FISHing FOR INDIGENOUS ANAMMOX BACTERIA

Report to the
Water Research Commission

by

W Stone[1], JA Wilsenach[2], M Mouton[1] & A Botha[1]
[1]The University of Stellenbosch
in association with
[2] Virtual Consulting Engineers (Pty) Ltd

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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.

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FISHing FOR INDIGENOUS ANAMMOX BACTERIA

The Steering Committee responsible for this project consisted of the following persons:

Dr Valerie Naidoo	:	Water Research Commission (Chairperson)
Prof Alfred Botha	:	Stellenbosch University (Project leader)
Dr JA Wilsenach	:	Virtual Consulting Engineers (Project Team)
Prof Fanus Venter	:	University of Pretoria
Prof Faizal Bux	:	Durban University of Technology
Prof George Ekama	:	University of Cape Town
Prof Evans Chirwa	:	University of Pretoria
Dr Francois Talbot	:	Talbot & Talbot (Pty) Ltd
Ms Annalien Toerien	:	Golder Associates Africa (Pty) Ltd

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1. INTRODUCTION

In many countries research is being conducted to improve biological nitrogen removal from wastewater. Current biological nitrogen removal processes yield high amounts of "unwanted" sludge and an unwanted byproduct of the process require external carbon sources to function adequately. The anammox process, comprising the anaerobic oxidation of ammonium, is an improvement on the current biological nitrogen removal process employed by wastewater treatment works (WWTW) the world over. Anammox bacteria are slow-growing autotrophs, thus during this nitrogen removal process, high amounts of sludge is not produced and external carbon sources are not utilized. In addition, these bacteria need 25% less metabolic energy than conventional nitrifiers and heterotrophic denitrifiers. Therefore, anammox process is being actively pursued for implementation in the wastewater treatment industry across the globe.

Anammox bacteria were discovered in a number of anaerobic habitats all over the world, however, their enrichment from these natural habitats is considered difficult, since anammox activity is cell density-dependent and anammox cultures are only robust and effective once almost pure cultures are obtained. Nevertheless, the benefits of this process have driven many researchers to attempt enrichment of anammox bacteria for industrial applications.

Based on the above motivations, the aim of our study was to screen for anammox bacteria from various South African habitats using an enrichment process, with small-volume reactors. In addition, we aimed to inoculate larger laboratory scale reactors with composite samples, for anammox biomass enrichment. Detection of anammox bacteria in the reactors, as well as in the natural environment, was conducted using molecular techniques such as FISH and PCR amplification of taxonomically relevant gene sequences. In the reactors, anammox activity was assessed using stoichiometric evaluation of the nitrogenous compounds. The enriched anammox biomass was to be characterized in terms of pH tolerance, oxygen tolerance and activity, in order to evaluate these South African strains for industrial application. However, the small-volume reactors were found to be ineffective, due to oxygen diffusion and toxicity. Larger laboratory scale reactors also demonstrated very slow enrichment rates. The focus shifted to the investigation of possible reasons for this slow enrichment of anammox bacteria by using pyrosequencing of the total genomic DNA to elucidate the competition within the reactor consortia that seemed to be limiting anammox growth.

The above mentioned aims are addressed in this report; beginning with a broad literature overview of the discovery, characteristics, industrial applications and ecological impact of anammox bacteria and followed by a description of the anammox sampling, enrichment and detection procedures followed in this work. Finally, a discussion of the feasibility of anammox enrichment and the detection of anammox bacteria in various South African habitats are described, with suggestions on the application of these discoveries to the South African wastewater treatment industry as well as suggestions for future work on anammox reactors.

