Conclusion

Biological denitrification is used widely to remove high nitrate concentrations from the wastewaters. Higher nitrate concentrations are produced by some fertilizer, chemical and explosive industries. When these nitrates are untreated and released into the environment, it causes eutrophication of lakes, or the nitrate percolates into the drinking water. Consumption of high level of nitrates causes various diseases including methemoglobinamia in the infants. The nitrates are removed by different processes like the activated sludge process (suspended growth process) or the biofilm-based reactors (attached growth process). However, there is very little understanding of the microbiology in these processes although the degradation job is done by these organisms, and more importance is generally given to the engineering design of the reactor. Knowing the denitrifying community composition of these nitrate removal processes will reinforce a different perspective of the reactor design to increase its efficiency. A top-down to bottom-up approach was used in this study, where the denitrifying bacterial community in different activated sludge were assessed and single population biofilm as well as community biofilm structure of denitrifying reactors were analysed with an attempt to correlate it with activity.

The denitrifying bacterial composition in the four different activated sludge samples were studied by both culture-dependent and independent approaches. *Pseudomonas* sp. and *Alcaligenes* sp. are found to be numerically dominant by the culturable methods, whereas the culture independent techniques targeting the *nosZ* gene shows betaproteobacteria to dominate the sludge environment. Denitrification was observed to vary among the cultures isolated from sludge, with some accumulating nitrite while reducing nitrate, and the others not accumulating. Difference in denitrification pattern among the cultivated bacteria, and presence of truncated denitrifiers with high numbers in the sludge as observed in this study could possibly be one of the reasons for inefficient nitrate removal and accumulation of intermediates during denitrification in wastewater treatment processes.

Denitrification studies between two isolates *Diaphorobacter* sp. D1 and *Paracoccus* sp. W1b, indicated two different kinds of electron transfer to occur in the bacteria. One being a sequential like the *Diaphorobacter* sp. D1, which accumulates nitrite at higher nitrate concentrations, and a branched electron transfer like the *Paracoccus* sp. W1b, which denitrifies efficiently without accumulation of the intermediates. The branched

electron transfer strategy also helps the organism to tolerate higher nitrate concentrations, as in the *Paracoccus* sp. W1b.

Biofilm studies on *Paracoccus* sp. W1b showed that the nutrients influence the biofilm formation, as well as its architecture significantly. Plackett-Burman statistical design was used to screen the effect of various nutrients on the biofilm formation, which showed high concentrations of succinate, Mg^{++}, Ca^{++} and Mn^{++} to enhance biofilm formation, whereas higher concentration of iron decreased biofilm formation. The divalent cations and succinate were also observed to modulate the biofilm architecture. Moreover, the Mg^{++} and Ca^{++} - induced biofilms, though increased biofilm formation by the cohesion of cells, formed contrasting architectures with subpopulations of pillar-like protruding cells distributed on a mosaic form of monolayer cells in the former and the latter induced a dense confluent biofilm. However, the subpopulations also differed in the fatty acid composition showing diversification in the *Paracoccus* sp. biofilm. Chelator treatment of various biofilm ages indicated that divalent cations are important in the initial stages of biofilm formation and the results obtained for *Paracoccus* biofilm also seems apparent for the ecological adaptation model proposed by Klausen et al (2006). Increased biomass and thickness of the biofilm, as induced by magnesium and calcium, increased the denitrification activity, and this could possibly be exploited in nitrate removal processes to provide anoxic conditions for increasing the denitrifying efficiency.

A laboratory-scale biofilm reactor was operated with different carbon sources in denitrifying conditions because exogenous carbon sources, which act as electron donors are provided for denitrification to occur in nitrate removal processes. This study suggests that acetate as a sole carbon source is efficient in nitrate removal by denitrification. Glucose as a carbon source supports the growth of nitrate ammonifying bacteria, which compete with the denitrifiers in anoxic zones leading to the accumulation of nitrite and ammonium. Though methanol is used widely for nitrate removal purposes, the methanol-fed biofilm accumulated high nitrite. Ethanol supports the growth of denitrifying bacteria in comparison to the nitrate ammonifiers and shows higher specific denitrification activity, however, the process of nitrate removal is relatively slow. *Pseudomonas* sp. dominated the biofilm community in presence of the different carbon sources used, possibly because of its strategy of colonizing the substratum surface from the initial period of biofilm development. Increasing the nitrate concentration increased

the population of *Paracoccus* sp., however the *Pseudomonas* sp. were found to be adhered to the substratum refurbishing that they colonize the substratum efficiently.

Denitrification studies with pure cultures have been generally carried out in *Paracoccus denitrificans, Pseudomonas stutzeri* and *Alcaligenes faecalis*. Nevertheless, this study found betaproteobacteria to dominate the sludge samples and especially the Comamonadaceae family. It is envisaged that further studies with more members from this group would provide better insight in the process design. Also, an extensive screening for enumeration of truncated denitrifiers in the sludge environments would help to model out their significance in the nitrate removal processes and the impact they have on the nitrogen cycle. This study also showed that the nutrients play an important role in determining the structure of biofilms, which importantly influence the activity. Thus, it would be interesting to develop statistical designs like Plackett-Burman, for high thoroughput screening of environmental impacts on biofilm structure to activity for various applications, including monitoring of substrates qualitatively and quantitatively to improve reactor efficiency in wastewater treatment processes.