Chapter 4

General Discussions and Conclusions

The two sequencing batch reactors (SBRs) operating with artificial wastewater containing 100 mg NH_4-N/l (15 mg NH_4-N/l final concentration in the SBR) and 25 ppt salinity were successfully used to enrich AOB and NOB from different seed sources (Chapter 3, part 1). Nitrification in the SBRs was studied at both low (7 d HRT) and high (3.5 d HRT) nutrient loading. Both AOB and NOB increased in number over the long-term operation of both SBRs indicated by chemical analysis and most probable number (MPN) technique. The ammonia removal 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 while the values were 71% and 83%, respectively, for the SBR with commercial seed. According to quantitative fluorescence *in situ* hybridization (FISH) probing with NSO190 and NSO1225, *Nitrosomonas* were the presumed abundant AOB from the natural and commercial seeds, comprising 21 ± 2% and 30 ± 2%, respectively of all bacteria. *Nitrosomonas* is a broad group of AOB encompassing many different species with varying ammonia oxidising capacities. It was concluded that the shrimp farm sourced seed might contain more efficacious AOB as more ammonia was removed by fewer AOB in this seed compared to the commercial seed. This could have significant practical ramifications.

Artificial wastewater was used in this study since there was great variation in the content of the outlet water from shrimp farm which was the consequence of different loading and growth of shrimp. The initial concentration of ammonia of 15 mg NH_4^+-N/l was high compared to actual aquaculture water (0.4 mg NH_4^+-N/l). However, in order to compare the actual ammonia loading in a recirculating system to the continuous-type operation used in the SBR experiment, the ammonia concentration was diluted to 15 mg

similar to that of Kim *et al.* (2000), where immobilized nitrifying bacteria were used in an aquaculture system.

The SBR was inoculated with water and sediment from a shrimp farm and was called the "nitrifying bacteria sequencing batch reactor" (NFSBR). The microbial community in the NFSBR was studied by FISH and 16S rRNA gene cloning and analysis (Chapter 3, part 2). FISH showed that two dominant bacterial groups were the *Cytophaga-Flavobacterium* group of *Bacteroidetes* (25% according to CF319a probe) and the *Betaproteobacteria* (24% according to BET42a probe). The abundant AOBs were concluded to be *Nitrosomonas* as 21± 2% of all bacteria bound NSO190 and NSO1225 for *Nitrosomonas*. Potentially novel AOBs were also deemed to be present in the NFSBR biomass since clusters of cells similar to the morphology of nitrifying bacteria clusters bound BET42a but none of the AOB-specific probes. Most AOB belong to *Betaproteobacteria*. No cells bound any of the published NOB-specific probes. Therefore, the NOBs present in this bioreactor were considered to be novel. Cloning using bacterial specific primers (27f and 1492r) and using AOB specific primers (190f and 1492r) revealed five clones that were highly identical to *Nitrosomonas nitrosa* in the *Nitrosomonas communis* clusters and these organisms would have been targeted by NSO190 and/or NSO1225. None of the clone sequences shed any light on the novel AOBs or NOBs hypothetically present in the NFSBR.

Since none of the published NOB-specific probes bound with NFSBR biomass, two SBRs using an autotrophic feed supplemented with low and high concentrations of nitrite were constructed in order to enrich autotrophic nitrite oxidizing bacteria (NOB) (Chapter 3, part 3). The sludge from NFSBR (Chapter 3, part 1) was taken and enriched separately in two SBRs containing modified artificial wastewater feed with KNO_2 at a low nitrite concentration of 0.24 mg NO_2-N/l (LNOBSBR) and at a high nitrite concentration of 50 –mg NO_2^--N/l (HNOBSBR) as the feed. The biomass from both SBRs confirmed that NOB were present according to the process performance data and results of MPN, FISH, and 16S rRNA gene cloning and analysis.

FISH results with NIT3 probing illustrated that the genus *Nitrobacter* comprised3% and 10% of all the bacteria in the LNOBSBR and HNOBSBR enrichments, respectively. These organisms are highly likely to be responsible for much of the nitrite oxidation occurring in these bioreactors. Large clusters of non-*Nitrobacter Alphaproteobacteria* were also observed in both biomasses along with clusters of other bacteria that might also be responsible for nitrite oxidation as the abundance of NIT3-binding cells was deemed to be too low to account for all the nitrite oxidation. The most abundant bacteria in LNOBSBR and HNOBSBR were *Alphaproteobacteria.* Cloning using bacterial specific primers (27f and 1492r) revealed 37 OTUs from the LNOBSBR biomass. One of these OTUs comprised 4 sequences which had a high match by BLAST with the 16S rRNA gene of *Nitrobacter alkalicus* (strain AN4, AF069958, a known nitrite oxidizer) and comprised 5% of the library. A total of 24% of clones from the HNOBSBR clone library demonstrated high BLAST matches with a clone sequence MPN2 (AY135357), closely related to *Nitrobacter.*

Evidence of *Nitrobacter* was found in all three SBRs - NFSBR, LNOBSBR and HNOBSBR (Table 17). However, it was also hypothesized that there were other nitrite oxidizers.