2. LITERATURE REVIEW

2.1 Introduction

Nitrogen removal from wastewater is crucial in preventing ecological catastrophes such as ammonia toxicity, eutrophication in receiving watercourses and high nitrate concentrations in drinking water. The conventional process associated with nitrogen removal forms one of the overall wastewater treatment process bottlenecks, i.e. the process cannot run faster than the rate of nitrogen removal. This aspect determines the physical size of the process, the energy requirement, sludge production and ultimately effluent quality. The current processes used for this task and the exploration for new methods of nitrogen removal are an active research domain. Currently, nitrogen removal is achieved by nitrification, involving oxidation of ammonium to nitrate via nitrite (nitrification) followed by denitrification of nitrate to dinitrogen gas (Van Dongen et al., 2001). Nitrification is an autotrophic aerobic process, whilst denitrification is carried out by anaerobic or anoxic heterotrophs. The biological removal of nitrogen gas over nitrate has been in place for many years and is the default option for wastewater treatment. However, because denitrifiers are heterotrophic, wastewater with a high nitrogen:COD ratio will require dosing of external carbon sources such as methanol – at greater costs – in order to remove nitrogen to acceptable concentrations. Furthermore, growth on carbon leads to higher sludge production, which adds to the cost of waste sludge management. Therefore, new means of nitrogen removal, are actively sought. Just such a biological catalyst was discovered nearly a decade ago (Strous et al., 1999). These organisms, known as anammox bacteria, are autotrophic and slow growing, eradicating the need for an external carbon source, eliminating the problem of a high sludge production and allowing for a 25% metabolic energy saving according to thermodynamic principles (Warakomski et al., 2007). These industrial benefits, as well as the unique metabolic, physiological and ecological characteristics of anammox bacteria have stimulated tremendous interest and have led to the generation of substantial and significant findings.

2.2 Discovery

More than forty years ago Broda used thermodynamic principles (Table 1) to predict the existence of microorganisms that can anaerobically convert ammonium to dinitrogen gas using nitrite as an electron acceptor (Broda, 1976). The existence of such organisms would solve the above-mentioned problems associated with the current nitrogen removal process, by eliminating the chemical oxygen demand. By comparing the energy yield of ammonium oxidation with different electron acceptors (Table 1), the anammox process is shown to be energetically comparable to aerobic ammonium oxidation. However, the biological anammox catalysts remained elusive until 1999, when the first organism able to carry out the anammox reaction was discovered in a denitrifying pilot plant and provisionally identified as *Candidatus* 'Brocadia anammoxidans' (Strous et al., 1999). Since this discovery, the physiology and metabolism of anammox (anaerobic ammonium oxidizing) bacteria have been extensively studied and three genera, *Candidatus* 'Brocadia', *Candidatus* 'Kuenenia' (Schmid et al., 2000) and *Candidatus* 'Scalindua' (Schmid et al., 2003), were discovered in anaerobic sludge from wastewater reactors and in anoxic oceanic sediments (Jetten et al., 2005). These three genera have similar metabolism and ultrastructure, indicating that the capability for anaerobic ammonium oxidation evolved only once (Strous et al., 2006). *Candidatus* "Anammoxoglobus propionicus" has been recently discovered in a laboratory reactor (Kartal et al., 2007). An even newer genus, discovered in a granular sludge anammox reactor in Korea, was named *Candidatus* "Jettenia" (Quan et al., 2008). Anammox nomenclature is always preceded by 'Candidatus' due to the fact that no strain has yet been isolated in pure culture, rendering official classification impossible. Anammox activity in the above-mentioned anoxic oceanic water columns was shown to contribute up to 67% of total nitrogen production in these natural regions, demonstrating the significance of anammox organisms in the global nitrogen cycle (Thamdrup and Dalsgaard, 2002).

