1991). Data on the composition of shrimp pond waters from this study were presented in Table 8. They demonstrate that 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. Chaiyakam and Songsangjinda, (1992) found 7 of 15 studied ponds contained NH_3-N more than 0.4 mg/l. Chaiyakam *et al.* (1992) and Tunvilai *et al.* (1993) (cited by Dierberg and Kiattisimkul, 1996) reported that all 7 studied shrimp ponds contained > 0.004 mg/l NO_2-N. All these data strongly support the notion that the shrimp farm wastewaters exceed natural water levels of ammonia and nitrite and they should be treated before being discharged to the environment.

Table 7. Information relevant to FISH oligonucleotides probes bound specifically to nitrifying bacteria.

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)
NSO1225	35	CGCCATTGTATTACGTGTGA	1224–1243	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)
Ntspa662	35	GGAATTCCGCGCTCCTCT	662-679	All *Nitrospira* spp.	Daims *et al.* (2001)

Table 8. Data of the composition of shrimp farm water from six ponds in Songkhla Province and Nakhon-Sri-Thammarat Province

Parameter	Ponds number					
	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-Sri-Thammarat Province.

3.1.4.2. Enrichment of nitrifying bacteria population in SBR

SBRs were specially operated to enrich autotrophic nitrite oxidizing bacteria by employing organic-carbon-free synthetic wastewater and nitrite. Only bacteria capable of fixing carbon dioxide, or utilizing bicarbonate, were able to grow (autotrophically). Therefore, the synthetic wastewater forced the microbial community of the activated sludge toward one strongly dominated by autotrophic nitrifying bacteria.

Minimal biomass was produced in both SBRs (Figure 10), likely due to the relatively slow growth rate of autotrophic nitrifiers (Painter, 1970; Biston, 1994). The microbial community in the biomass from the 3.5 d HRT SBRs contained both Gram positive and Gram negative bacteria of many different shapes (e.g. cocci, bacilli, and spiral). Gram negative bacteria were more abundant in both samples (Figure 11). Nitrifying bacteria are typically Gram-negative (Madigan *et al.*, 2000) and in the case of the SBRS, were assumed to be the abundant nitrifiers in the system. Phase-contrast microscopy demonstrated a floccular nature of the developed biomass and tight clustering of microorganisms (Figure 11). Nitrifying bacteria in other enrichment cultures are often reported as tightly packed or clustering (Allison and Prosser, 1992; Daims *et al.*, 2001).

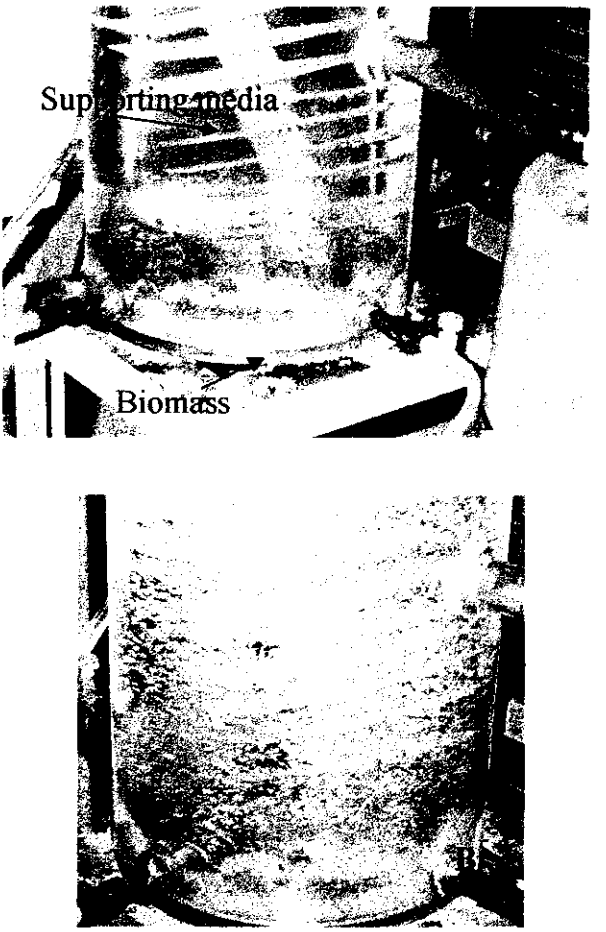

Figure 10. Photographs of the slow growing autotrophic nitrifiers biomass in nitrifying bacteria sequencing batch reactor (NFSBR) at the settling (A) and aeration (B) SBR phase.

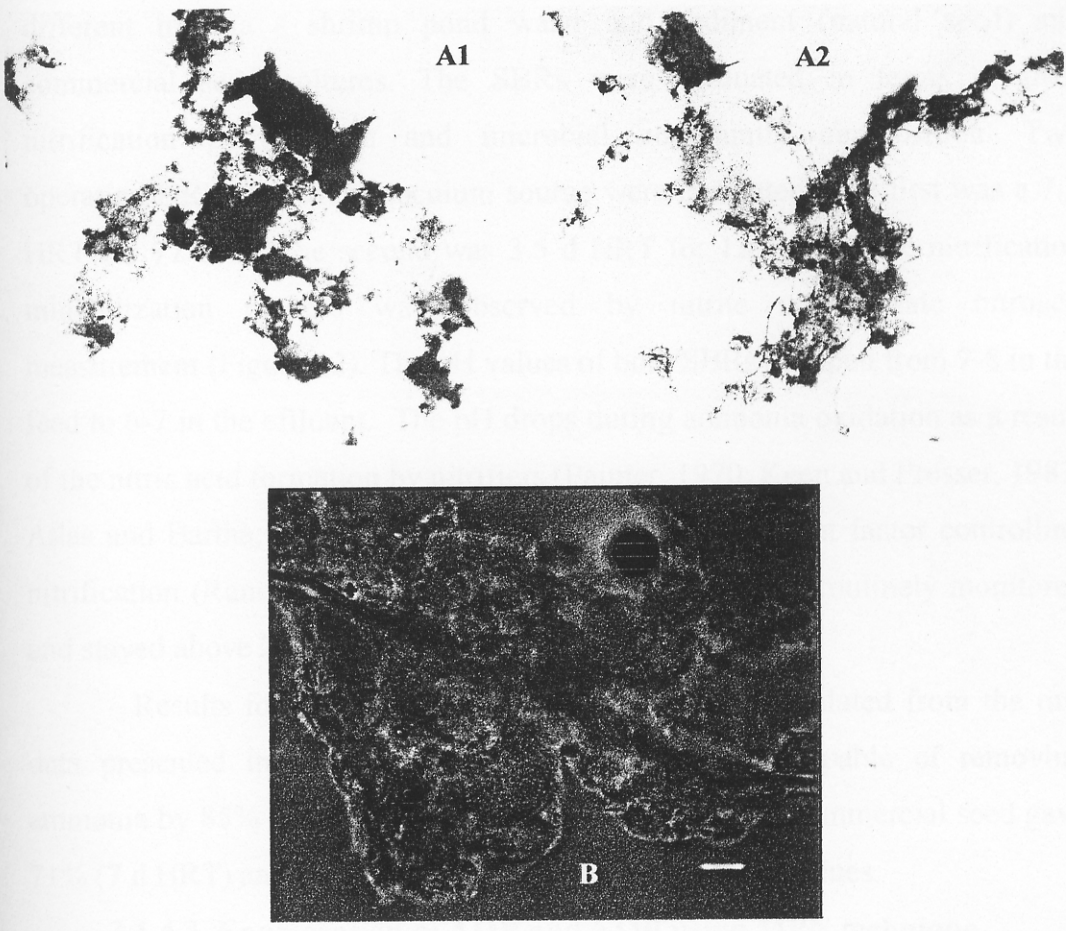

Figure 11. Micrographs of cultures from the 3.5 d HRT SBRs. (A) Gram staining demonstrating the Gram negative bacteria were found to be abundant in both SBRs; SBR the source was from shrimp farm (A1) and commercial seed (A2). (B) Phase-contrast micrograph of biomass from the biomass from the 3.5 d HRT SBR inoculated with shrimp farm water and sediment demonstrating the floccular nature of the developed biomass and the characteristic of microorganisms arranged in tightly packed clusters.

The operation of SBRs was to enrich AOB and NOB from two different 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. The nitrification mineralization process was observed by nitrite and nitrate nitrogen measurement (Figure 12). The pH values of both SBRs dropped from 7-8 in the feed to 6-7 in the effluent. The pH drops during ammonia oxidation as a result of the nitric acid formation by nitrifiers (Painter, 1970; Keen and Prosser, 1987; Atlas and Bartha, 1993). DO concentration is an important factor controlling nitrification (Randall, 1992), thus the DO of effluent was routinely monitored and stayed above 2 mg/l.

Results for ammonia removal capacity were calculated from the raw data presented in Figure 12. The natural seed was capable of removing ammonia by 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.

3.1.4.3. Enumeration of AOB and NOB using MPN technique

Data for AOB and NOB in the SBRs using MPN are presented in Figure 13. It was clearly demonstrated that both of these populations increased in number over the operational time of the SBRs. These results can be correlated with chemical analyses. The ammonia nitrogen concentrations decreased as a result of ammonia oxidation by AOB. NOB presence was confirmed by increases in NO_3-N concentrations. Not unexpectedly, NO_2-N build-up was not observed (Figure 12).

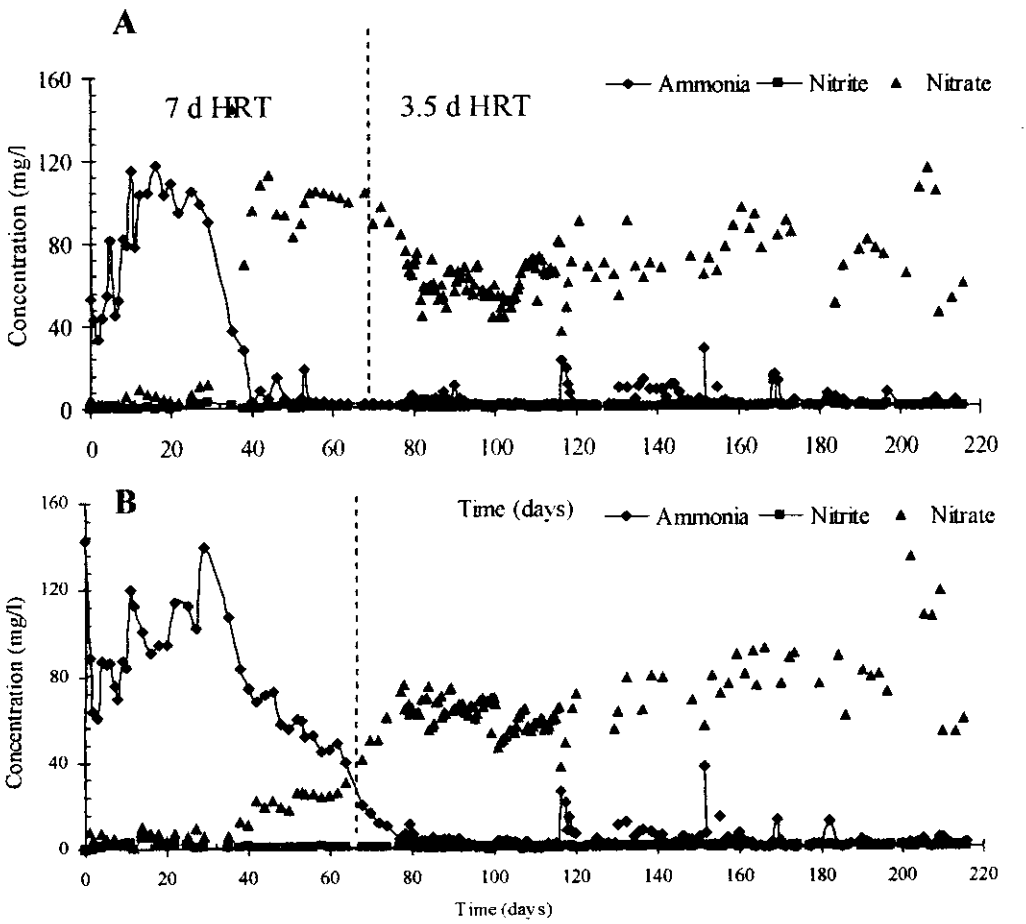

Figure 12. Data for effluent values of ammonia, nitrite and nitrate determined during the long-term operation of the SBRs at 7 d HRT and 3.5 d HRT. The source for data given in A was a shrimp farm and for the data given in B, the source was from commercial seed

The quantity of AOB in the wastewater treatment process and water environment is an important factor questioning nitrogen removal (Konuma *et al.*, 2001). The MPN technique has been used for enumeration of AOB (Matulevich *et al.*, 1975). However, due to their long generation time, their cluster formation and the difference in the culturability of different AOB species, AOB are likely underestimated by this technique (Wagner *et al.*, 1995; Araki *et al.*, 1999).

3.1.4.4. Enumeration of nitrifying bacteria by FISH technique

Some bacteria enriched from the natural seed and the commercial seed hybridized with the *Betaproteobacteria* AOB probes NSO190 and NSO1225 (an example is shown in Figure 14). No microorganisms hybridized with an additional *Betaproteobacteria* AOB probe NEU, which targets halophilic and halotolerant members of *Nitrosomonas*, including *N. mobilis*. No microorganisms in either enrichment hybridised with NIT3 (for the NOB *Nitrobacter* in the *Alphaproteobacteria*), NSR1156 and Nstpa662 (both for different NOB *Nitrospira* groups in the *Nitrospira* phylum (Daims *et al.*, 2001)). Collectively, this suite of probes should detect NOB from diverse habitats. According to the chemical analyses (Figure 12) and MPN results (Figure 13), 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 were present and responsible for the nitrite oxidation. Access to 16S rRNA gene sequence data for designing and evaluating FISH probes for NOB identification, can be obtained from 16S rRNA gene clone libraries from nitrifying enrichments. The enrichments containing NOB in this research were exploited for this purpose (part3).

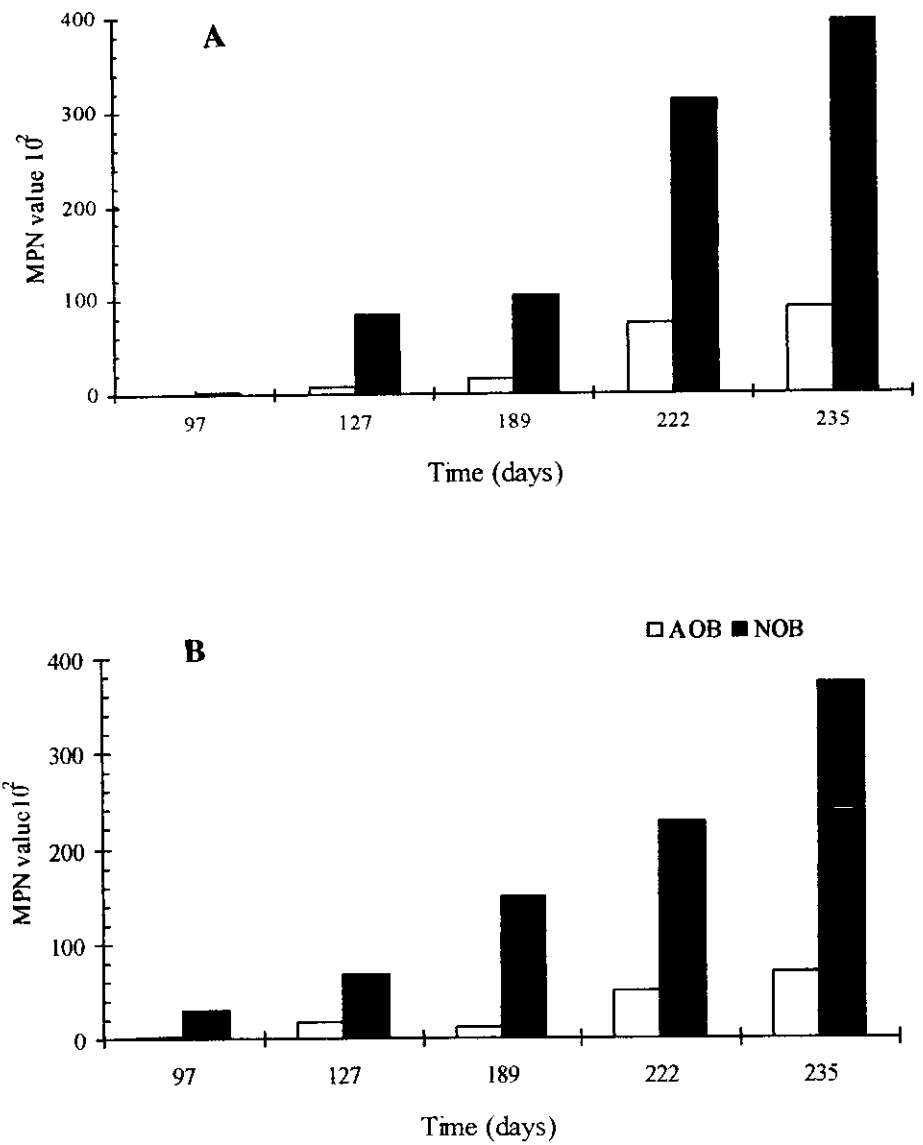

Figure 13. MPN data for AOB and NOB in the SBRs. The source for data given in A was a shrimp farm and for the data given in B, the source was from commercial seed.

