EXECUTIVE SUMMARY

Conventional biological nitrogen removal processes in wastewater treatment works (WWTW) depend on carbohydrates (COD) as both energy and carbon source. Therefore, this process normally does not allow primary sedimentation and methane generation. In addition, these processes yield high amounts of sludge that have to be disposed of. An alternative nitrogen removal process, which does not require such costly manipulations, is the so-called anammox process.

Anammox (anaerobic ammonium oxidizing) bacteria are slow-growing, anaerobic autotrophs that convert ammonium and nitrite to dinitrogen gas. Consequently, these bacteria which require 25% less metabolic energy than the bacteria employed in conventional biological nitrogen removal processes, do not need an organic carbon source and have a low sludge production. However, inhibition by nitrite and oxygen, the slow growth rate and difficulty in establishing an enriched culture, are all significant drawbacks to the anammox process. In contrast, enriched anammox cultures are robust and therefore commercial processes based on anaerobic ammonium oxidizing bacteria (e.g. Anammox and CANON) have been patented and implemented. These anammox processes have drawn tremendous interest across the globe and, following the first experimental anammox reactor set-up in Delft, and better yet, the first commercial anammox reactor in Rotterdam, Netherlands, a number of reactors have been established. Such reactors could also improve the nitrogen removal capacity and efficiency of South African WWTW.

Based on these developments, the primary aim of this project was to identify and verify the presence of anammox bacteria in samples obtained from various anaerobic habitats in South Africa. This would have been achieved by first enriching for these bacteria using 1 L gas-lift reactors inoculated with samples taken from different anaerobic aqueous habitats. Stoichiometry would then be used to obtain evidence for anammox activity within these reactors. In addition, fluorescence *in situ* hybridization (FISH) and 16S RNA gene sequence analyses would be used to confirm the presence of anammox bacteria in the microbial consortia within the reactors. However, as the project developed, it became clear that screening different habitats for the presence of anammox by enriching for anammox bacteria in 1 L reactors was impractical and ineffective. Therefore, where possible, samples taken from different anaerobic aqueous habitats were directly screened for the presence of

anammox bacteria using molecular probes for taxonomic informative 16S RNA gene sequences.

Originally, the second aim of the project was to obtain sufficient anammox biomass in 5 L laboratory scale, gas-lift reactors to allow for physiological characterization of the anammox activity. The stoichiometry of the anammox process would then be analysed in batch tests at different temperatures, pH values, as well as at different nitrite and oxygen concentrations. However, because of the inefficiency of the 1 L gas-lift reactors to enrich for anammox bacteria, these larger laboratory scale reactors were used for anammox biomass enrichment. Although enrichment was later shown to occur within these larger laboratory scale reactors, the rate of enrichment was unsatisfactory. A lack of sufficient biomass once again lead to an adjustment of the project aims. Without obtaining sufficient biomass within the time-frame of the project, adequate physiological characterization of the biomass was impossible and therefore the focus shifted towards the investigation of reasons for the slow biomass production. Thus, to identify the predominant competing organisms in the reactors and their possible role in inhibiting the sufficient enrichment of anammox bacteria, pyrosequencing of the total genomic DNA in the reactors was included in the scope of the project. Confirming the data obtained for the stoichiometric analyses of the reactors it was found that Planctomycetes, containing anammox bacteria, represented only the minor bacterial populations within the consortia of the reactors. The dominant bacterial populations within the reactors either belonged to the Proteobacteria or Firmicutes, both containing taxa known to utilize the nitrogen compounds included in the anammox enrichment medium and therefore potentially able to outcompete the anammox bacteria in the reactors.

Literature and experiences reported in Delft and across the world indicate that enriching from industrial and natural environments where anammox bacteria are in low concentrations is particularly difficult, and that enriching anammox bacteria by seeding a reactor from a previously enriched anammox culture is more effective. Nevertheless, seeding from previously enriched cultures was not attempted in this study due to the difficulty of obtaining an enriched culture and because the scope of the project emphasized screening specifically indigenous habitats in order to determine the distribution of these bacteria in South Africa.