Table 1 A comparison of the energy yield of ammonium oxidation with various electron acceptors

Electron Acceptor	Reaction Stoichiometry	Gibbs Free Energy Change[a]
Oxygen	$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 2H_2O + 4H^+$	-241[b]
Nitrite	$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$	-335[b]
Nitrate	$5NH_4^+ + 3NO_3^- \rightarrow 4N_2 + 9H_2O + 2H^+$	-278[b]
Oxygen	$NH_4^+ + 2O_2 \rightarrow NO_3^- + H_2O + 2H^+$	-349[c]

[a] kilojoules/mole ammonium at pH 7, 25ºC, substrate and product conc. 0.1mM (except water and H^+)
[b] Strous et al. 2002
[c] Jetten et al. 1999

2.3 Physiology and Metabolism

Anammox bacteria have interesting physiological characteristics that support their unique metabolism. They form part of the order *Planctomycetales* and are characterized by budding reproduction and a proteinaceous cell wall that lacks peptidoglycan (Van Niftrik et al., 2004). They are most notably distinct from the other *Planctomycetes* in their chemolithoautotrophy (Chamchoi et al., 2007). These bacteria are renowned as slow-growing, with a doubling rate of 11 days under optimal conditions (Van der Star et al., 2007), although if inoculated from a pre-culture, this doubling rate is drastically reduced (Op den Camp et al., 2006). In addition, their anammox activity seems to be dependent upon cell density (Strous et al., 1999). Due to these characteristics, anammox bacteria are incompatible to current isolation protocols and therefore Percoll density centrifugation, in combination with enrichment, is the best means of obtaining a mostly pure culture.

Anammoxosome. The anammox catabolism is localized in a unique organelle, the anammoxosome (Van Niftrik et al., 2004). The anammoxosome has shown to be vertically inherited by means of a novel cell division protein (Van Niftrik et al., 2009). The cytoplasm of these organisms is divided into three compartments, the anammoxosome being the largest and innermost compartment (Fig 1). This organelle is delimited by a single bilayer membrane that is ether-linked (reminiscent of the *Archaea*) as well as ester-linked (reminiscent of the *Bacteria* and *Eucarya*) and contains ladderane lipids which appear to be unique to anammox bacteria, and are therefore often used as a target for detection in natural environments (Kuypers et al., 2003).

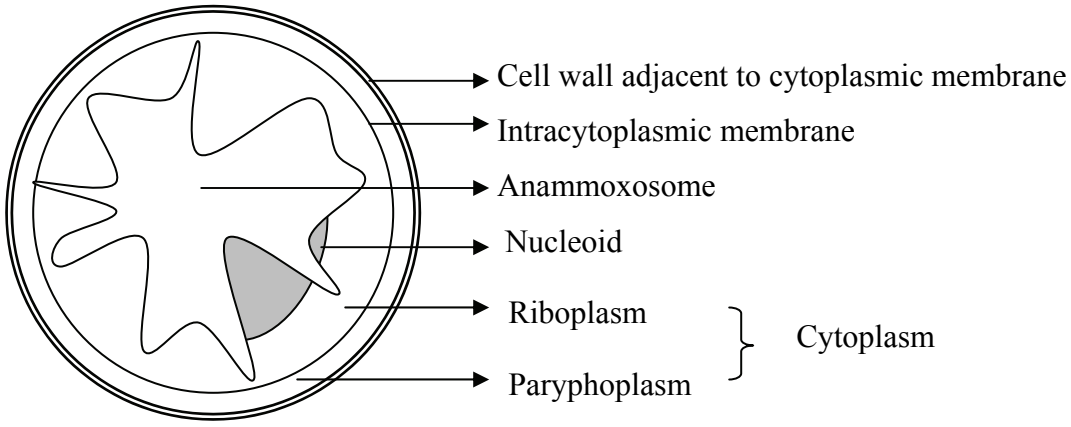


Fig 1 Schematic representation of membrane compartmentalization in anammox bacteria (Adapted from Van Niftrik et al., 2004).

A number of proteins were found to occur only in annammox bacteria, including a hydrazine hydrolase, a hydrazine-oxidizing enzyme and a membrane-bound ATP synthase (Karlsson et al., 2009). These were all found to be confined to the anammoxosome, thereby localizing the anammox metabolism to the anammoxosome.