According to Image Analysis from FISH images (Figure 14), AOB from the SBR operated with the natural seed comprised 21± 2% of all bacteria. The AOB comprised 30 ± 2% 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 (85% (7 day HRT) – 92% (3.5 day HRT) ammonia removal) with fewer AOB than the SBR inoculated with commercial seed (71% (7 day HRT) – 83% (3.5 day HRT) ammona removal). 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.

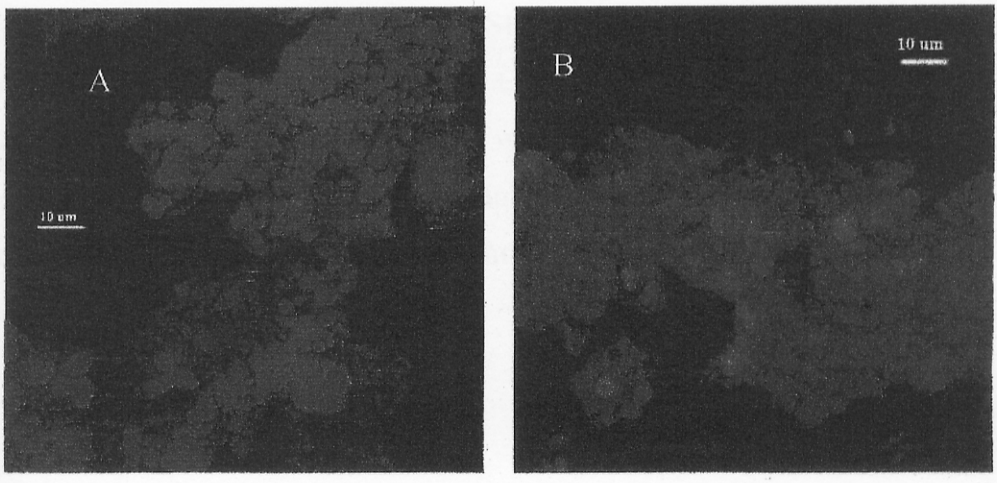

Figure 14. Confocal laser scanning micrograph of culture from the 3.5 d SBRs inoculated with shrimp farm water and sediment (A) and commercial seed (B). Image of FISH is dual hybridised with EUBMIX (blue) for all bacteria and NSO190 (red) for *Betaproteobacteria* AOB. Magenta coloured cells have bound both probes and are AOB.

3.1.5. Conclusions

SBRs operating with artificial wastewater containing 100 mg NH_4-N/l and 25 ppt salinity were able to enrich AOB and NOB from different seed sources. Both AOB and NOB increased in number over the long-term operation of both SBRs indicated by chemical analysis and most probable number (MPN) determinations. Both high (3.5 d HRT) and low nutrient loading (7 d HRT) were evaluated in the SBRs and nitrification was recorded for both. The ammonia removed from the influent in the SBR with natural seed (shrimp farm sediment and water) 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 removal for 7 d HRT and 3.5 d HRT, respectively.

According to quantitative FISH probing, AOB from the natural seed and commercial seed comprised 21 ± 2% and 30 ± 2%, respectively of all bacteria, therefore, the shrimp farm water and sediment seed might contain highly efficacious AOB as more ammonia was removed by fewer AOB compared to those sourced from a commercial seed. This could have significant practical ramifications.

Chapter 3 Results and Discussions

Part 2: The Microbiology of Bacteria in Sequencing Batch Reactor for Nitrifying Bacteria (NFSBR)

3.2.1. Introduction

In order to understand the nitrifiers ecology in shrimp farm, a previously enrichment (Part 1) of nitrifying bacteria composed of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) from shrimp farm inoculum in sequencing batch reactors (NFSBR) was studied by molecular method. The initial identification and partial quantification of the nitrifying bacteria (AOB and NOB) were carried out by fluorescence *in situ* hybridization (FISH) using previously published 16S rRNA-targeting oligonucleotide probes. The published NOB probes for *Nitrobacter* and *Nitrospira* did not hybridize to any bacterial cells. Therefore new communities of NOBs, differing from previously reported ones, exist in the enrichments. Molecular genetic techniques such as cloning, sequencing and phylogenetic analysis of the 16S rRNA genes from the nitrifying enrichments were performed in efforts to identify the AOB and NOB.

3.2.2. Objectives

To provide an insight into the bacterial diversity of nitrifying bacteria sequencing batch reactor (NFSBR) use molecular genetic techniques. Three clone libraries with the different primers set will be constructed and compared; one from the bacteria universal primers (27f and 1492r) and others from AOB and NOB specific primer regarding to focus on the present of member of the nitrifying bacteria.

3.2.3. Materials and Methods

3.2.3.1. Sample collection

The biomass in the following studied came from The NFSBR which operating stably for full nitrification from ammonia to nitrate and AOB and NOB were clearly present in the bioreactor biomass (Chapter 3, part 1).

3.2.3.2. Culture dependent analysis

Mixed liquor was taken from NFSBR after 1 year and 2 years SBR operation and washed twice with phosphate buffer saline (PBS; 135 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 1.75 mM K_2HPO_4 [pH 7.5] (Burrell *et al.*, 1998) resuspended in PBS and serially diluted to a final dilution of 10^{-3}. A volume of 100 µl of cell suspension from each dilution was spread onto modified artificial wastewater (as same with media in the SBR) solid agarose plates (1% w/v). The plates were then incubated at 28°C until growth occurred. Colonies were picked followed a range of colonies with different morphologies and cultivation time.

Individual colonies were picked and sub-cultured until they were seemed to be pure by being observed via Gram staining. The nitrifier isolates were investigated by Fluorescence *in situ* hybridization (FISH) techniques using AOB specific probe (NSO 190), and NOB specific probe (NIT3 or NSR662). The 16S rDNA from isolates which showed positive with AOB or NOB probes will be sequenced for identification purposes. Each of the sequences was compared to publicly available databases using the basic local alignment search tool (BLAST) to determine approximate phylogenetic affiliations.

3.2.3.3. Fluorescence *in situ* hybridization (FISH)

Samples from the NFSBR were fixed in 4% paraformaldehyde and analysed the microbial ecology by FISH using oligonucleotide probes detailed in Table 9 and methods described in the section 2.2.2, Chapter 2.

3.2.3.4. Clone library preparation and analysis

Biomass from the NFSBR (2 ml) was centrifuged at 12,000 x g for 5 min. The DNA was extracted following the protocol described in Chapter

2 (2.2.3.1). Amplification of the near complete 16S rRNA genes from the extracted NFSBR DNA was done by the methods described in the Chapter 2, (section 2.2.3.2) using the conserved bacterial primers 27f and 1492r in a PCR. A second AOB specific PCR was carried out with the extracted NFSBR DNA using Nso190 (Table 9) as a forward primer (190f) and 1492r in a PCR. The third clone library was not possibly constructed because none of known NOB probes were detected NOB in the sample.

The PCRs were separately cloned immediately following the description in section 2.2.3.3, Chapter 2. The two libraries were prepared – a bacterial library and an AOB library. Clones containing expected length inserts (ca. 1,500 bp for the bacterial library and 1,300 bp for the AOB library) were determined, picked, homogenized in 100 µl of sterile 20% glycerol and stored at –70°C until required.

Clones were screened and partial 16S rDNA sequences as described in the section 2.2.3.4, Chapter 2. The RFLP was analysed using *Hin*P1 (BioLabs Inc., New England), *Hin*P1 is restriction enzyme that recognized and cuts the tetranucleotide sequence -5' G-CGC 3'.

The partial 16S rDNA sequences were analysed as described in the section 2.2.3.5, Chapter2.

Table 9. Oligonucleotide probes used in the microbial ecology study of the NFSBR

Probe	%FA	Probe sequence (5'-3')	Target site	Specificity	Reference
EUB338 [a]	0-70	GCTGCCTCCCGTAGGAGT	16S rRNA (338-355)	most Bacteria	Amann *et al.* (1990)
EUB338-II	0-50	GCAGCCACCCGTAGGAGT	16S rRNA (338-355)	Bacterial domain (Planctomycetes)	Daims *et al.* (1999)
EUB338-III	0-50	GCTGCCACCCGTAGGAGT	16S rRNA (338-355)	Bacterial domain (Verrucomicrobia)	Daims *et al.* (1999)
NSO190	55	CGATCCCCTGCTTTTCTCC	16S rRNA (190-208)	ammonia oxidizing *β*-Proteobacteria	Mobarry *et al.* (1996)
NSO1225	35	CGCCATTGTATTACGTGTGA	16S rRNA (1224–1243)	ammonia oxidizing *β*-Proteobacteria	Mobarry *et al.* (1996)
NEU	40	CCCCTCTGCTGCACTCTA	16S rRNA (653-670)	halophilic and halotolerant members of the genus *Nitrosomonas*	Wagner *et al.* (1995)
Nsv443	30	CCGTGACCGTTTCGTTCCG	16S rRNA (444–462)	*Nitrosospira* spp.	Mobarry *et al.* (1996)
NmV	35	TCCTCAGAGACTACGCGG	16S rRNA (174–191)	*Nitrosococcus mobilis* ("*Nitrosomonas*") lineage	Juretschko *et al.* (1998)
NIT3	40	CCTGTGCTCCATGCTCCG	16S rRNA (1035-1048)	*Nitrobacter* spp.	Wagner *et al.* (1996)
NSR1156	30	CCCGTTCTCCTGGGCAGT	16S rRNA (1156-1173)	Freshwater *Nitrospira* spp.	Schramm *et al.* (1998)
Ntspa662	35	GGAATTCCGCGCTCCTCT	16S rRNA (662-679)	4 sublineages of *Nitrospira*	Daims *et al.* (2001)
NSR827	20	GTAAMCCGCCGACACTTA	16S rRNA (827-847)	*Nitrospira* genus	Schramm *et al.* (1998)
Ntspa714	55	CCTTCGCCACCGGCCT	16S rRNA (714-729)	Phylum Nitrospira	Daims *et al.* (2001)
ALF1b	20	CGTTCGCTCTGAGCCAG	16S rRNA (19-35)	alpha subclass of Proteobacteria	Manz *et al.* (1992)
BET42a	35	GCCTTCCCACTTCGTTT	23S rRNA (1027-1043)	beta subclass of Proteobacteria	Manz *et al.* (1992)
GAM42a	35	GCCTTCCCACATCGTTT	23S rRNA (1027-1043)	gamma subclass of Proteobacteria	Manz *et al.* (1992)
CF319a	35	TGGTCCGTGTCTCAGTAC	16S rRNA (319-336)	*Cytophaga-Flavobacterium* cluster of CFB phylum	Manz *et al.* (1996)
HGC69a	25	TATAGTTACCACCGCCGT	23S rRNA (1901-1918)	gram-positive bacteria with high DNA G+C content	Roller *et al.* (1994)
LGC354a [b]	35	TGGAAGATTCCCTACTGC	16S rRNA (354-371)	part of Firmicutes	Meier *et al.* (1999)
LGC35b	35	CGGAAGATTCCCTACTGC	16S rRNA (354-371)	part of Firmicutes	Meier *et al.* (1999)
LGC354c	35	CCGAAGATTCCCTACTGC	16S rRNA (354-371)	part of Firmicutes	Meier *et al.* (1999)

[a] EUBMIX comprised equal amounts of EUB338, EUB338-II and EUB338-III. [b] LGC354 combined equal amounts of LGC354a, LGC354b and LGC354c .

3.2.4. Results and Discussions

3.2.4.1. Culture dependent analysis

Although 16S rRNA studies combined with genomic analyses of naturally suggested the existence of metabolic functions, a comprehensive understanding of the physiology of these organisms, and of the complex environmental processes in which they engage, will undoubtedly require their cultivation. Twelve colonies from the both sources of biomass (4 colonies of 1 year cultivation, 8 colonies of 2 years cultivation) were selected regarding to a range of colonies with different morphologies at the different cultivation times. All 9 colonies were sub-cultured more than 3 times to ensure that the cultures were pure. Then the isolates were found both Gram positive and negative bacteria, but mainly were Gram negative bacteria.

The AOB investigation of the isolates using FISH technique was shown that NSO190 (AOB specific probe) bound with none of them, thereby, no traditionally recognized ammonia oxidizing *Betaproteobacteria* were isolated from the NFSBR inoculation spread plates. The NOB isolates were not currently possibly investigated according to the known NOB specific probes were not bound with the NFSBR biomass.

The 16S rDNA of some isolates were randomly sequenced for identification purposes. Two isolates from the 2 years NFSBR biomass were sequenced. One of them was 99 percent similarity with closet BLAST match to *Vibrio* sp. QY102, accession number AY174868. Another isolate was 97 percent similarity with closest BLAST match to *Marinobacter excelentus* strain KMM 3809, accession number AY180101. The one isolate from 1 year NFSBR biomass was 99% closest BLAST match to *Vibrio diazotrophicus* (ATCC 33466T), accession number X74701.

Vibrio spp. are Gram-negative, facultative aerobic rods and curved rod that posses a fermentative metabolism. 16S rDNA sequence analysis revealed the phylogenetic affiliation to the members of the family *Vibrionaceae*, subdivision of *Gammaproteobacteria* (Priest and Austin, 1995). Most of Vibrio

are aquatic (either fresh or marine), found wildly habitat (Madigan *et al.*, 2000). Therefore, it was possible that these microorganisms could have been growing by any number of mechanisms in the aerobic environment of the reactor but they were not actively growing.

Marinobacter excelentus is a novel marine bacterium, isolated from green alga *Ulva fenestrata*, seawater and bottom sediment sample collected in the Gulf Peter the Great of the Sea of Japan by Gorshkova *et al.*, (in press_ published online 17 October 2003). It is heterotrophic, Gram-negative, yellow pigmented, aerobic, oxidase and catalase positive bacteria. 16S rDNA sequence analysis revealed that the strains studied were the members of the family *Flavobacteriaceae*. The phylum *Cytophaga Flavobacterium-Bacteroides* is found abundant in the environment, according to broad physiological repertoire and the ability to synthesize a variety of either cell-bound or extracellular degradative enzyme like cellulase, chitinase, or protease. Therefore they are very important members of microbial food chain and commonly found in high abundance from various habitats by tools of microbial ecology (Weller *et al.*, 2000). Glockner and other (1999) found that members of the *Cytophaga-Flavobacterium* cluster were the most abundant group detected in the marine systems, accounting for 18% (range, 2 to 72%) of the 4', 6-diamidino-2-phenylindole (DAPI) counts. Thereby, it was not surprise that this bacterium was found in the reactor feeding with 25 ppt artificial wastewater.

The results from FISH and 16S rDNA sequence illustrated that no autotrophic nitrifiers were successfully isolated. There were heterotrophs obtained on the plate both one and two years NFSBR biomass even the organic carbon sources were not present in the media. Prosser and Embley (2002) mentioned that omission of organic carbon discourages, but does not eliminate heterotrophs, which grow on organic by-products of ammonia oxidiser growth and on volatile organic compounds or contaminants of media and culture vessels.

Nitrifying bacteria were realized that very difficult to isolate in pure culture (Allison and Prosser, 1992; Fouratt *et al.*, 2003). Allison and Prosser (1992) described two main reasons for this; firstly, colony development on solid medium is slow and colony sizes are small, making transfer difficult; and secondly, heterotrophs growing on organic compounds produced by nitrifiers grow at faster rates and are therefore difficult to remove, even when cultures are grown in an inorganic liquid medium with no organic contaminants.