NFSBR and HNOBSBR clone libraries had sequences affiliated with MPN2 (accession number AY135357) recently reported by Fouratt *et al.* (2003) from a nitrifying bioaugmentation product was closely related to *Nitrobacter alkalicus* strain AN4 (AF069958) found in LNOBSBR biomass. *N. alkalicus* (AN4, AF069958) was isolated from an alkaline habitat by Sorokin *et al.* (1998). These two sequences (MPN2 and AN4) were thus the most common *Nitrobacter*-affiliated sequences found to be present in the SBR biomasses. Both these sequences are similar to *Nitrobacter* sp TH21 (AF080257) and *Nitrobacter winogradskyi* ATCC 25381 (L35506). All the *Nitrobacter*-affiliated sequences found in the biomasses from the different SBR clone libraries (Table 17) contained the NIT3 probe target and therefore organisms from which the clone sequences had been obtained should have bound the NIT3 probe.

Table 17. Evidence for the presence of *Nitrobacter* in NFSBR, LNOBSBR and HNOBSBR by 16S rRNA gene library and FISH.

SBR biomass	16S rRNA Clone Library	NIT3 probing
NFSBR	2% of all clones	no bacteria observed
LNOBSBR	5% of all clones	3% of all bacteria
HNOBSBR	24% of all clones	10% of all bacteria

It is not surprising that NOB found in all three SBRs appear to be closely related to *Nitrobacter* species. Most nitrification studies have been carried out with *N. winogradskyi* since they have been easily isolated to pure culture (Prosser, 1989). However, recent analyses of the bacterial populations found in activated sludge systems or wastewater biofilms suggest that *Nitrospira* is more commonly maintained as the NOB in these environments compared to *Nitrobacter* (Burrell *et al.*, 1998; Juretschko *et al.*, 1998; Burrell *et al.*, 1999; Blackall, 2000; Daims *et al.*, 2001).

It had already been hypothesized that the novel NOB could be *Alphaproteobacteria* or other bacteria. The latter could be bacteria closely related to *Nitrospira marina* as these are marine sourced NOB. Very few 16S rRNA gene sequences have been reported that are phylogenetically affiliated with *N. marina*. Thus, any FISH probes designed for *Nitrospira* would likely be relatively poorly designed specifically for *N. marina* and consequently many *N. marina* organisms might not be targeted by the *Nitrospira* probes and could be overlooked in FISH experiments. Currently, there are two 16S rRNA gene sequences for *N. marina* available in GenBank. These two sequences are also affiliated with some clone sequences obtained from microbial communities in Nullabor Plains Caves in Australia. However, the nitrite oxidation capacity of these cave microbial communities was only conjectured. Therefore, in an effort to determine if the hypothesized novel NOB in the saline SBRs were affiliated with *N. marina*, three 16S rRNA gene clone libraries were constructed using

the bacterial conserved 27f primer in combination with a primer designed to amplify *N. marina* (SNtsp1010r) (Chapter 3, part 4). 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. 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. Primers ChanyF and ChanyR designed from the non-16S rRNA gene sequences from the 27f-1010r libraries was thus deemed also unlikely to reveal anything specific about potential *N. marina*-related NOBs.

A preliminary attempt to isolate the hypothesized novel NOB in the different SBRs recovered 37 colonies of organisms that grew on nitrite-containing, organic carbon-deficient agarose-solidified media. Interestingly, one "pure culture" (called HA1) of the 37 generated a PCR amplificate of the appropriate size (250 nt) with ChanyF and ChanyR and, additionally the sequence of this product was identical to those generated from OTUs from which the ChanyF and ChanyR primers were designed. HA1 was found to not be a "pure culture" as it comprised a mixture of bacteria of which the most abundant were *Alphaproteobacteria* (according to ALF1b and ALF969 probing) and *Gammaproteobacteria* (according to GAM42a probing). However, neither of these is in any way related to *N. marina*, the target of 27f-1010r and consequently also the target of ChanyF and ChanyR. This HA1 "isolate" was found to be able to consume 100 mg NO_2^--N/l and the 16S rRNA gene sequence of an amplificate from HA1 generated by 27f-1492r PCR was highly identical to *Alcanivorax* sp. in *Gammaproteobacteria*. The *Alphaproteobacteria* in the "isolate" HA1 have not been able to be further identified and nor has confirmation of the capacity for nitrite oxidation of the "isolate" been possible since there was no analytical data confirming that the growth medium lacked organic carbon, although none was intentionally added.

Nitrite and nitrate were not detected, when checking the activity of HA1, due to the fact that *Alcanivorax* sp. was able to reduce nitrate (Bruns and Berthe-Corti, 1999). So the special characteristic of NOB in HA1 was to provide the complete nitrification. Therefore, HA1 may apply to remove nitrite perfectly in the environment because it removes nitrite without nitrate production to the system.