Catabolism. The ladderane lipids are composed of cyclobutane and cyclohexane ring systems, both of which have a low degree of rotational freedom, affording the membrane increased rigidity and impermeability (Van Niftrik et al., 2004). In addition, the membranes contain the hopanoids typical of the *Planctomycetes* (Damste et al., 2004). This impermeability of the anammoxosomal membrane prevents leakage of protons and metabolic intermediates, including hydrazine which is very reactive and therefore toxic to the cell. Should the dissipation of protons occur, the proton motive force (PMF), which is essential to the catabolism of these bacteria (Fig 2) will decrease. The prevention of the dissipation of the PMF is especially critical due to the slow catabolism of these organisms, as 15 moles of ammonium must be oxidized for the formation of 1 mole biomass according to the following stoichiometry (Van der Star et al., 2007):

$$1NH_4^+ + 1.3NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3 + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O$$

It has been suggested that this conversion of ammonium and nitrite to dinitrogen gas occurs via the formation of the intermediates hydrazine (N_2H_2) and hydroxylamine (NH_2OH), two metabolic intermediates that are regularly added to reactors to kick-start anammox enrichment (Strous et al., 2006). Elucidation of the *Candidatus* 'Kuenenia stuttgartiensis' genome has however thrown doubt upon the role of hydroxylamine and has suggested nitrous oxide as an intermediate. Nevertheless, electron transport along this path produces a proton gradient across the anammoxosome membrane (Fig 2a). This PMF stimulates the production of ATP which is used in the autotrophic formation of biomass via the acetyl-Coenzyme A carbon fixation pathway. Acetyl-CoA synthetase obtains electrons from hydrazine via ferrodoxin, and the reduced hydrazine pool is replenished by reverse electron transport (Fig 2b). This catabolic model has been supported by the demonstration, for only the second time in chemotrophic bacteria, of an intracellular pH gradient in *Candidatus* 'K. stuttgartiensis' (Van der Star et al., 2009).

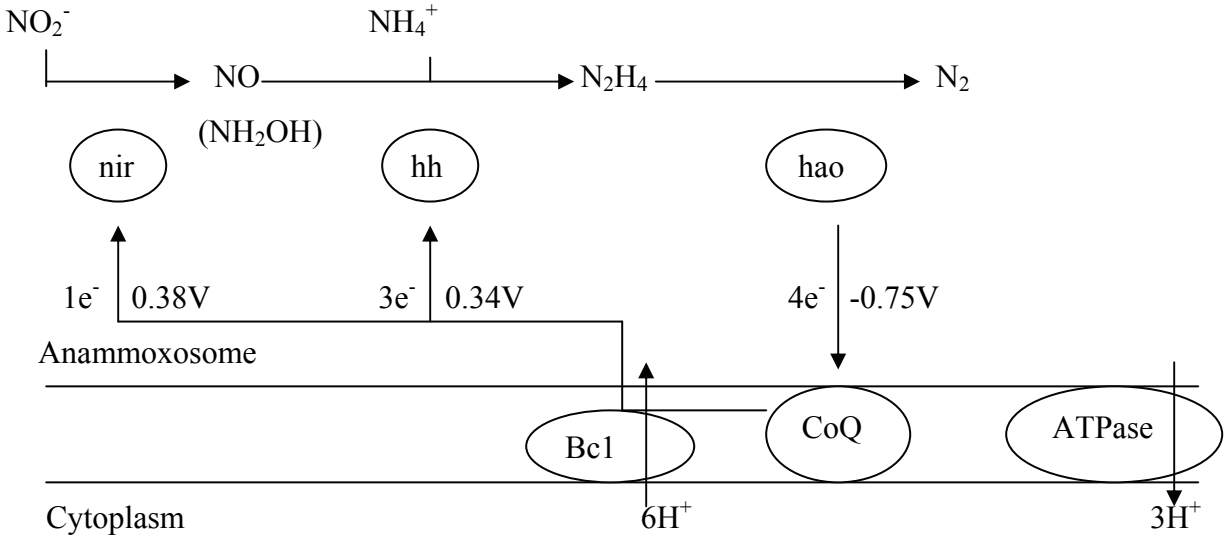


Fig 2a The central catabolism of anammox organisms, including electron transport and energy conservation (Adapted from Strous et al., 2006). nir – nitrite reductase, hh – hydrazine hydrolase, hao – hydroxylamine oxidoreductase, Bc1 – bacterial cytochrome, CoQ – Coenzyme Q.