3.2.4.2. Fluorescence *in situ* hybridization (FISH)

Nso1225 and Nso190 theoretically target most of the recognized ammonia-oxidizers of the *Betaproteobacteria* with the exception of *Nitrosococcus mobilis* (Mobarry *et al.*, 1996). In our enrichments, Nso1225 and Nso190 collectively targeted about 87% of cells also targeted by BET42a (for *Betaproteobacteria*, Table 10, and Figure 15). Many of the cells targeted by Nso1225, Nso190 and BET42a appeared to be relatively small and formed spherical, tightly packed clusters, whereas others were slightly larger and formed looser aggregates. No cells bound NEU, Nsv443 or NmV probes for other recognised AOB. Therefore, neither *Nitrosospira* (targeted by Nsv443) nor *Nitrosococcus mobilis* (targeted by NmV) existed in our enrichment. The inconsistent binding of several reported AOB probes has been documented by others and there also appears to be a broad phylogenetic diversity within the AOB from a number of different environmental settings (Purkhold *et al.*, 2000). The approximately 13% of BET42a targeted cells not targeted by Nso1225 or Nso190 in our nitrifier enrichment could be novel AOBs. Bacterial clusters were also found that did not bind any of Nso1225, Nso190 or BET42a probes (Figure 15). These clusters could have been nitrite oxidizers, denitrifiers or, less likely, unknown AOBs. We therefore used 16S rRNA gene cloning in attempts to identify the putative other AOB in our nitrifier enrichment.

Table 10. Percentages of EUBMIX-targeted cells in the NFSBR sludge hybridizing group-specific probes by FISH analysis.

Probe	FISH (%)
Nso190[a] + Nso1225[a]	21 ± 2
ALF1b[b]	19 ± 2
BET42a[b]	24 ± 2
GAM42a[b]	8 ± 1.3
CF319a[b]	25 ± 2.5
HGC69a[b]	1.3 ± 0.3

[a] Probes specific for some *Betaproteobacteria* AOB. No cells were targeted by NEU, Nsv443, or NmV, which are specific for recognised *Betaproteobacteria* AOBs. No cells were targeted by NIT3, NSR1156, Ntspa662, Ntspa714 or NSR827, which are specific for some for recognised NOBs (*Nitrobacter* and *Nitrospira*).

[b] Probes specific for different phyla and subphyla of Bacteria – see Table 9. No cells were targeted by LGC354 probe.

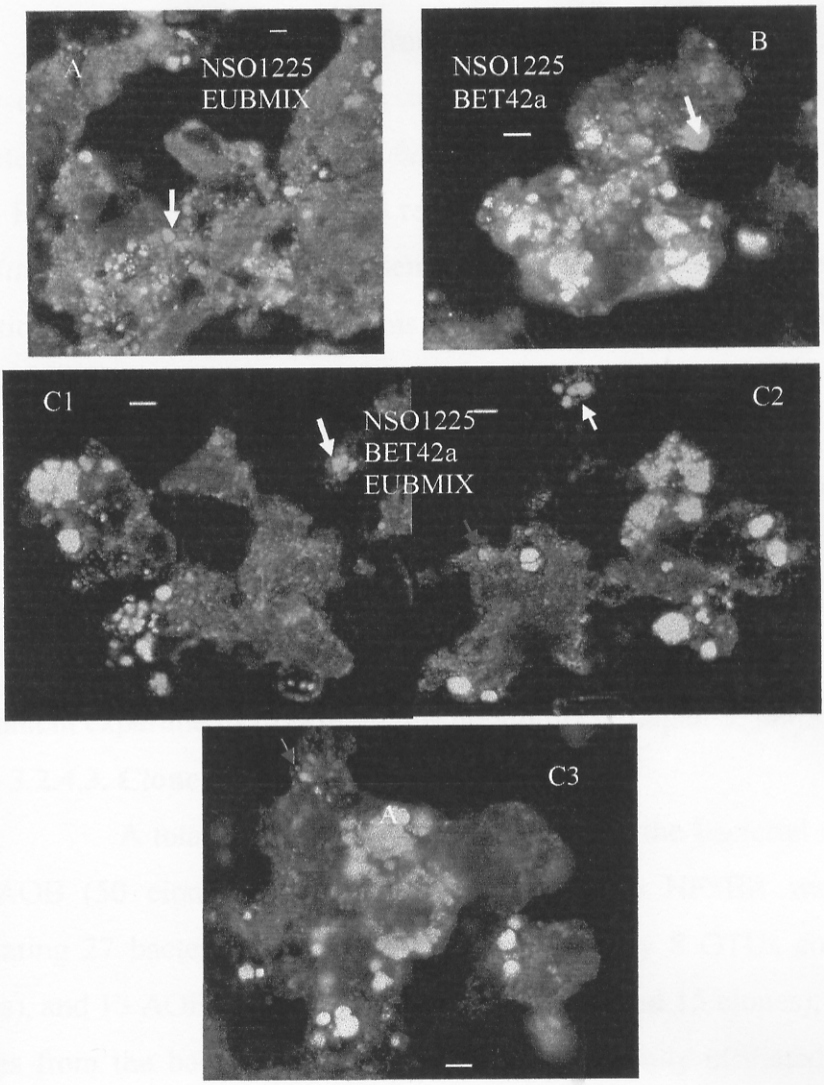

Figure 15. Confocal laser scanning micrographs of FISH from a laboratory-scale NFSBR. Probes are indicated in Table 9; the bar on all images are represents 10 um. In all cases the colors of each probe are indicated in parentheses after the probe name. In superimposed images, the overlap between green and blue is cyan, between red and green is yellow, and between red, green and blue is white. Images (A), (B) and (C) show different probe combinations in the same image of biomass from the laboratory-scale NFSBR. (A) NSO1225(red) and EUBMIX (green). The majority of the ammonia betaproteobacteria-binding cells also bind either NSO1225 and EUBMIX (yellow), for unknown cluster bacteria bind only EUBMIX (green, arrowed). (B) NSO1225 (red) and BET42a (green). There are some of the *betaproteobacteria*-binding cell are not bind NSO1225 (green, arrowed). (C1, C2 and C3) NSO1225 (red); BET42a (blue) and EUBMIX (green). Clusters of white cells are *Betaproteobacteria* AOBs (have bound all probes), cyan-colored cells are other *Betaproteobacteria* (have bound EUBMIX and BET42a, pink arrowed) and green-colored cells are all other bacteria (have only bound EUBMIX, white arrowed).

Process performance data from the SBR and MPN analyses confirmed nitrite oxidation activity in the system and the presence of nitrite oxidisers (Chapter 3, part 1). However, *Nitrobacter* cells were not detected by FISH with NIT3. Recently, much of the NOB research in freshwater systems have shown that *Nitrospira* are more likely present and potentially are responsible for nitrite oxidation activity in water systems than *Nitrobacter* (Burrell *et al.*, 1998; Juretschko *et al.*, 1998; Daims *et al.*, 2001). However, no cells in enrichment bound NSR1156, Ntspa662, Ntspa714 or NSR827 (Table 10), which collectively target most known *Nitrospira*. Therefore the hypothesis has been made that new communities of marine NOBs, possibly related to *Nitrospira marina* and differing from freshwater nitrite oxidizers, may exist in our marine water enrichments. Further investigations were carried out using NOB enrichment experiments to explore this possibility (Chapter 3, part3).

3.2.4.3. Clone library analysis

A total of 150 16S rDNA clones from the bacterial (100 clones) and AOB (50 clones) libraries prepared from the NFSBR were selected, generating 27 bacterial library OTUs (of which only 8 OTUs contained > 3 clones), and 13 AOB library OTUs (2 OTUs comprised 15 clones), (Figure 16). Clones from the bacterial library were phylogenetically affiliated with seven distinct bacterial phyla (50-60 clones sequenced, Table 11), whilst 16 clones from 26 of the clones from the AOB library were *Betaproteobacteria* (5 clones sequenced from 3 OTUs).

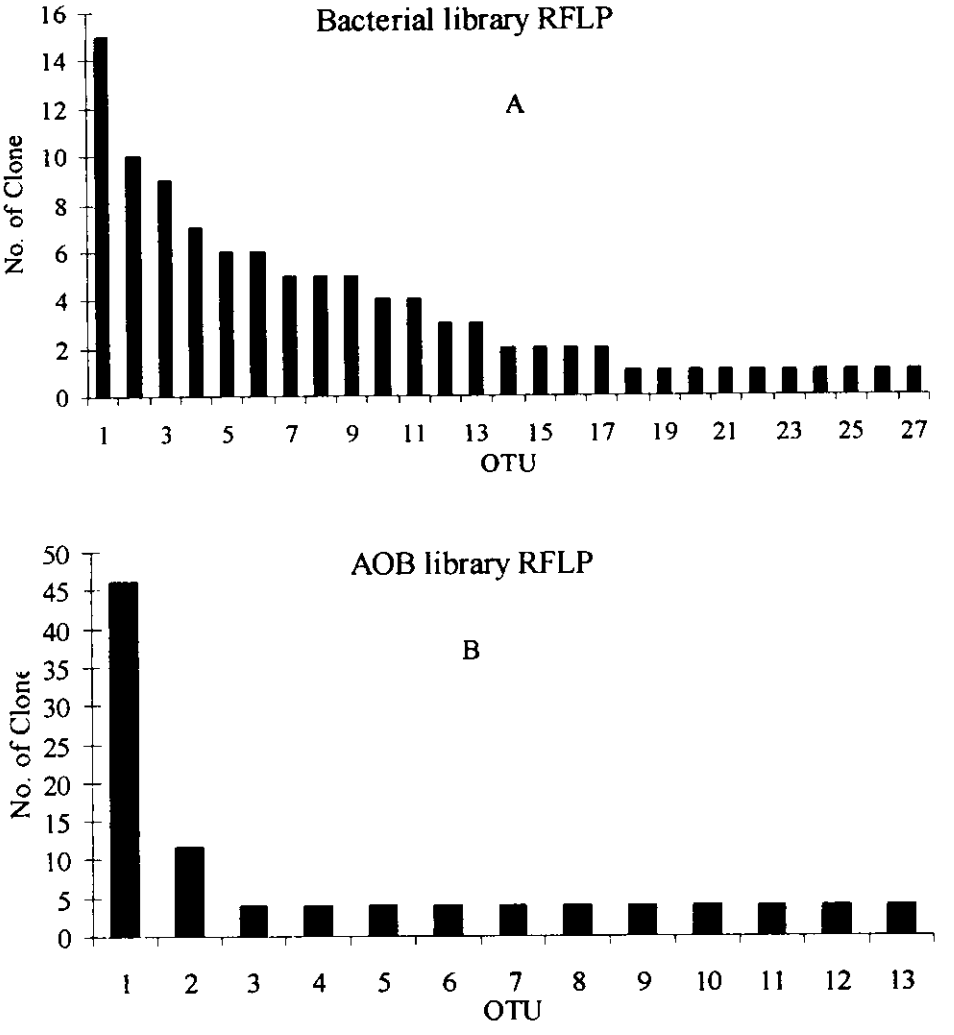

Figure 16. The percentage of clones in operational taxonomic units (OTUs) from (A) Bacterial and (B) AOB clone libraries.

Table 11. Phylogenetic affiliations of the bacterial clone library (27f-1492r) from the NFSBR

Bacterial group	Percentage of all clones
1. Proteobacteria	
Alphaproteobacteria	31
Betaproteobacteria	3
Gammaproteobacteria	11
2. Chlorobi	2
3. Cytophaga-Flavobacterium of Bacteroidetes	32
4. Firmicutes	1
5. Actinobacteria	11
6. OP10	4
7. Fibrobacter	1
Total clones	100

Nearly half of the bacterial library clones belonged to the *Proteobacteria* phylum. Two 16S rDNA clone sequences (Bact0471 and Bact0442) were fully sequenced-see the full 16S rDNA sequence in Appendix 7, according to BLAST were most closely related to *Nitrosomonas nitrosa*, in the *Betaproteobacteria* (Figure 17). Analysis of the AOB clone library revealed that most of clones (e.g. AOB012, AOB025 and AOB030-see full 16S rDNA sequences of these clones in appendix 8) were also closely related to *Nitrosomonas nitrosa*. The full 16S rDNA sequencing of representative five AOB clones are most closely related to *Nitrosomonas nitrosa* and fallen within the *Nitrosomonas communis* cluster. They were the only AOB-like clones in either library. All these five sequences contained the Nso1225 probe target, while three clones (AOB030, Bact0442 and Bact0471) did not contain the

Nso190 probe target. Therefore, these sequences are not useful for determining who are the potential novels AOBs in the enrichment.

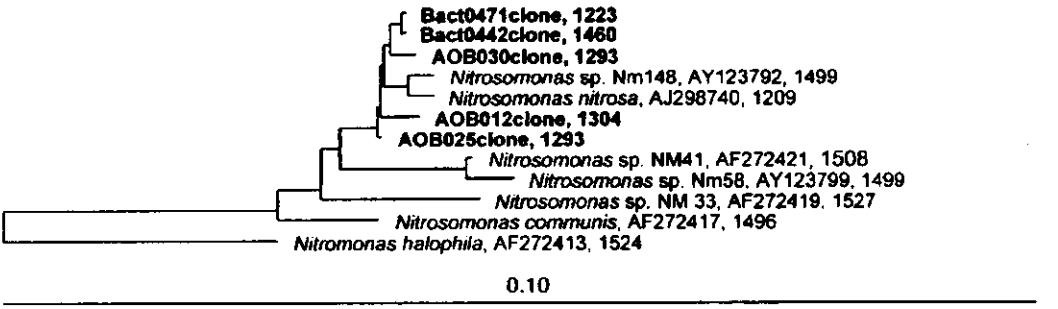

Figure 17. Evolutionary distance tree of the five *Nitrosomonas* clones found in bacterial library ("Bact" coded sequences) and the AOB library ("AOB" coded sequences) together with selected members of the *Nitrosomonas* genus. The bar represents 0.1 estimated changes per nucleotide.

Additionally, the digestion profile of the *Nitrosomonas*- affiliated OTU of Bacterial clone library (Bact0442, Bact0471) and AOB clone libraries (e.g. AOB030, Bact0442 and Bact0471) was also shown the same digested 16S rDNA pattern (Figure 18). From this observation, it was confirmed the right of the sequencing and analysis of the sequence result.

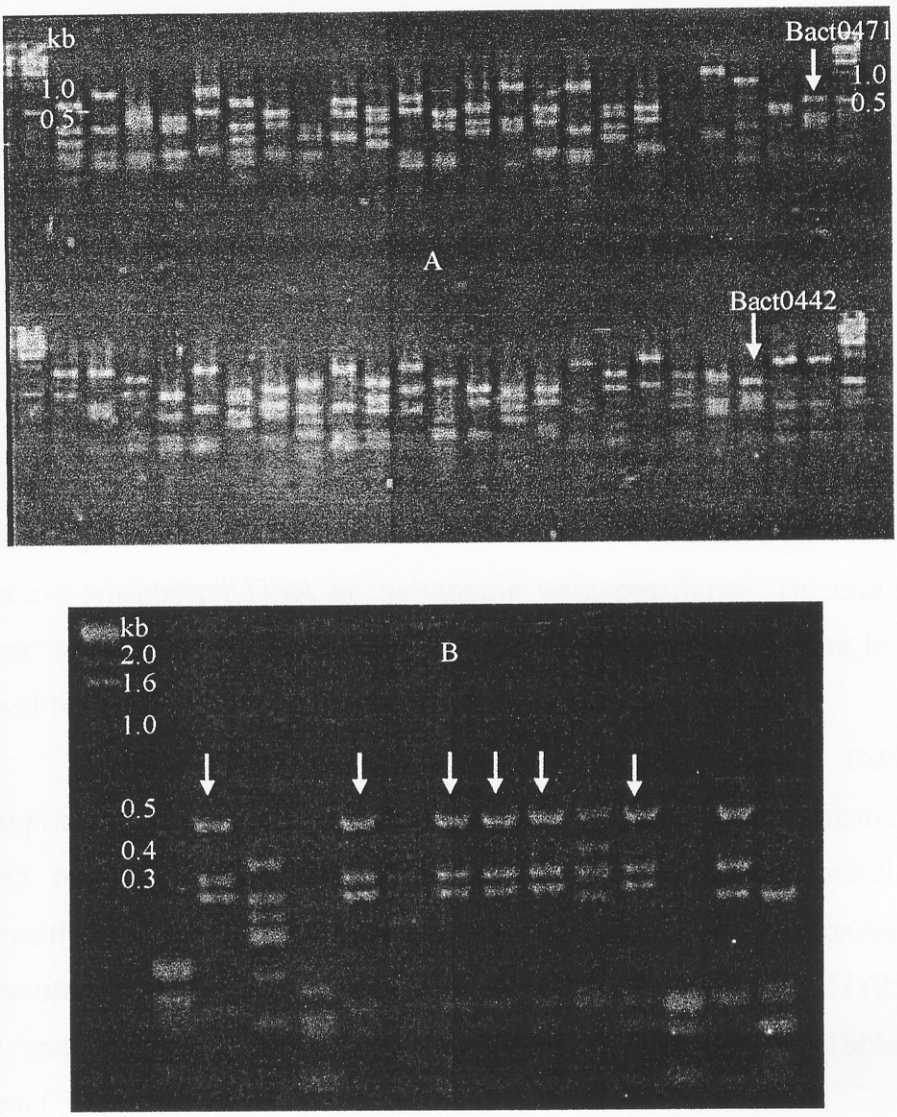

Figure 18. Digestion profiles of the operational taxonomic units (OTUs) by restriction enzyme *HinP* I, of the Bacterial (A) and AOB (B) clone libraries. The digested 16S rDNA was running on 3% agarose gel electrophoresis. Bact0471 and Bact0442 have the same OTU digestion pattern with the mainly AOB clones (arrowed).