Another aim of the project was to transfer the necessary skills to students for the identification and characterization of bacteria, including FISH, 16S RNA gene sequence

analyses, as well as different physiological and biochemical characterization procedures and to further hone these techniques. Knowledge from this project was also to be disseminated to create awareness of the anammox process, through a peer reviewed article and conference presentations. In terms of capacity building, the project equipped two post-graduate students at the University of Stellenbosch, Ms. Wendy Stone and Mr. Ferdinand Postma, in anammox reactor maintenance, as well as in molecular techniques for the detection of anammox bacteria. Ms. Wendy Stone received an Honours degree *cum laude* on the project. She also gave oral presentations of her findings at two national conferences. In addition, an undergraduate student at the University of Stellenbosch was exposed to the laboratory environment, working on the project as laboratory assistant during vacations. Furthermore, Grade 11 learners in the Stellenbosch area were selected based on their performance in Science. These learners were then exposed to the department of Microbiology at Stellenbosch University and introduced to the challenges facing South Africa regarding water conservation and management. The members of this group who performed the best in Science during the following year then received bursaries for their final school year. In summary, the project exposed a number of young potential scientists to the wastewater industry of South Africa.

The last aim of this project was to stimulate inter-institutional research in environmental microbiology between the CSIR and Stellenbosch University that would lead to greater exposure of CSIR employees to academic research in microbiology. In addition, Stellenbosch University's potential to impact on the water sector would be increased through co-operation with the CSIR where the focus will be more on development and commercialization of the technology. Both these goals were reached since the employees of the CSIR were actively involved in every aspect of the project and Stellenbosch University's potential to impact on the water sector was greatly enhanced.

In summary, enrichment screening for anammox bacteria in small-volume reactors can be considered ineffective, since oxygen toxicity has a greater impact due to diffusion. Larger volume reactors are more effective in anammox enrichment, however, yet again oxygen leakage seemed to have a significant impact on reactor consortia, since small oxygen leakages early in enrichment seemed to stimulate consortium competition, slowing down pure anammox enrichment significantly. Nevertheless, molecular techniques were employed to conclusively demonstrate the presence of anammox bacteria in anoxic oceanic mud zones off the coast of South Africa. In addition, these oceanic anammox bacteria were enriched in

reactors, although at a notably slow rate. Although anammox bacteria could not be directly detected in wastewater, low concentrations of anammox bacteria were shown to be present after long periods in enrichment reactors inoculated with wastewater.

With regard to application of the anammox process in the South African wastewater treatment sector, the biomass enriched in this work was not sufficient for industrial applications. Literature indicates that anammox activity is cell density-dependent, and it has been demonstrated that although enrichment of anammox bacteria to an almost pure culture is a sensitive process, near pure cultures are nevertheless robust and efficient, well-suited to application in the wastewater industry. The importance of a number of parameters for anammox enrichment have been demonstrated in this work, including oxygen toxicity, the addition of vitamins and growth factors, nitrite toxicity and biomass washout and shear force due to mixing. Nevertheless, the primary suggestion based on this work would be the inoculation of reactors using enriched, active anammox sludge from functioning anammox reactors. The presence of anammox bacteria was conclusively demonstrated in South African environments; however the enrichment process is less feasible than inoculating from robust, active anammox cultures, since anammox bacteria are so sensitive to growth inhibition at low concentrations.

This report describes some of the difficulties of enriching for anammox bacteria from the environment, with several suggestions for biotechnologists planning to set up anammox reactors in South Africa. Nevertheless, the benefit of this biological nitrogen removal process remains attractive, and the establishment of viable anammox populations from the environment still remains the most important step in this work. Since the presence of anammox bacteria was demonstrated in natural and manmade environments in South Africa, the effective harnessing of these indigenous bacteria to improve wastewater treatment would be an ideal outcome, if this work is further pursued.