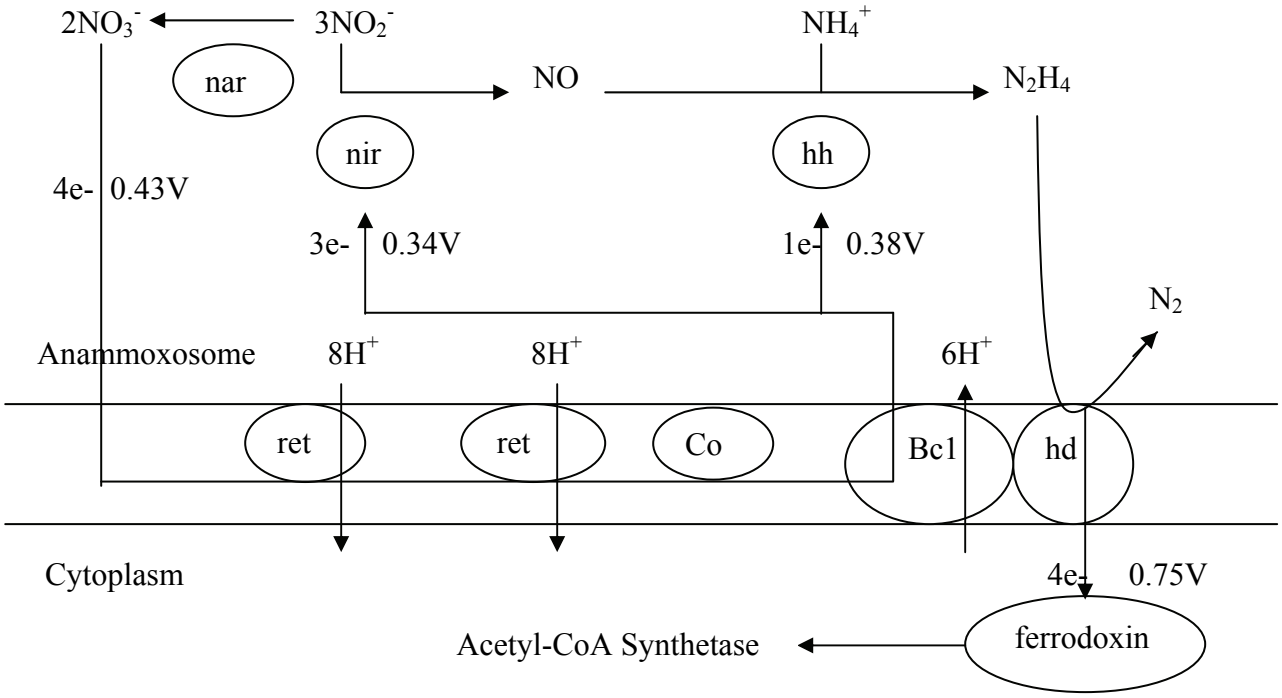


Fig 2b Anammox central catabolism combined with reverse electron transport to provide electrons for acetyl-CoA synthetase and anabolism (Adapted from Strous et al., 2006). nar – nitrate reductase, ret – reverse electron transport chain proteins, hd- hydrazine dehydrogenase.