It has been acknowledged that clone frequency does not necessary accurately describe community structures (Snaidr *et al.*, 1997; Crocetti *et al.*, 2000). This must be considered in the light of the 2 clones belonged to *Nitrosomonas* from Bacterial clone library. Only a quantitative method such as FISH will support the dominant of AOB in the NFSBR sludge (21± 2% of all bacteria, Table 10).

With respect to known nitrite oxidizing bacteria, only two *Nitrobacter* clones (from *Alphaproteobacteria*; Table 11) were found in the bacterial clone library and no *Nitrospira* clones were detected. Nevertheless, *Nitrobacter*-specific probe (NIT3) was not bound with the NFSBR sludge, thus, amplified inactive-Nitrobacter DNA in the cloning were possibility. Therefore, none of these sequences were useful for determining the identity of the hypothetical novel NOBs.

Clones from the *Cytophaga-Flavobacterium* of *Bacteroidetes* comprised 32% of the bacterial library. However, these organisms have not been reported to be neither AOB nor NOB in any microbial systems. Nevertheless, it was interesting to note that even though cloning is not a quantitative method, the most common bacteria detected by FISH (25%, Table 10) and the most abundant clones (32% of all bacterial clones, Table 11) were from *Cytophaga-Flavobacterium* of *Bacteroidetes*.

3.2.5. Conclusions

The nitrifying bacteria isolates from both NFSBR were not bound with any of known ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) specific probes, therefore, none of traditionally recognized AOB and NOB were isolated from the NFSBR inoculation spread plates.

From the identification of NFSBR microbial community by FISH demonstrated that two dominant bacterial groups were *Cytophaga-Flavobacterium* of *Bacteroidetes* and *Betaproteobacteria* while the abundant

AOBs were Nso1225 and/or Nso190 binding cells (21± 2% of all bacteria). However, potentially novel AOBs are present in the NFSBR biomass since clusters of cells similar to the morphology of nitrifier clusters bound BET42a but none of the AOB-specific probes. No cells bound any of the published NOB-specific probes. Therefore, the NOBs present in this bioreactor are all novel. However, none of the clone sequences shed any light on the hypothetical novel AOBs or NOBs present in the NFSBR.

The cloning results of bacterial clone library- using bacterial specific primers (27f and 1492r) and AOB clone library- using AOB specific primers (190f and 1492r) recovered most of clones that were highly identical to *Nitrosomonas nitrosa* in the *Nitrosomonas communis* cluster.

Chapter 3

Part 3: The Microbiology of Nitrite Oxidizing Bacteria in Low and High Nitrite Sequencing Batch Reactor (LNOBSBR, HNOBSBR)

3.3.1. Introduction

In part 2 of Chapter 3, involving the operation of a saline ammonium oxidizing sequencing batch reactor (nitrifying sequencing batch reactor or NFSBR), it was observed that the enriched nitrifying biomass did not contain either *Nitrobacter* or *Nitrospira* cells when employing *Nitrobacter* and *Nitrospira* specific fluorescent *in situ* hybridization (FISH) oligonucleotide probes. Therefore an hypothesis has been made that a novel marine nitrite oxidizing bacteria (NOB) consortium, possibly containing *Nitrospira marina* or different bacteria to freshwater nitrite oxidizers, may exist in this marine water enrichment. Investigations were carried out using NOB enrichment experiments to explore this possibility.

3.3.2. Objectives

There were 2 aims of this study:-

1. to generate an enrichment of autotrophic nitrite oxidizing bacteria (NOB) in two sequencing batch reactors (SBRs) using an organic-carbon-free mineral salts medium feed supplemented with low and high concentrations of nitrite, and

2. to analyze the enriched NOB culture by both pure-culture-dependent (MPN) and pure-culture-independent (16S rRNA gene cloning and analysis) methods to explore the NOB communities.

3.3.3. Materials and Methods

3.3.3.1. NOB enrichment in sequencing batch reactors (SBRs)

The sludge from a previously studied nitrifying sequencing batch reactor (NFSBR) was taken and further enriched separately in two SBRs (Figure 19) containing nitrite and modified artificial wastewater feed (Chapter 3, part 1). A low nitrite oxidizing SBR (LNOBSBR) was operated with 0.24 mg/l NO_2-N and a high nitrite oxidizing SBR (HNOBSBR) was operated with 50 mg/l NO_2-N in the feed. The SBRs were fed 230 ml of feed per day, mixed and aerated with air (to 3-6 mg/l DO) and operated in a 12 h-cycle time (3.5 days HRT, infinite SRT) consisting of 20 min filling, 10.5 h aeration reaction, 40 min settling, and 30 min decanting. The pH of the effluent was monitored regularly to ensure that the pH of the SBRs remained stable (7-8) and this was achieved by the addition of 10% $NaHCO_3$ solution.

In addition, supporting substrata made from perforated plastic were placed in the system to encourage bacterial attachment. The rate of nitrification was monitored daily by measuring the concentrations of NO_2-N and NO_3-N from both the influent and effluent using a Spectroquant NOVA 60 (Merck Ltd Co, Germany). After the system had reached a steady state (no detectable nitrite in the effluent) at Day 48, the nitrite concentrations of the feed for the LNOBSBR and HNOBSBR were increased to 5 mg/l and 100 mg/l NO_2-N, respectively.

pH (pH meter, WTW Germany) and dissolved oxygen (DO meter, WTW, Germany) were measured as previously described. Mixed liquored volatile suspended solids (MLVSS) measurements were done after the NOBSBR had been operating for 50 days. Duplicate 10 ml samples of mixed liquor were taken during the aeration period. These were filtered onto predried Whatman GC/F filters and then dried to a constant weight at 105° C. This residue was ignited at 550° C for 15 min in a muffle furnace (APHA, 1992).

Cycle studies of the SBRs were performed by sampling the mixed liquor at regular periods (every 15 minutes until nitrite concentration was almost undetectable then hourly). The specific nitrite oxidation rates were

calculated from NO_2-N oxidized/hr and MLVSS data to give mg NO_2-N oxidized/hr/g VSS.

Gram stains and wet mounts were observed the morphologies and clustering characteristics of the nitrite oxidizing biomass from LNOBSBR and HNOBSBR. Photomicrographs were taken on an OLYMPUS DP50 (Japan) microscope.

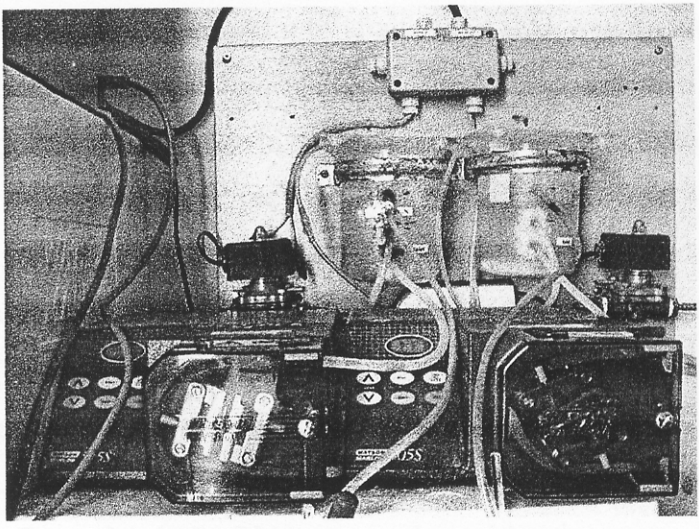

Figure 19. Photograph of the low nitrite oxidizing bacteria sequencing batch reactor (LNOBSBR) and high nitrite oxidizing bacteria sequencing batch reactor (HNOBSBR)

3.3.3.2. Enumeration of NOB using Most Probable Number (MPN)

The MPN method for enumerating nitrifying bacteria in LNOBSBR and HNOBSBR was modified from McCaig *et al.* (1999) as previously reported (Chapter 2, section 2.2.1).

3.3.3.3. Attempted isolation of NOB using pure-culture-dependent method

Mixed liquor was sampled from the LNOBSBR and HNOBSBR at Day 72 and washed twice with phosphate buffered saline (PBS), resuspended in PBS and serially diluted to a final dilution of 10^{-3}. A volume of 100 µl of

cell suspension from each dilution was spread inoculated onto plates of modified artificial wastewater (basal medium composition as in LNOBSBR/HNOBSBR) solidified by agarose (1% w/v) and containing either 5 mg/l NO_2-N or 100 mg/l NO_2-N. The plates were incubated at 28°C. Individual colonies were picked and sub-cultured until they were deemed to be pure (Gram staining).

The 16S rRNA gene of putative pure cultures was amplified by PCR after DNA extraction, sequenced and analyzed. FISH was also carried out on the "pure cultures" using known NOB-specific probes (Table 12). The DNA was extracted from lysed cells in sterile MQ water at 94° C for 10 min in a thermocycler (name the machine). The 16S rRNA genes were amplified by PCR as described in Chapter 2, section 2.2.3.2 and sequenced by 530f. The sequences were compared to those in publicly-available databases using the Basic Local Alignment Search Tool (BLAST, Altschul *et al.*, 1990) to determine approximate phylogenetic affiliations

3.3.3.4. Community 16S rRNA gene cloning and analysis

Bacterial 16S rRNA gene clone libraries were prepared from genomic DNA extracted from the biomass taken from LNOBSBR and HNOBSBR after 4 months of enrichment. The methods of DNA extraction, amplification of the 16S rRNA genes, cloning, sequencing and analysis of sequences were described in the Chapter 2, section 2.2.3.

3.3.3.5. Fluorescence *in situ* hybridization (FISH)

Biomass samples from both SBRs were analysed by FISH using oligonucleotide probes detailed in Table 12 using the FISH method previously described (Chapter 2, section 2.2.2).

Table 12. Information relevant to FISH oligonucleotides used in this LNOBSBR and HNOBSBR

Probe	%FA	Probe sequence (5'-3')	*E. coli* rRNA position	Specificity	Reference
EUBMIX	20		16S rRNA (338-355)	Domain *Bacteria*	
-EUB338		GCTGCCTCCCGTAGGAGT			Amann *et al.* (1990)
-EUB338-II		GCAGCCACCCGTAGGAGT			Daims *et al.* (1999)
-EUB338-III		GCTGCCACCCGTAGGAGT			Daims *et al.* (1999)
ALF1b	20	CGTTCGCTCTGAGCCAG	16S rRNA (19-35)	*Alphaproteobacteria*	Manz *et al.* (1992)
ALF969	20	TGGTAAGGTTCTGCGCGT	16S rRNA(969-986)	*Alphaproteobacteria* and *Fibrobacter*	Neef (1997)
BET42a	35	GCCTTCCCACTTCGTTT	23S rRNA (1027-1043)	*Betaproteobacteria*	Manz *et al.* (1992)
GAM42a	35	GCCTTCCCACATCGTTT	23S rRNA (1027-1043)	*Gammaproteobacteria*	Manz *et al.* (1992)
Ntspa714	55	CCTTCGCCACCGGCCT	16S rRNA (714-729)	Phylum Nitrospira	Daims *et al.* (2001)
NIT3	40	CCTGTGCTCCATGCTCCG	16S rRNA (1035-1048)	*Nitrobacter* spp.	Wagner *et al.* (1996)
NSR1156	30	CCCGTTCTCCTGGGCAGT	16S rRNA (1156-1173)	Freshwater *Nitrospira* spp.	Schramm *et al.*(1998)
Ntspa662	35	GGAATTCCGCGCTCCTCT	16S rRNA (662-679)	All *Nitrospira* spp.	Daims *et al.* (2001)
NSR827	20	GTAAMCCGCCGACACTTA	16S rRNA (827-847)	genus *Nitrospira*	Schramm *et al.* (1998)

3.3.4. Results and Discussion

3.3.4.1. NOB enrichment in sequencing batch reactors (SBRs)

The operation of LNOBSBR and HNOBSBR were designed to enrich autotrophic nitrite oxidizing bacteria by providing the SBRs with a synthetic wastewater free of organic carbon but containing nitrite. Only bacteria capable of fixing carbon dioxide or utilizing bicarbonate autotrophically should be able to grow. The SBRs were operated in two stages; the first when the influent was fed at 0.24 and 50 mg/l NO_2-N and the second where the feed concentration was increased to 5 and 100 mg/l NO_2-N in the LNOBSBR and HNOBSBR, respectively (Figure 20). The two SBRs reached steady state at Day 48 as shown in Figure 20. To prevent salt interference, nitrate analyses were conducted on water samples that were diluted up to1000-fold with deionized water.

In the LNOBSBR, the effluent had a mean of 0.07 mg NO_2-N/l and 0.89 mg NO_2-N/l in stages one and two, respectively, and 13.97 mg NO_3-N/l. The data for the HNOBSBR were 0.12 mg NO_2-N/l in both stages, 110.38 mg NO_3-N/l in stage one and 343.76 mg NO_3-N/l in stage two.

It was observed that there was no significant increase in the settled sludge volume in either SBR biomass (Figure 21) and so no sludge was wasted giving an infinite sludge age. Burrell (1998) reported that wasting any nitrite oxidizing sludge, even at low levels, had a significant impact on the operation of the nitrite oxidizing SBR, given the very low growth rate observed in the biomass. This is typical for the growth of autotrophic bacteria as they are recognized for their slow growth and low cell yield (Bock and Heinrich, 1969; Keen and Prosser, 1987; Prosser, 1989).

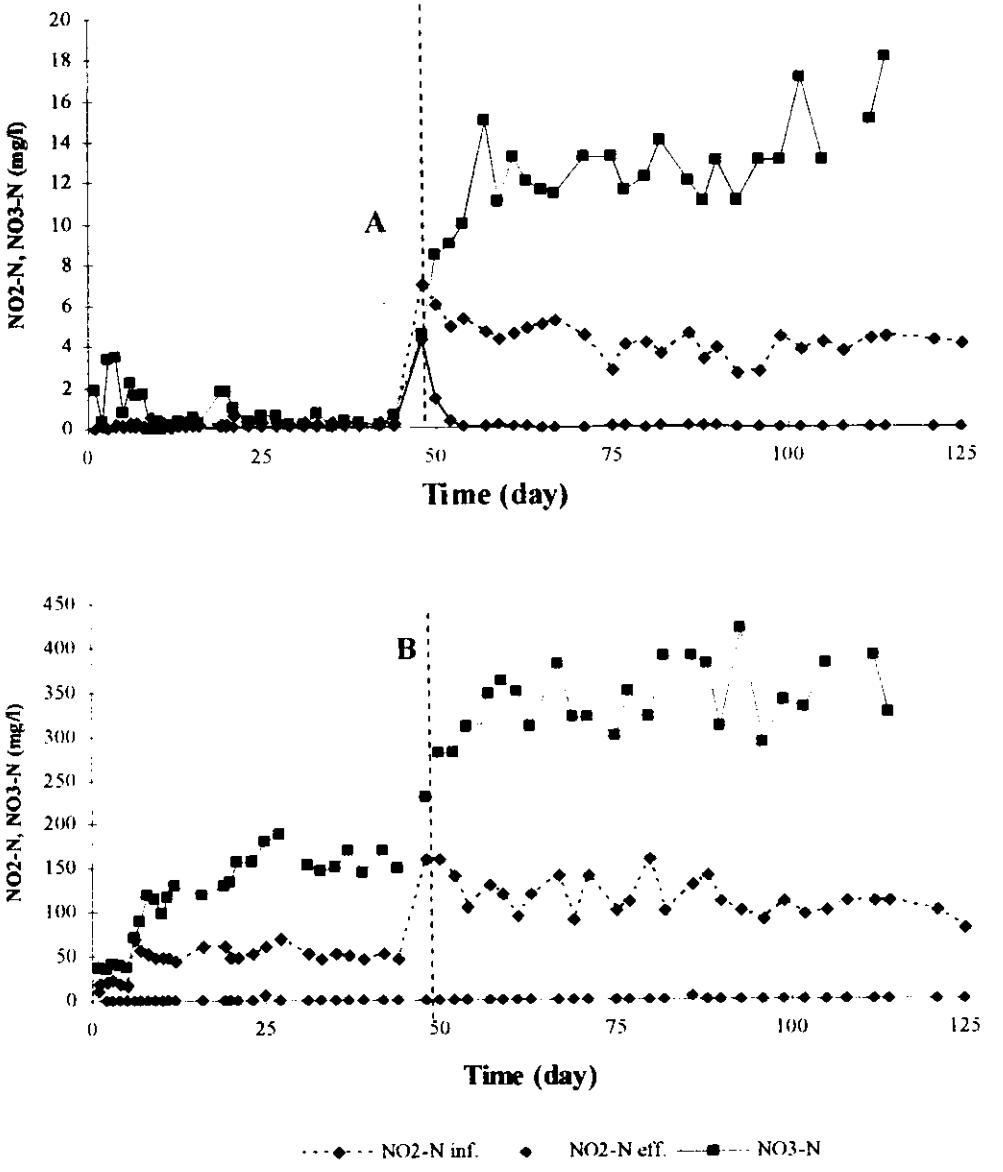

Figure 20. Influent and effluent NO_2^--N and NO_3^--N concentrations during the long-term operation of the SBR containing low NO_2^--N (A) and high NO_2^--N (B) at 3.5 d HRT and infinite sludge age (SRT)

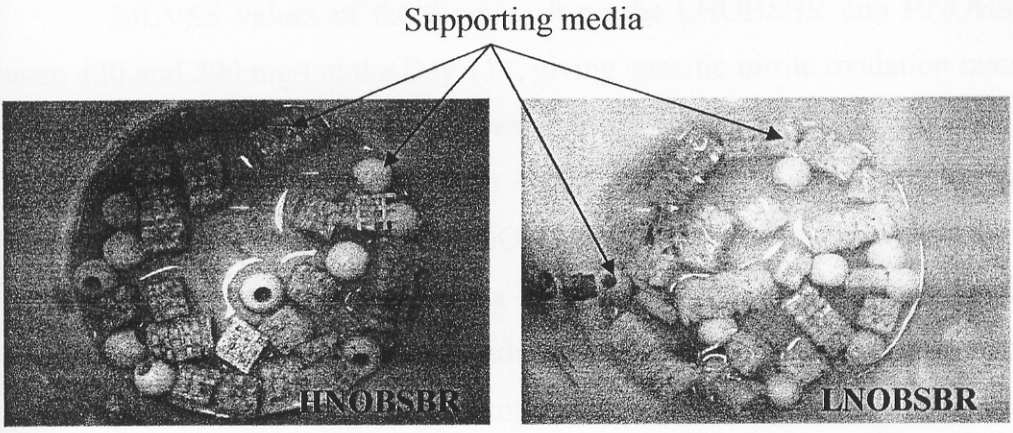

Figure 21. Photographs of the slow growing autotrophic nitrite oxidizing bacteria biomass in HNOBSBR and LNOBSBR

The nitrite oxidizers in the SBR were dependent on the nitrite concentrations provided for growth and the enrichment of the biomass in the SBRs was evaluated by their capacity oxidize nitrite to nitrate and by an increase in the rate of nitrite oxidation. Both SBRs were operated for 125 days. The nitrite removal capacities of both SBRs were presented in Figure 21.

Nitrite was oxidized to nitrate stoichiometrically in both SBRs according to cycle study data (Figure 22). In the LNOBSBR 4.4 mg NO_2-N/l was completely oxidized in 5 h generating 4.4 mg NO_3-N/l.The 13.6 mg/l of residual NO_3-N from the previous cycle increased to 18 mg/l after the 4.4 mg NO_2-N /l was completely oxidized. In the HNOBSBR, 110 mg NO_2-N/l was oxidized in 6 h generating 102 mg NO_3-N/l. The 215 mg/l of residual NO_3-N from the previous cycle increased to 317 mg NO_3-N/l after the 102 mg NO_3-N/l was produced.

The LNOBSBR biomass was capable of 100% nitrite removal at a nitrite oxidation rate of 1.03 mg NO_2-N/l/h at Day 114. The HNOBSBR had a higher nitrite oxidation rate of 19.04 mg NO_2-N/l/hr. This shows that both SBRs systems were able to successfully remove nitrite from the synthetic saline nitrite-rich wastewater.

MLVSS values of the biomass from the LNOBSBR and HNOBSBR were 420 and 590 mg/l at the Day 114, giving specific nitrite oxidation rates of 2 and 30 mg NO_2-N/g MLVSS/h, respectively. These rates for two different nitrite concentration systems are in broad agreement with data from other studies of *Nitrobacter* of 2.5 mg NO_2-N/g MLSS/h (Surmacz-Gorska *et al.*, 1997) and activated sludge of 61 mg NO_2-N/g MLSS/hr (Vazquez-Rodriguez and Rols, 1997). In none of these studies is the actual nitrite oxidising bacterial component known, so only broad comparisons can be made.

Determination of half saturation constant (Ks)

The Ks (half saturation constant for nitrite) was determined by finding the NO_2-N concentration that is equivalent to half the maximal substrate uptake rate. The substrate uptake rate is shown in Figure 23. The Ks values could not be determined accurately but were estimated to be approximately 0.03 and 0.3 mg NO_2^--N/l for the LNOBSBR and HNOBSBR respectively. The Ks of the nitrite oxidizers in LNOBSBR was one order of magnitude lower than that for the HNOBSBR, and this may be because of different communities of nitrite oxidizers. These data are comparatively lower than the Ks values of a pure culture of *Nitrobacter* of 0.06-8.4 mg NO_2^--N/l (Bitton, 1994) demonstrating that the nitrite oxidizing enrichments have a very high affinity for nitrite. Ks values for nitrite among nitrite oxidizers have typically been reported to be in a range from 0.2 to 5 mg NO_2^--N/l with the average being quoted as 1.4 mg NO_2^--N/l (Tchobanoglous and Burton, 1991). Vazquez-Rodriguez and Rols (1997) presented the Ks of a nitrite-oxidizing population in a fresh water activated sludge system to be 3.04 mg NO_2^--N/l. Nitrifiers commonly grow more slowly in sewage than in pure culture or other natural environments (Painter, 1986), which is possibly due to the presence of growth inhibitors like heavy metals.

Microscopic study

Bacterial clusters were found in both HNOBSBR and LNOBSBR biomass, but were observed to be more abundant, and to comprise more tightly packed clusters, in the HNOBSBR. The reason might be because of the higher nitrite nitrogen concentrations in the HNOBSBR, allowing for more rapid

growth of nitrifiers, thus forming larger, more dense and numerous clusters. There were both Gram positive and negative bacteria in both enrichments. The Gram negative bacteria were found to be more abundant and arranged in tightly packed clusters (Figure 24).

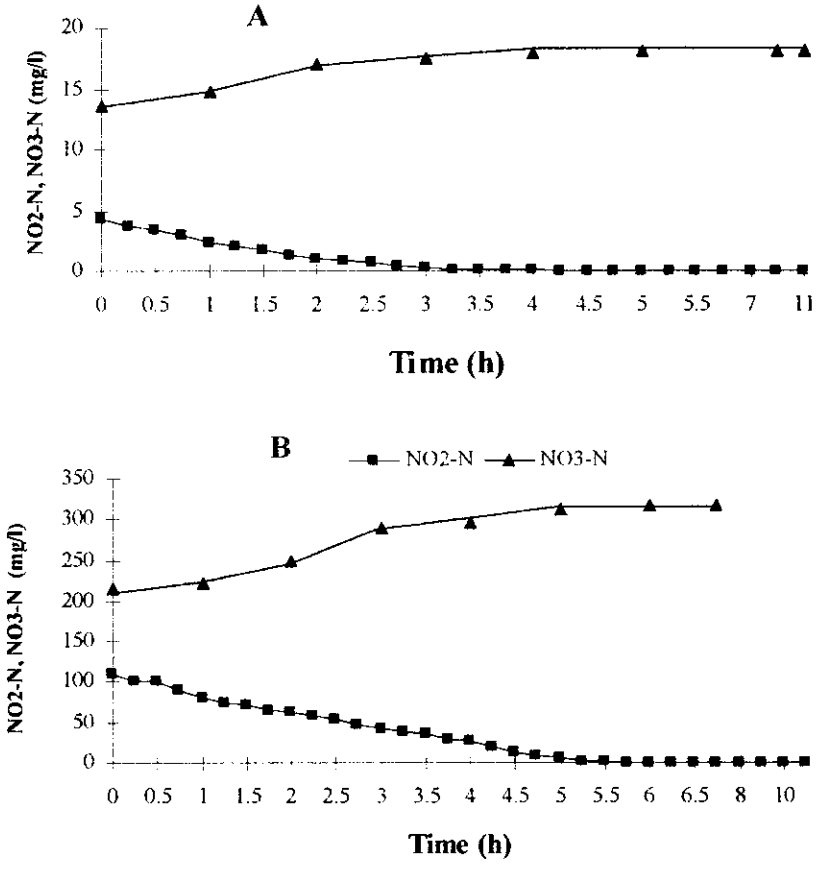

Figure 22. SBR cyclic operation of SBR on Day 125; LNOBSBR (A) and HNOBSBR (B)

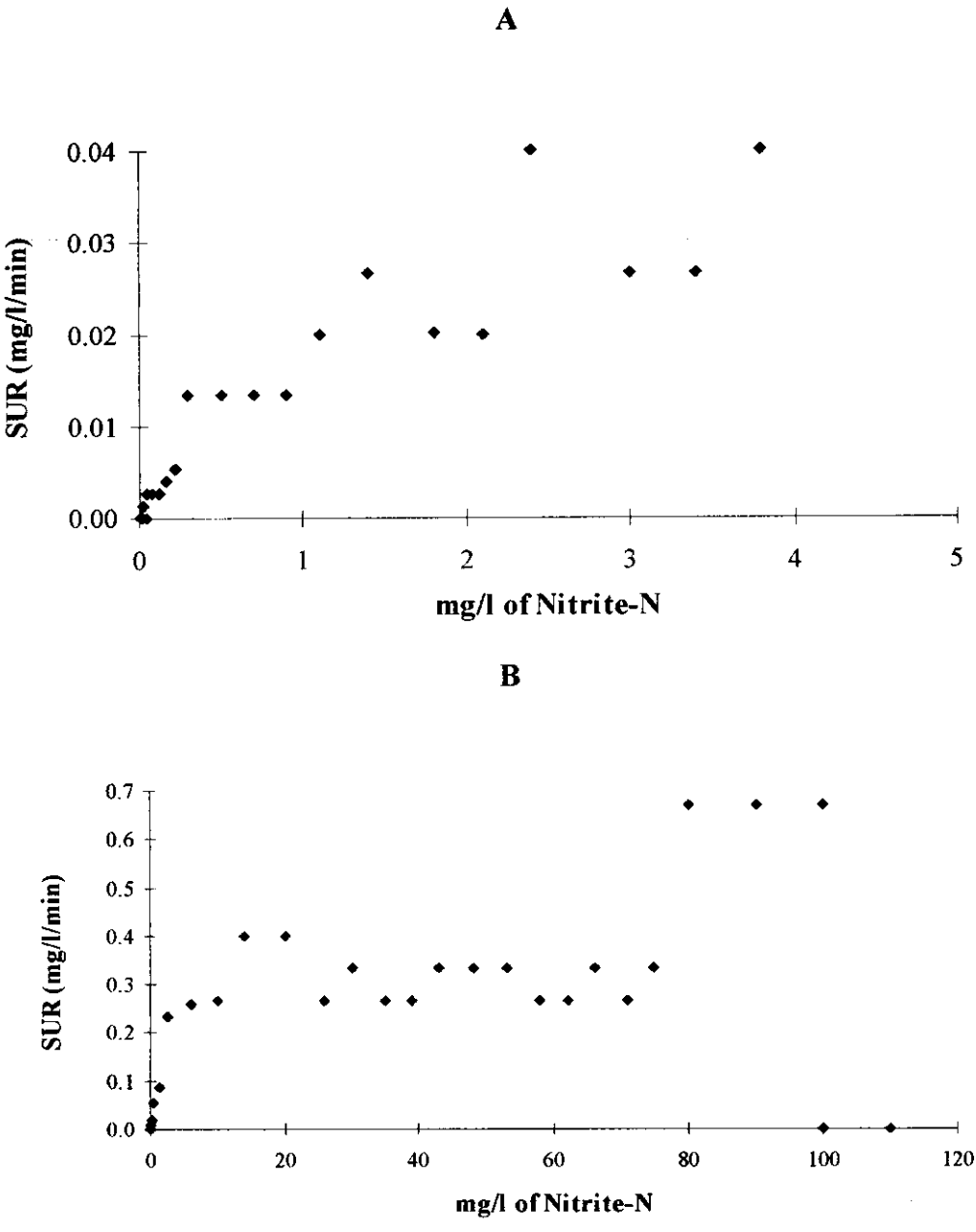

Figure 23. Substrate uptake rate versus the NO_2^--N concentrations in mixed liquor of LNOBSBR (A) and HNOBSBR (B) at Day 114

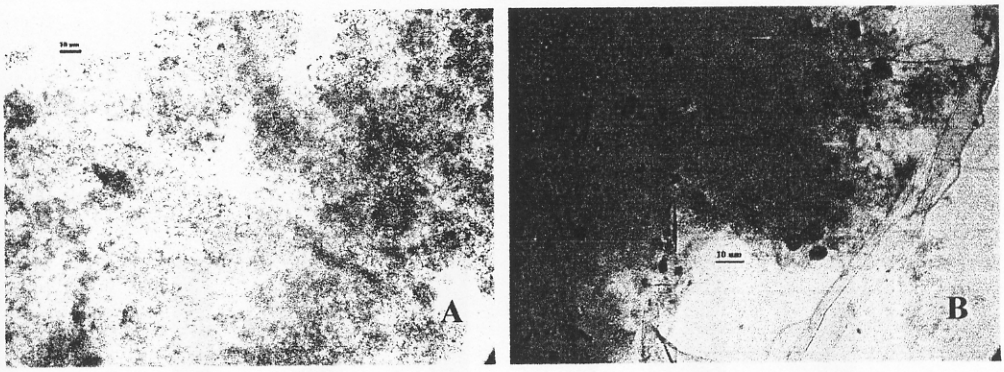

Figure 24. Micrographs of cultures from the HNOBSBR. (A) Bright field micrograph of biomass demonstrated the floccular nature of the developed biomass. (B) Gram staining micrograph demonstrating the gram negative bacteria which were found to be more abundant and arranged in tightly packed clusters.

3.3.4.2. MPN determination

The numbers of NOBs from both SBRs using MPN are presented in Figure 25. It was clear that the NOB populations from both SBRs increased over the 125 days of SBR operation.

Monitoring the population of NOB by MPN was relatively straightforward and demonstrated the presence of significantly larger numbers of these organisms (Hall, 1986). MPN determinations can be augmented by specific nitrification rate measurements, but the range of methods and the requirement for laboratory incubation makes data generated from these methods difficult to interpret in population ecology terms (Hastings *et al.*, 1998).