The possible catabolic pathway of the anammox process, which takes place in the anammoxosome, as described by Van Niftrik et al. (2004), was discussed in more detail by Chamchoi et al. (2007). Hydrazine hydrolase, the hydrazine forming enzyme, oxidizes ammonium with hydroxylamine as an electron acceptor (Fig 2a; Jetten et al., 1999; Van Niftrik et al., 2004). Subsequently, the hydrazine oxidizing enzyme converts hydrazine to dinitrogen gas (Jetten et al., 1999; Schmid et al., 2003; Van Niftrik et al., 2004). This oxidation generates four electrons that combine with five protons to reduce nitrite to hydroxylamine via the nitrite reducing enzyme (Schmid et al., 2003; Van Niftrik et al., 2004).

As described in above-mentioned anammox metabolic equation (Van der Star et al., 2007), the ratio of ammonium to nitrite consumption is theoretically 1:1.3. The anaerobic oxidization of nitrite to nitrate accounts for the excess 0.3 moles of nitrite, and yields electrons for CO_2 fixation, reducing it to the level of cell material for cell growth (Van de Graaf et al., 1996; Strous et al., 1998; Van Dongen et al., 2001; Schmid et al., 2003). It must be emphasized that this pathway is hypothetical, determined *in silico,* but is the most appropriate pathway thermodynamically consistent with Ockham's razor, involving the fewest number of biochemical reactions (Jetten et al., 2009). This metabolic pathway has clearly been described as strictly autotrophic, however it is not comprehensive in terms of anammox catabolism as the more recently discovered species *Anammoxoglobus propionicus* has been shown to grow on organic acids such as acetic acid, and has even been demonstrated to out-compete heterotrophs for propionate (Kartal et al., 2007). During this metabolic study, it was shown that alcohols (most potently methanol), inhibit anammox activity, whereas short-chain fatty acids are converted by these organisms. Propionate consumption and anammox took place simultaneously, and these bacteria were even shown to reduce nitrate to nitrite by a still unknown mechanism. Although the focus of anammox exploration is on autotrophic nitrogen removal, this research clearly demonstrates the substantial versatility of the anammox metabolism.

2.4 Enrichment and Industrial Applications

Enrichment reactor. Although the potential to harness their unique metabolism in the wastewater treatment industry was immediately identified upon discovery of anammox bacteria (Strous et al., 1999), the practical application of these organisms in wastewater treatment works (WWTW) proved to be less encouraging. As previously mentioned, anammox bacteria have a reported enrichment doubling time of 11 days, in the most optimal conditions yet found (Schmid et al., 2003). Hence, enrichment reactor design is crucial in order to effectively obtain anammox biomass from an inoculum. Long-term

enrichment, biomass retention and quantitative analysis are three aspects that should be kept in mind during reactor design (Chamchoi et al., 2007). Schmid et al. (2003) also reported a biomass production of 0.13 g dry weight/g NH_3-N oxidized. This low sludge production is a benefit in the wastewater treatment industry, but emphasizes the importance of biomass retention during enrichment. A number of reactor types have thus been explored to find the most appropriate reactor for the anammox process (Chamchoi et al., 2007). They include the biofilm, or granular sludge reactor (Van Dongen et al., 2001); the fluidized bed reactor (Mulder et al., 1995; Van de Graaf et al., 1996); rotating biological contactors (Egli et al., 2001); gas-lift reactors (Dapena-Mora et al., 2004) and the sequencing batch reactor (Van Dongen et al., 2001). Among these, much research seems to have eventually established the sequencing batch reactor as the most popular design for anammox enrichment, combining the affectivity of the gas-lift reactor with the benefit of biomass retention. However, the highest nitrogen removal rate has thus far been achieved in gas-lift reactors (Dapena-Mora et al., 2004), illustrated in Fig 3.

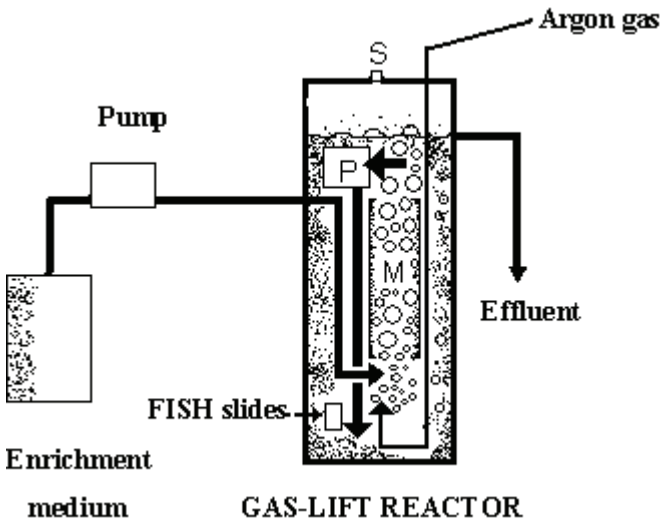


Fig 3. Representation of a gas-lift reactor, adapted from Chamchoi et al., 2007. The design includes a sampling port at the top (S) and a central tube (M) allowing for enhanced mixing as the gas flows upwards. The reactors used in the present study also contained slides to be used for FISH analyses. A pump (P) circulating the culture fluid from the top to the bottom was later installed. A sequencing batch reactor has a similar design, but the media is batch fed, with the sludge allowed to settle before effluent removal, improving biomass retention.

Enrichment and scale up. Despite specific enrichment demands, anammox bacteria were reported to be successfully enriched from various conventional sludge inocula; including upflow anaerobic sludge blanket, activated sludge and anaerobic digestion sludge; all in as little as four months (Chamchoi et al., 2007). The anammox process has a reported saving of 90% of the operation costs, in comparison to

the conventional nitrogen removal processes (Jetten et al., 2001), outweighing the difficulty of a long-term enrichment process. In addition, if preceded by partial nitrification to nitrite, the anammox process can replace conventional denitrification, reducing nitrification aeration costs by 50% (Op den Camp et al., 2006). In particular, the most prolific research and application has taken place with regards to the nitrogen removal from livestock manure digester liquor (Kunz et al., 2007; Molinuevo et al., 2008; Dong et al., 2010; Qiao et al., 2010). The anammox process is relevant in this case because of the notably high nitrogen load in this particular waste category.

The anammox process was successfully scaled up from laboratory-scale to full-scale for the first time in Rotterdam, Holland, in 2007. The scale-up procedure, including pitfalls, was comprehensively described by Van der Star and colleagues (2007), making the technology accessible to all wastewater treatment industries. The full scale-up was reported as successful, with the industrial-scale reactor treating 750 kg-N/day upon stabilization. The problems they reported have a significant impact on the implementation of this technology. These challenges include nitrite toxicity, biomass washout, methanol (originating from nitrification) toxicity, and failing of mixing apparatus. Biomass washout is due to a slow growth rate (Van der Star et al., 2007). Nitrite toxicity was reported at a threshold level of as low as 50-150 mg-N/L (Strous et al., 1999). This poses another enrichment challenge, as nitrite is a metabolic substrate (Strous et al., 2006), resulting in the demand of a fine balance during enrichment. A problem not reported by Van der Star and colleagues is the sensitivity of anammox bacteria to oxygen. Low levels reversibly inhibit the anammox reaction, whereas high levels are irreversibly toxic to the organisms (Strous et al., 1999). This is less of an obstacle during scale-up, but a significant obstacle during enrichment, since anammox activity is cell density dependent (Strous et al., 1999). However, as already mentioned, the process has been successfully implemented industrially, with a significant cost benefit, and these problems are only reported in order to speed up the process, rather than to discourage implementation. Since the implementation of the first full-scale reactor in Holland, at least five other full-scale reactors were effectively implemented (Op den Camp et al., 2006).