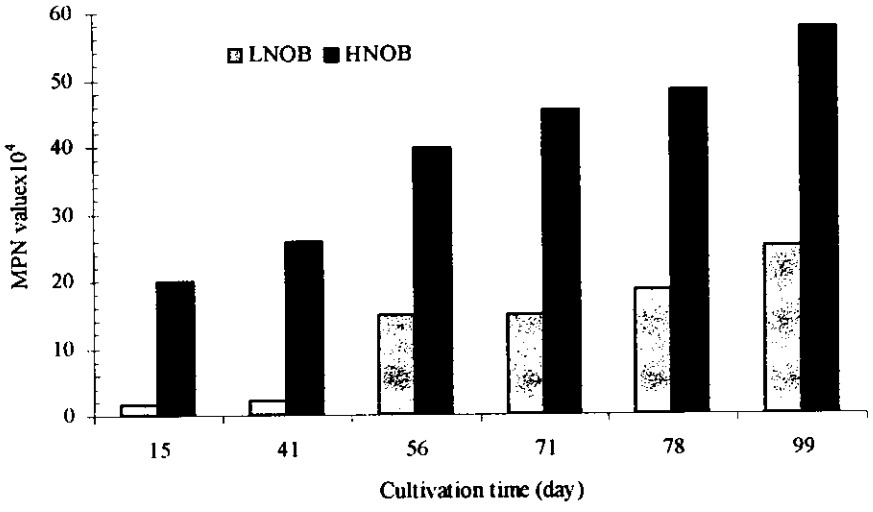

Figure 25. MPN data for the enumeration of NOB in the LNOBSBR and HNOBSBR enrichments at different time point during their operation

3.3.4.3. Pure-culture-dependent method

The NOB needed to be obtained in pure-culture because a comprehensive understanding of the physiology of these organisms is required. Seventeen isolated colonies from the HNOBSBR and twenty isolated colonies from LNOBSBR, grown on agarose nitrite oxidation medium plates were selected. All 37 colonies were sub cultured more than 3 times to ensure that the cultures were pure. Most of the bacterial isolates that appeared to be pure were Gram negative according to Gram stains.

Investigation of the isolates using FISH (Table 12) showed that none of the NOB-specific probes (NIT3, NSR1156, Ntspa714, NSR662 and NSR827) bound to them. Therefore, the isolates were not able to be characterized by NOB specific probes. However, the 16S rRNA genes from 2 HNOBSBR isolates and 1 LNOBSBR isolate were amplified and sequenced in attempts to determine their identity.

The BLAST result from one HNOBSBR isolate and one LNOBSBR isolate showed high identity with *Alcanivorax* sp. (accession number AB055207) and another of HNOBSNR isolate had 89% identity with *Cytophaga sp* (AB098581). *Alcanivorax* sp was not only found in isolates from HNOBSBR but was also found in isolate from LNOBSBR.

Alcanivorax is a *Gammaproteobacteria*, Gram-negative, aerobic, rod-shape with variable cell size. They are found in marine environments (Bruns and Berthe-Corti, 1999; Syutsubo *et al.*, 2001). Bruns and Berthe-Corti (1999) studied the physiological characteristics of this bacterium and found that it is able to reduce nitrate. Therefore, it is vaguely possible that this bacterium has an ecological niche in the LNOBSBR and HNOBSBR due to nitrate production by oxidation of nitrite and due to its ability to survive in marine environments. However, these denitrifiers require an organic carbon compound as carbon and electron source and none was deliberately added to the growth medium. *Cytophaga* sp. is heterotrophic, Gram-negative bacteria, belonging to members of the family *Flavobacteriaceae* in the *Bacteriodetes* phylum. *Bacteriodetes* are found commonly in the environment, partially due to their broad physiological repertoire and the ability to synthesize a variety of either cell-bound or extracellular degradative enzymes like cellulase, chitinase, or protease. Therefore they are important members of the microbial food chain and found in high abundance in various habitats by different microbial ecology methods (Weller *et al.*, 2000). Thus, it was perhaps not surprising to find this bacterium among the microbial communities in the bioreactor.

3.3.4.4. 16S rRNA gene cloning and analysis

A total of 79 clones from the LNOBSBR biomass and 79 clones from the HNOBSBR biomass 27f-1492r PCR generated libraries were selected and analysed. A total of 37 LNOBSBR operational taxonomic units (OTUs), containing 7 OTUs with greater than 3 clones, were generated by RFLP analysis. The HNOBSBR clone library generated 27 OTUs containing 11 OTUs with greater than 3 clones by RFLP analysis (Figure 26). The high

diversity of OTUs in both reactors was shown that a dominant bacterial group was not detected.

One OTU (OTU5; Figure 26), which comprised 5% (4 clones) of the LNOBSBR library was phylogenetically 97% affiliated to a known nitrite oxidizer, *Nitrobacter alkalicus* strain AN4 (AF069958) which was isolated from alkaline habitats;, sediments of three soda lakes (Kunkur Steppe, Siberia; Crater Lake and Lake Nakuru, Kenya) and from a soda soil (Kunkur Steppe, Siberia) by Sorokin *et al.* (1998). Appendix 9 shows the full insert sequence of one OTU5 clone (LNOBSBR078). A total of 24% of clones from the HNOBSBR clone library (19 clones from 2 OTUs) were 99% affiliated with the nitrite-oxidizing bacterium MPN2 (AY135357). The full 16S rRNA gene insert sequence of representatives of these OTUs (HNOBSBR025 and HNOBSBR015) are shown in Appendix 10. The 16S rRNA gene sequence from a nitrite-oxidizing "bacterium" called MPN2 was recently reported by Fouratt *et al.* (2003) in a nitrifying bioaugmentation product (NBP). An alignment of the 16S rRNA gene sequence of MPN2 with previously published sequences shows a close relationship to *Nitrobacter* sp TH21 (AF080257); *Nitrobacter winogradskyi* ATCC 25381 (L35506); and *Nitrobacter alkalicus* strain AN1 (AF069958). The AY135357 sequence and the highly identical sequences to AY135357 obtained in this current study on shrimp aquaculture all have a perfect match with the FISH probe NIT3 for *Nitrobacter*.

It is not surprising to find NOB in both reactors highly 99% identical to *Nitrobacter* species. Many studies on nitrification have been carried out with *Nitrobacter winogradskyi* since they are relatively easy to grow in the laboratory as pure cultures (Prosser, 1989). However, recent analyses of the bacterial populations found in activated sludge systems or wastewater biofilms suggest that *Nitrospira* could be more commonly maintained in these environments as the NOB versus *Nitrobacter* (Burrell *et al.*, 1998; Juretschko *et al.*, 1998; Burrell *et al.*, 1999; Blackall, 2000; Daims *et al.*, 2001).

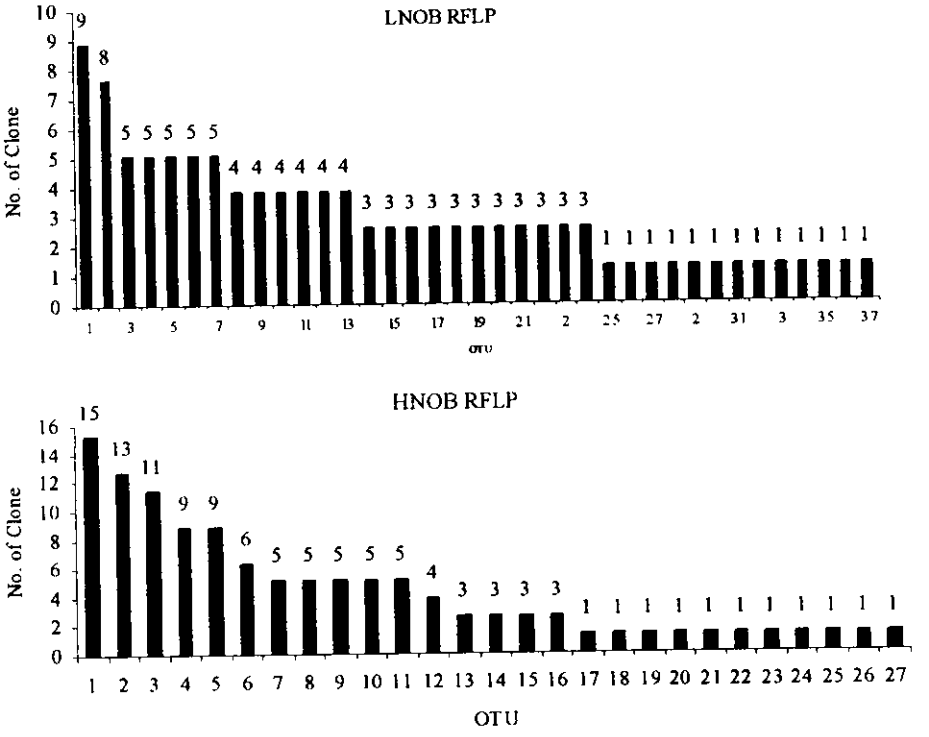

Figure 26. The percentage of clones in operational taxonomic units (OTUs) from the LNOBSBR (low nitrite SBR) and HNOBSBR (high nitrite SBR) clone libraries

3.3.4.5. Fluorescence *in situ* hybridization (FISH)

Data for all FISH probing of LNOBSBR and HNOBSBR are presented in Table 13. The NIT3 probe for known nitrite oxidizers *Nitrobacter* in the *Alphaproteobacteria*, hybridized to 3% and 10% of all bacterial cells in the LNOBSBR and HNOBSBR enrichments, respectively. The white coloured cells in Figure 27 are *Nitrobacter* as they hybridized with all 3 probes (EUBMIX, ALF969 and NIT3). No organisms in either of LNOBSBR or HNOBSBR bound any of NSR1156, Ntspa662, NSR827 and Ntspa 714 for different *Nitrospira* sp. A relatively high abundance of *Alphaproteobacteria* in three bioreactors (19% of all bacteria in NFSBR (Chapter 3, part 2) and 5% and 30% in the LNOBSBR and HNOBSBR, respectively) according to a

combination of ALF1b and ALF969 probes. *Gammaproteobacteria* were also relatively highly abundant in LNOBSBR and HNOBSBR (13% and 3% of all bacteria, respectively) but lower than the abundance of *Alphaproteobacteria*.

Table 13. Percentage of EUBMIX-targeted cells in the LNOBSBR and HNOBSBR sludge hybridizing group-specific probes by FISH analysis

Probe	Specificity	LNOBSBR	HNOBSBR
NIT3	*Nitrobacter* spp.	3	10
NSR1156	Freshwater *Nitrospira* spp.	0	0
Ntspa662	Whole genus *Nitrospira*, including the 4 sublineages	0	0
Ntspa714	Phylum Nitrospira	0	0
NSR827	*Nitrospira* genus	0	0
ALF1b+ ALF969	Alpha subclass of Proteobacteria	5	30
GAM42a	Gamma subclass of Proteobacteria	13	3

Given the high rates of nitrite oxidation, particularly in the HNOBSBR enrichment, the numbers of *Nitrobacter* (3% and 10% in LNOBSBR and HNOBSBR enrichments respectively), might not explain all the nitrite oxidation. It is possible that the large clusters of magenta *Alphaproteobacteria* cells and/or the other blue bacterial cells in Fig. 27 may also be responsible for nitrite oxidation. Therefore, it is possible that there still exists some bacteria in each biomass (LNOBSBR and HNOBSBR) that did not bind to FISH probes targeting known nitrite oxidizing bacteria like *Nitrobacter* and *Nitrospira*. Additionally, many of the bacterial cells, hybridizing only to the EUBMIX probes (the blue bacterial cells), existed as large cell clusters typical of

nitrifiers. Therefore, potentially new and as yet unidentified nitrite oxidizing bacteria are likely present in these enrichments.

Sakairi *et al.* (1996) studied immobilized seawater nitrifiers and reported that the NOB was possibly a *Nitrobacter* spp. but this was simply assumed from cell morphology using transmission electron micrographs (TEM). In contrast, Hovanec and DeLong (1996) used nitrite-oxidizing specific oligonucleotide probes in freshwater and marine aquaria. Their data suggested that *Nitrobacter* species were not responsible for nitrite oxidation activity as they were unable to detect *Nitrobacter* cells. Johnson and Sieburth (1976) used scanning electron microscopy to investigate nitrifying bacteria obtained from biological filters and waters of three aquaculture systems (one freshwater and two seawater samples). Again, these researchers were unable to find *Nitrobacter winogradskyi* in any of the natural systems in which they were sampled, although this microorganism could be enriched for using traditional cultural techniques.

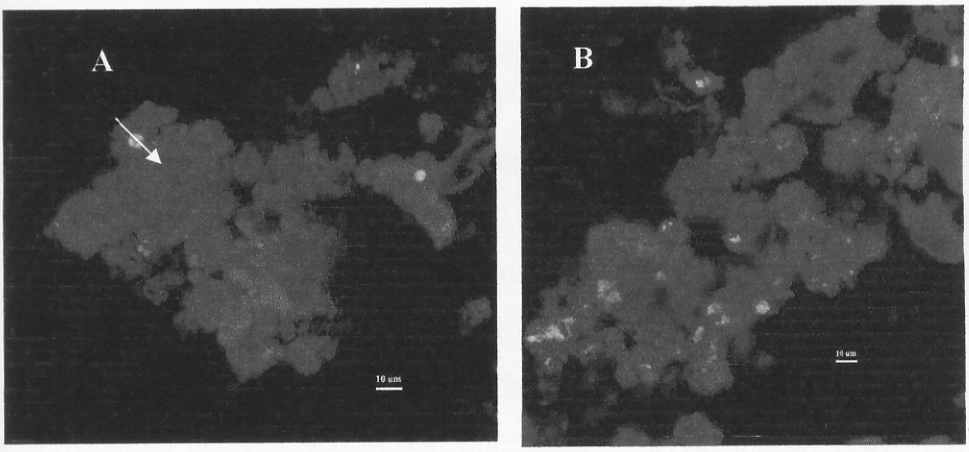

Figure 27. Confocal laser scanning micrograph of LNOBSBR enrichment (A) and HNOBSBR enrichment (B). Illustration are the multiple hybridizations of EUBMIX probes (blue) for all *Bacteria,* ALF1b+ALF969 probes (red) for *Alphaproteobacteria* and NIT3 probe (green) for *Nitrobacter* spp. Magenta coloured cells are *Alphaproteobacteria* that are not *Nitrobacter* spp. White cells are *Nitrobacter* spp. belonging to *Alphaproteobacteria.* Blue cells are Bacteria that do not belong to the *Alphaproteobacteria.*

Members of the genera *Nitrococcus*, *Nitrospina*, and *Nitrospira* are obligate lithoautotrophs isolated from marine habitats (Watson and Waterbury, 1971; Watson *et al.*, 1989). Although these *Deltaproteobacteria* nitrite-oxidizing bacteria were isolated from marine environments, the salinity of the water sample, from which *Nitrospina gracilis* was isolated was low (12.870 ppt) (Watson and Waterbury, 1971). However, most recent studies using molecular methods indicated that *Nitrospira* sp. plays an important role in nitrite removal (Burrell *et al.*, 1998; Schramm *et al.*, 1998; Schramm *et al.*, 1999; Daims *et al.*, 2000).

3.3.5. Conclusions

NOB phenotypes were present in the LNOBSBR and HNOBSBR biomass according to process performance data and according to FISH and MPN results. In addition, MPN results clearly indicated that the NOB populations in LNOBSBR and HNOBSBR increased over the operational time of the SBRs.

Cloning using bacterial specific primers (27f and 1492r) recovered 37 OTUs from the LNOBSBR, one of which comprising 5% of the LNOBSBR library was phylogenetically affiliated to a known nitrite oxidizer *Nitrobacter alkalicus* strain AN4 (AF069958). A total of 24% of clones from the HNOBSBR clone library were affiliated with a clone sequence MPN2 (AY135357) obtained from a nitrifying bioaugmentation product. MPN2 is phylogenetically in *Nitrobacter*. The biomass in LNOBSBR contained 3% *Nitrobacter* and the HNOBSBR contained 10% *Nitrobacter* according to NIT3 probing.

Thus *Nitrobacter* was clearly present in the biomass according to cloning and FISH and likely responsible for nitrite oxidation in both the LNOBSBR and in the HNOBSBR. It was however hypothesized that the abundances of *Nitrobacter* were too low to account for all the nitrite oxidation that was occurring in the bioreactors and that an as yet unidentified NOB was still likely to be present in the biomass. It is possible that the large clusters of

unidentified *Alphaproteobacteria* and/or other unidentified bacteria may be responsible for the additional nitrite oxidation activity in HNOBSBR and LNOBSBR. Some preliminary isolation attempts using agarose solidified media lacking added organic carbon were largely unconclusive.

Chapter 3 Results and Discussions

Part 4: The Exploration of Novel Nitrite Oxidizing Bacteria (NNOB) in NFSBR, HNOBSBR and LNOBSBR

3.4.1. Introduction

The overall observation in NOB enrichment experiments was that the biomass from each enrichment was very similar in microbial community composition to each other. The *Nitrobacter* spp. specific FISH oligonucleotide probe (NIT3) hybridized to approximately 10% and 3% of the bacterial cells from the HNOB and LNOB enrichments, respectively. However, there also existed other large bacterial clusters that did not bind with the nitrite oxidizing bacterial probes. It was questioned whether these relatively low numbers of *Nitrobacter*, in the presence of large unknown bacterial clusters reminiscent of other nitrifying biomasses, were likely to able to achieve these high rates of nitrite oxidation observed in both SBRs. Recently it has been shown that members of the *Nitrospira* genus are more likely responsible for nitrite oxidation process in freshwater systems than *Nitrobacter* genus (Burrell *et al.*, 1998; Juretschko *et al.*, 1998; Burrell *et al.*, 1999; Blackall, 2000; Daims *et al.*, 2001). Therefore, it was postulated that potentially novel nitrite oxidizing bacteria containing *Nitrospira marina* may be present in the enrichments.

3.4.2. Objectives

This study aims to prove the presence of potential novel nitrite oxidizing bacteria carried out by 16S rDNA clone libraries construction using the new designed *Nitrospira marina* specific primer.

3.4.3. Materials and Methods

3.4.3.1. Sample collection

Activated sludge samples were collected from 3 sequencing batch reactors (SBR) composed of NFSBR (as described in Chapter3, part 1, 2), LNOBSBR and HNOBSBR (as described in part 3, Chapter 3).

3.4.3.2. Primer design

- *Nitrospira marina* specific primer

Since no known *Nitrospira* spp. specific FISH probes (NSR1156, NSR662, NSR827 and Ntsp 714; detailed in Table 5, Chapter 2) were hybridized to any of the bacteria in any samples from NFSBR, LNOBSBR and HNOBSBR. Near complete 16S ribosomal RNA gene of *Nitrospira marina* was manually aligned (SeqEd) with all known *Nitrospira* spp. FISH probes (Paul Burrell, personal communication). All candidate oligonucleotides were checked for the specificity of *Nitrospira marina* by using the Basic Local Alignment Search Tool (BLAST) program (Altschul *et al.*, 1990). Oligonucleotides were synthesized at the Proligo Primers and Probes, Australia.

- Novel NOB specific primer

ChanyF and ChanyR primers set were derived from the representative of OTU1 sequence. The representative of OTU1 sequence were submitted to Basic Local Alignment Search Tool (BLAST) program (Altschul *et al.*, 1990) in order to select a part of oligonucleotides with no organisms specific (Figure 28). All candidate oligonucleotides were checked using BLAST to be ensured that none of any organisms were match. Oligonucleotides were synthesized at the Proligo Primers and Probes, Australia.

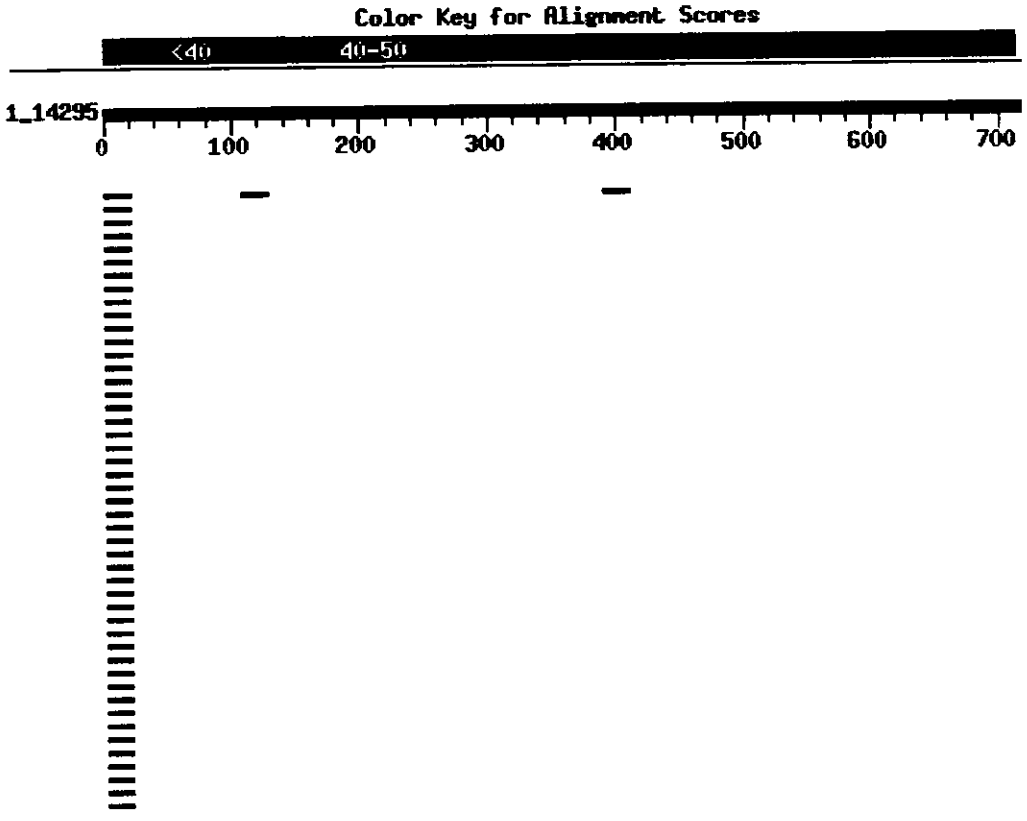

Figure 28. The BLAST result of representative OTU1 sequence using for OTU1 specific primer design

3.4.3.3. 16 rDNA clones sequencing

Three 16S rRNA gene (rDNA) clone libraries were constructed from the extracted DNA of NFSBR, LNOBSBR and HNOBSBR (as described in Chapter 3, part 2 and 3). Amplification of the *Nitrospira marina* 16S rRNA gene from all sources was achieved by using the conserved bacterial primer 27f [AGAGTTTGATCCTGGCTCAG, Lane (1991)] and *Nitrospira marina* specificity primer (SNtsp 1010r) obtained from previous section (3.4.3.2). Near completed *Nitrospira marina* 16S rRNA genes were cloned, sequenced and analyzed using methods as described in Chapter 2 section 2.2.3.

3.4.3.4. Pure culture of novel nitrite oxidizing bacteria (nNOB) investigation

Thirty-seven isolates from LNOBSBR and HNOBSBR (Chapter 3 part 3) were explored for novel NOB using culture dependent and independent techniques. Molecular approaches were carried out by 16S rDNA sequencing and FISH technique. DNA of the isolates was extracted by lysis the cells in the sterile MQ water at 94° C for 10 minutes in the thermocycler. Lysed cell-DNA were amplified for the 16S rDNA by polymerase chain reaction (PCR) techniques. PCR reaction was performed as described in Chapter 2 section 2.2.3.2, except the new designed primers for novel NOB obtained from this study (ChanyF: AATCCGGCCCGTATATAAGG and ChanyR: CCATGACACTTCATTAACAGATGC) were used. The PCR cycle on the thermocycler consisted of 1 cycle at 94° C for 5 minutes and 30 cycles of 94° C for 30 seconds, 55° C for 30 seconds, and 72° C for 15 seconds. This was followed by an extension cycle of 72° C for 5 minutes.

Amplified products were sequenced as method described in Chapter 2 section 2.3.3.4. FISH technique was done using probes as related to the affiliated group of nNOB. FISH was performed as described in Chapter 2 section 2.2.3.4 and 2.2.2.

The isolates which appeared positive with ChanyF and ChanyR primers set (novel NOB) were confirmed by cultivation-based approach, the experiment was performed in a 50 ml flask containing 5 ml of artificial wastewater as the same with media feeding in LNOBSBR and HNOBSBR (Chapter 3, part 3), and incubated on a rotary shaker (100 rpm) at 28° C until the cell growth was observed. Mixed liquor was assessed for NO_2-N, NO_3-N by Spectrometer NOVA 60 (Merck, Ltd., Germany). Biomass from HNOBSBR and fresh media were used for positive control and negative control of this experiment.

3.4.4. Results and discussions

3.4.4.1. Primer design

- *Nitrospira marina* specific primer

There were bases of *Nitrospira* spp. specific FISH probes (NSR1156, NSR662, NSR827) that did not match with *Nitrospira marina* 16S rRNA gene sequence found from the alignment done by Dr. Paul Burrell (personal communication) This may be the explanation why none of these probes with specific to *Nitrospira marina* were hybridized with the samples. The *Nitrospira marina* specific primer so called SNtsp 1010 (TCACCCTTTCAGGTTC) was chosen for the clone libraries construction.

- Novel NOB specific primers

Novel NOB specific primer so called ChanyF (AATCCGGCCCGTATATAAGG) and ChanyR (CCATGACACTTCATTAA CAGATGC) were designed to amplify specifically a 250 bp DNA fragment of the "OTU1" sequence. Both primers were not specific with any organisms checking by BLAST, excepted OTU1 sequence (detailed of OTU1 was shown in section 3.4.4.2).

Since OTU1 was found in all clone libraries sourced from NFSBR, LNOBSBR and HNOBSBR, therefore, positive PCR using ChanyF and ChanyR primers were performed to confirm the right of these primers. Genomic DNA of all sources from NFSBR, LNOBSBR and HNOBSBR were investigated for the presence of novel NOB by PCR using ChanyF and ChanyR primers. The results indicated that one positive band with the corrected size (250 bp) appeared in all sources, thereby, ChanyF and ChanyR primers were correct. In addition, this result illustrated that the novel NOB was present from the beginning of nitrifying bacteria from sediment and shrimp farm enrichment (NFSBR) and continue to the LNOBSBR and HNOBSBR which being inoculated with biomass from the NFSBR.

3.4.4.2. 16 rDNA clones sequencing

All clone libraries from three sources were randomly picked for eighty positive clones each. Clone library of HNOBSBR using primers set of 27f and SNtsp 1010r called nHNOBSBR library, performed the highest number of clone containing full size-inserted gene, 55 clones, while 22 clones from nLNOBSBR library (DNA from LNOBSBR) were obtained. Only 11 clones from nNFSBR (DNA from NFSBR) contained full size-inserted gene. A total of eighty-eight clones containing full size-inserted gene from nNFSBR, nLNOBSBR and nHNOBSBR libraries were selected for grouping by RFLP analysis.

All three clone libraries from NFSBR, LNOBSBR and HNOBSBR contained one OTU which was the most common OTU in the LNOBSBR and HNOBSBR libraries and was also present, but not the commonest OTU, in the NFSBR library. Representatives of all 27f-1010r generated clones were sequenced (Table 14). The sequences of several OTUs, including the most abundant OTU from the LNOBSBR and HNOBSBR, revealed no affiliation with any 16S rRNA gene sequence data and nor with any small subunit RNA data and were deemed to be sequences from non-rRNA genome fragments fortuitously amplified in the PCR with 27f-1010r. Sequences in some OTUs from the 27f-1010r generated libraries (72.7% in NFSBR, 63.7% in LNOBSBR and 98.2% in HNOBSBR libraries) showed affiliation by BLAST with the 16S rRNA gene of 27.3, 36.3, and 1.8 However, none of these organisms are closely affiliated with *N. marina*, and the amplificates are therefore the result of non-specific PCR amplification with these primers. Thus, this approach to explore the role of *N. marina*-like organisms in nitrite oxidation in the SBRs was unsuccessful.

Table 14. BLAST identities of the 27f-1010r generated clone member in nNFSBR, nLNOBSBR, and nHNOBSBR of all were sequenced

OTU	Clone library	% of clone	BLAST %	BLAST identity; phylum	Affiliation with 16S rRNA gene
1	NFSBR	9.1	0	Not significant match	No
	LNOBSBR	59.1	0	Not significant match	No
	HNOBSBR	60	0	Not significant match	No
2	NFSBR	18.2	0	Not significant match	No
	LNOBSBR	22.7	85	AL 162756 *Neisseria meningitides*; betaproteobacteria	Yes
	HNOBSBR	25.5	0	Not significant match	No
3	NFSBR	9.1	-		
	LNOBSBR	13.6	82	AP00598 *Bradyrhizobium japonicum*; Alphaproteobacteria	Yes
	HNOBSBR	3.6	-		
4	NFSBR	18.2	91	AY234519 Bacterium Ellin5102: Verrucomicrobia	Yes
	HNOBSBR	5.5	0	Not significant match	No
5	NFSBR	9.1	97	Not significant match	No
	LNOBSBR	4.5	-		
	HNOBSBR	1.8	97	*Marinobacter exelentus* AY180101; Gammaproteobacteria	Yes
6	NFSBR	9.1	0	Not significant match	No
	HNOBSBR	1.8	-		
7	HNOBSBR	1.8	-		
8	NFSBR	9.1	0	Not significant match	No

Table 14. BLAST identities of the 27f-1010r generated clone member in nNFSBR, nLNOBSBR, and nHNOBSBR of all were sequenced (continued)

OTU	Clone library	% of clone	BLAST %	BLAST identity; phylum	Affiliation with 16S rRNA gene
9	NFSBR	9.1	80	AP00598 *Bradyrhizobium japonicum*; Alphaproteobacteria	Yes
10	NFSBR	9.1	0	Not significant match	No

The performance of the dominant pattern of the LNOBSBR library (60% abundance) was identical with the dominant pattern of the HNOBSBR library (60% abundance) and with the second dominant pattern of the nNFSBR library (Figure 29, 30). The dominant group of nNFSBR, nLNOBSBR and nHNOBSBR obtained by OTU1 plays an important role of nitrite consumption according to their abundance in all libraries.

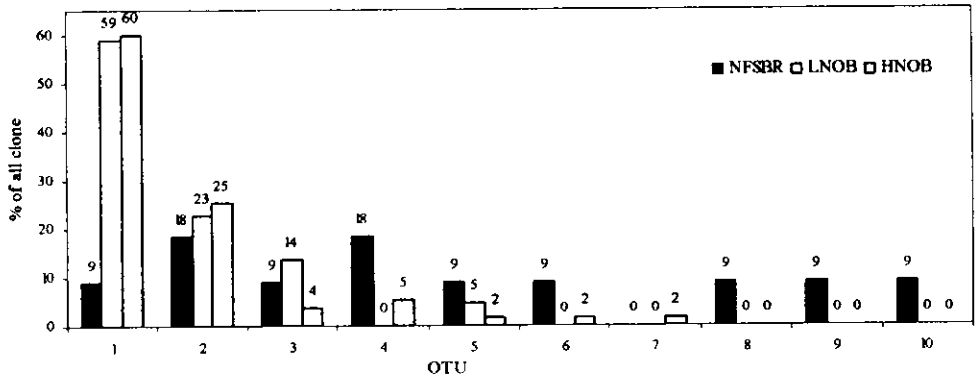

Figure 29. The percentage of clones which grouped in operational taxonomic units (OTUs) found in nNFSBR, nLNOBSBR and nHNOBSBR libraries constructed by 27f and Sntp1010r primers set

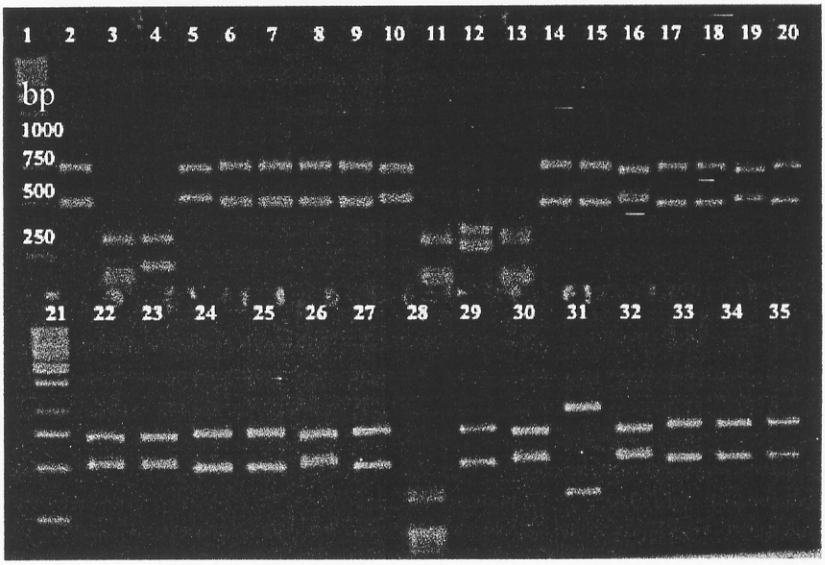

Figure 30. The digestion profile of 16S rDNA found in nNFSBR, nLNOBSBR and nHNOBSBR clone libraries with 27f and Sntpa1010r primers set running on 3% agarose gel electrophoresis OTU 1 (lane 2, 6,7, 8, 9,14,15, 17,18, 20, 24, 25, 2729, 33, 34, 35) was dominant in all libraries.

One clone from OTU1 of each library was sequenced, N102 for nNFSBR library, L5 for nLNOBSBR library, and H1 for nHNOBSBR library. The sequences of N102, L5 and H1 were identical so called "OTU1" sequence- see full 16S rDNA sequence in Appendix 11. The result from BLAST with OTU1 did not give significant homology with any organism. Therefore, two possibilities were hypothesized. Firstly, these sequences belonged to a new bacterium. One explanation was that the 16S rDNA sequence of OTU1 did not carry any conserved 16S regions except 27f and SNtsp1010r. The second hypothesis was that it was not a new bacterium if the primers set of 27f and SNtsp1010r amplify a region of DNA that did not belong to 16S rDNA. Since the length of the PCR product (1000 bp) corresponds to the size expected from 16S rDNA amplification using 27f and SNtsp1010r primer set, therefore, this hypothesis was quite improbable. However, the sequence of OTU1 was not

contained the conserved sequence of 16S rRNA, thus, it was confirm that primer set of 27f and SNtsp1010r amplify a region of DNA that did not belong to 16S rDNA.

Since, the potential novel NOB was still not known, the experiment to explore the novel NOB was required. Therefore, new primers set (called ChanyF and ChanyR) based on OTU1 sequence need to be designed.

3.4.4.3. Pure culture of novel nitrite oxidizing bacteria (nNOB) investigation

ChanyF and ChanyR primers were used to screen 37 presumed single colonies isolated from LNOBSBR and HNOBSBR by spread plate technique (Chapter 3, part 3). Only one colony (HA1, the fist colony of HNOB growing on the plate) was positive with these primers. The sequence of the HA1-PCR product was homologous to "OTU1". Therefore, the HA1 isolate was assumed to be the novel NOB which dominant in all sources. It was taken to culture in the artificial wastewater, as the same media feeding to HNOBSBR (Chapter 3, part 3), in order to confirm the characteristic of nitrite consumption.

Cultivation of HA1:

Cultivation of HA1 in the artificial wastewater revealed that this bacterium was able to remove NO_2-N (100 mg/l) within 6 days and no nitrate was observed. The HA1 isolate consumed nitrite as efficient as the positive control but no nitrate was observed (Table 15). Theoretically, nitrite oxidizing bacteria oxidizes nitrite to nitrate, so nitrate should be observed. There were two main possibilities of the disappearance of nitrate. Firstly, HA1 was able to oxidize nitrite to nitrate and consumed nitrate at the same time. The other explanation was that there were 2 bacteria in the HA1 isolate; one oxidized nitrite to nitrate and the other consumed that nitrate.

Table 15. The nitrite and nitrate nitrogen concentrations of mixed liquor in HA1, positive and negative control flasks after 6 days cultivation

Sample	NO_2-N (mg/l)	NO_3-N (mg/l)
HA1	0.9	0
Positive control	1	81
Negative control	110	5

The microscopic study of the HA1 isolate illustrated that it is Gram negative bacteria with 2 shapes; cocci and rod (Figure 31). This result gave two possibilities; the isolate was not pure, or it was the characteristic of this isolate. Rod-shaped bacteria generated the floc formation to cocci-shaped bacteria.

To prove the purity of HA1, it was re-streaking on agar plate (containing the same HA1 culture media (as above) solidified by adding 1% (w/v) agarose) and incubated at 28°C until single colonies appeared (two days). Ten single colonies (HA1-1 to HA1-10) were cultured in liquid media containing 100 mg/l NO_2-N. The growths of all colonies were observed which meaned that they could consume nitrite in the media. Seven of them were positive with ChanyF and ChanyR. Gram staining displays cocci and rod shapes, both were Gram negative bacteria. This result confirmed that the isolate HA1 consisted of more than 1 bacterium and composed of symbiotic cocci and rod-shaped bacteria.

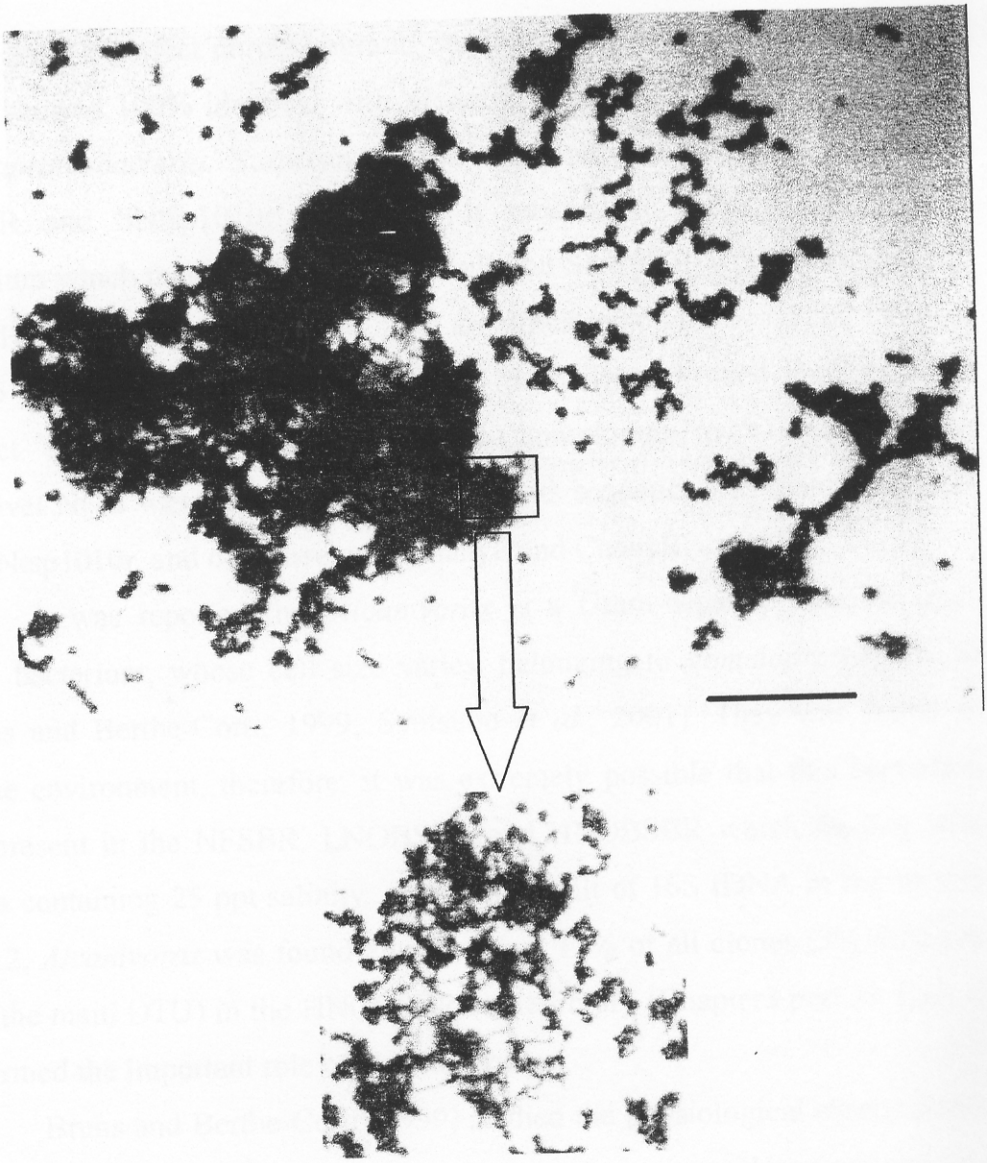

Figure 31. Phase contrast micrograph of HA1 represents the small cocci and rod shape of HA1. The rod-shaped bacteria generated the floc formation to the cocci-shaped bacteria as a result of the HA1 performed the cluster (small picture). Bar = 10 um.

Sequencing of HA1

PCR amplification on the lysed cell DNA of HA1-5 (positive with ChanyF and ChanyR) using 27f and 1492r primers set gave the corrected size of a 1.5 kb product. The sequence of this product (using 27f, 530f, 926f and

1492r) gave a perfect chromatogram. Sequences were determined by BLAST which showed 100% identical with *Alcanivorax* sp (AB055207) belonging to *gammaproteobacteria*. Surprisingly, this sequence did not contain ChanyF, ChanyR and SNtsp1010r sequences. It meaned that there was another bacterium which matched ChanyF, ChanyR and SNtsp1010r primers contained in the HA1 but was not amplified. PCR amplification on the lysed cell DNA of HA1-5 using 27f and SNtspa1010r gave the corrected sized band of 1 kb product. The sequence of this product was homologous to "OTU1". Hereby, the novel NOB was existed in the HA1 and its sequence was match with 27f and SNtsp1010r, and of course with ChanyF and ChanyR.

It was reported that *Alcanivorax* is a Gram-negative, aerobic, rod-shape bacterium, whose cell size varies, belonging to *gammaproteobacteria* (Bruns and Berthe-Corti, 1999; Syutsubo *et al.*, 2001). They was found in marine environment, therefore, it was extremely possible that this bacterium was present in the NFSBR, LNOBSBR and HNOBSBR which feeding with media containing 25 ppt salinity. From the result of 16S rDNA in the section 3.4.4.2, *Alcanivorax* was found abundantly at 11% of all clones (3% different with the main OTU) in the HNOBSBR clone library (Chapter3 part 3), thus, it confirmed the important role of this bacterium.

Bruns and Berthe-Corti (1999) studied the physiological characteristic of this bacterium and found that it is able to reduce nitrate. However, there is no research report on the oxidation of nitrite by this bacterium. So, it was clearly illustrated that the isolate HA1 composed of more than 1 bacteria, one of them is *Alcanivorax* sp. which consume nitrate from oxidation of nitrite by others. Due to *Alcanivorax* sp. is a rod-shaped bacterium, hence, cocci-shaped bacterium was assumed to be a novel NOB.

FISH result of HA1 study:

According to FISH technique, there were at least two bacteria in the HA1; one belonging to *Alphaproteobacteria* (maybe the new one?) and the other belonging to *Gammaproteobacteria* (*Alcanivorax* sp. + other?) (Figure 32). *Gammaproteobacteria* specific probe (GAM 42a) bound with rod-shape

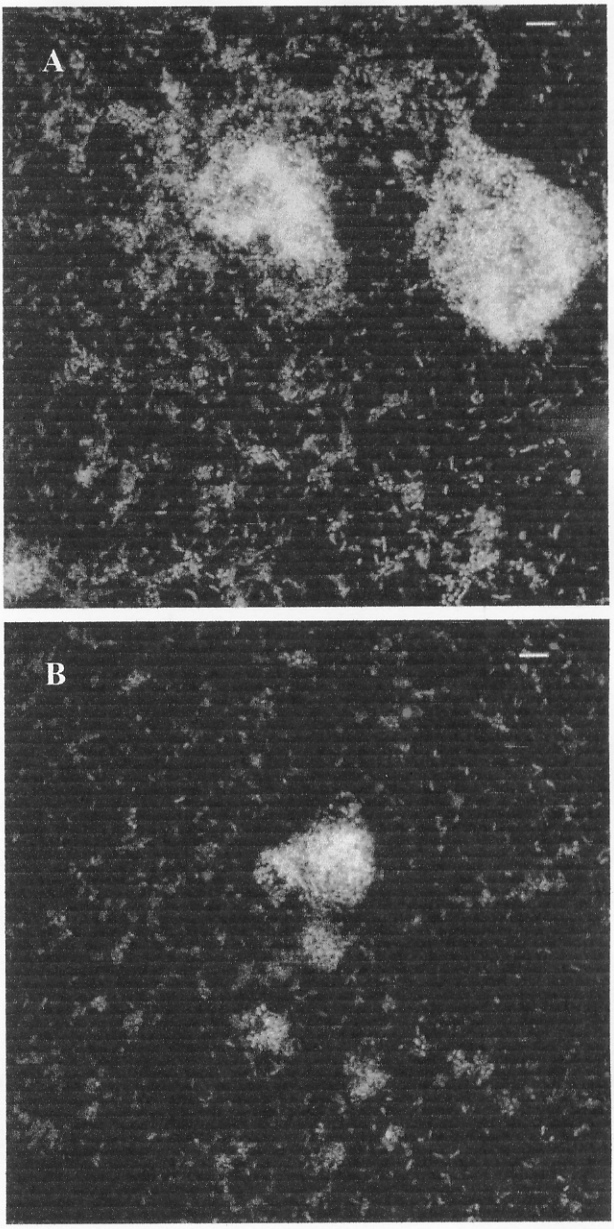

Figure 32. FISH micrograph of HA1 culture. (A) Image of FISH is dual hybridised with EUBMIX (green) for all bacteria and ALF968 (red) for *alphaproteobacteria.* Yellow coloured cells bound both probes and are the *alphaproteobacteria.* (B) Image of FISH is dual hybridised with EUBMIX (blue) for all bacteria and GAM 42 (red) for *gammaproteobacteria.* Yellow coloured cells bound both probes and are the *gammaproteobacteria.* All photographs, bar = 10 um.

bacteria, and *Alphaproteobacteria* bound with cocci-shape bacteria. *Alphaproteobacteria* were found more abundant than *Gammaproteobacteria.*

FISH results carried on HA1-5 indicated the presence of Proteobacteria (*Gammaproteobacteria* and *Alphaproteobacteria*) and also demonstrated that *Nitrobacter* (NIT3 probe), *Nitrospira* (Ntspa714 probe) and *Betaproteobacteria* (BET42a) specific probes were not present in the sample. *Nitrobacter* is *Alphaproteobacteria* and it was not present in the HA1, so this result strongly confirmed that *Alphaproteobacteria* in the HA1 is not *Nitrobacter.* Therefore, the conclusion at the moment could be that the cocci-shaped bacteria belonging to *Alphaproteobacteria* is the novel nitrite oxidizing bacteria, whereas the *Gammaproteobacteria* is *Alcanivorax* sp.

The dominance of *Alphaproteobacteria* in the HA1 is corroborated with previous FISH and 16S rDNA experiments, as illustrated in Table 16 (concluded from Chapter 3, part 2 and 3). As the result in Table 16, there were three bacterial groups composed of *alphaproteobacteria, gammaproteobacteria* and *Cytophaga Flavobacterium- Bacteroides* (CFB) that always found abundantly in LNOBSBR, HNOBSBR and NFSBR.

Table 16. Percentage of dominant bacterial groups found by FISH technique and 16S rDNA sequencing in LNOBSBR, HNOBSBR and NFSBR

Bacterial group	LNOBSBR		HNOBSBR		NFSBR	
	Sequencing	FISH	Sequencing	FISH	Sequencing	FISH
Alphaproteobacteria	33	5	24	30	31	19
Gammaproteobacteria	2	13	24	3	11	8
CFB	1	nd	9	nd	32	25

nd = not determined

The fact that the growth of halophiles requires at least some NaCl, but optimum varies with the organism; thus the terms mild halophile and moderate halophile and used to describe halophiles with low (1-6%) and moderate (6-15%) NaCl requirements, respectively (Madigan *et al.*, 2000). Therefore, HA1 may belong to the group of the mild halophile because it grows in the media with 2.5% salinity. However, the further experiment of cultivation HA1 in the media with the variation of salinity is needed for halophile group confirmation.

3.4.5. Conclusions

The OTU1 was the dominant group of nNFSBR, nLNOBSBR and nHNOBSBR clone libraries, so it could plays an important role of nitrite consumption according to their abundance in all libraries. The result from BLAST with OTU1 did not give significant homology with any organism, so it could be a novel NOB. The new primers ChanyF and ChanyR specifically to OTU1 sequence (novel NOB sequence) were designed in this study.

Only 1 isolate from 37 presumed single colonies isolated from LNOBSBR and HNOBSBR was matched to ChanyF and ChanyR, and able to consume 100 mg/l NO_2-N, so it was assumed to be the novel NOB which dominant in all sources. The result of 16S rDNA sequencing, FISH and the HA1 isolate microscopy study confirmed that HA1 composed of more than one bacterium. An unknown bacterium oxidizes nitrite to nitrate and *Alcanivorax* sp. consumes that nitrate. It was not possible to assign the NOB in HA1 to known genus because its sequence did not match with any known organisms. It is found to be a Gram negative, able to consume nitrite, grow in the 25 g/l salinity, and assumed to be a cocci-shaped, belongs to *alphaproteobacteria*.