Industrial reactors. Anammox bacteria have been successfully enriched with a number of reactor designs, and the process has been implemented in an industrial scale, dedicated reactor. In addition to these developments, the potential of the anammox process in combination with current nitrogen treatment processes has also been extensively explored (Van Dongen et al., 2001; Sliekers et al., 2002; Vazquez-Padin, 2009). Two effectively-combined reactor systems involving the anammox process have been comprehensively described. These are the Completely Autotrophic removal of Nitrogen

Over Nitrite (CANON) and the Single reactor system for High Ammonium Removal Over Nitrite (SHARON) processes. The CANON process involves partial nitrification of ammonium to nitrite by aerobic nitrifiers, and further oxidation of ammonium to dinitrogen gas by anammox bacteria (Sliekers et al., 2002). This occurs in a single reactor under oxygen limitation, as anammox bacteria are reversibly inhibited by low oxygen concentrations (Van de Graaf et al., 1996). The process thus occurs in separate phases to compensate for the different oxygen tolerances of the various species (Gong et al., 2007). The CANON process is particularly suitable for the treatment of ammonium-rich wastewater that has an organic load too low to support the conventional nitrification/denitrification process (Sleikers et al., 2003). A challenge of the CANON process, however, involves preventing growth of nitrite oxidizers, with dual competition for oxygen and nitrite being the best means of inhibiting the enrichment of such contaminants (Op Den Camp et al., 2006). Despite the need for a well-balanced nitrogen load and aeration, CANON has recently been demonstrated as an efficient means of treating anaerobic digester effluent at room temperature (Vazquez-Padin, 2009).

The combined SHARON-Anammox process involves two reactors, the first containing aerobic nitrifiers and the second containing anammox species (Van Dongen et al., 2001). Partial nitrification is achieved in the first reactor by operation at temperatures higher than 25°C. A high temperature increases the growth rate of bacteria which oxidize ammonium to nitrite, such as those of the genus *Nitrosomonas*. It also decreases the growth rate of species which oxidize nitrite to nitrate, such as those of the genus *Nitrobacter*. The flow rate of the reactor can be set to select for the rapidly growing ammonium oxidizers, washing out the nitrite oxidizers and successfully preventing nitrification from proceeding to the end. The effluent of this reactor then contains the ideal substrates for complete nitrogen removal by anammox in the subsequent reactor. Van Dongen et al. (2001) showed that the ammonium oxidizers from the SHARON effluent did not accumulate in the anammox sequencing batch reactor, and evaluated the process as sufficiently effective for industrial applications. The two processes mentioned above are the most popular for industrial upscale in the practical application of the anammox process.

Anammox as part of a diverse microbial consortium within the reactor. Aside from the above-mentioned two classic implementations of anammox reactors, anammox has been extensively demonstrated in combination with other processes and as a stable part of various consortia. Reactors with significant nitrogen removal capacities have been shown to contain anammox as a stable part of a phylogenetically diverse reactor population (Qiao et al., 2009). Particularly, aerobic ammonium

oxidizers, of the β-*Proteobacteria,* were found to occur in oxygen-limited environments alongside anammox bacteria (Li et al., 2009, Li et al., 2010). Even after a year-long anammox enrichment, a reactor consortium has still been reported to contain aerobic ammonium oxidizers (Quan et al., 2008). The contribution of the various components of the microbial community of a nitrogen removal process, involving the presence of CH_4 and 0_2, was evaluated (Waki et al., 2009). Anammox activity contributed up to 8.7% of total nitrogen removal, in conjunction with methane oxidation and microbial assimilation by denitrifiers and aerobic ammonium oxidizers. Reactors with concurrent anammox activity and denitrification were also found to remove nitrogen efficiently (Chamchoi et al., 2008); however, the organic load was found to have a negative impact on anammox activity. A COD concentration of over 300 mg/L was observed to completely eradicate anammox biomass, and a reactor in which denitrifiers and anammox coexist is therefore not ideal. It seems that, although anammox bacteria are difficult to enrich due to the need for very specific conditions, they are widely present in wastewater environments, even in those environments that may not seem ideal.

2.5 Detection and Molecular Analyses

Anammox enrichment and application are shown to be feasible without pure cultures. However, in order for such enrichment and application studies to take place, crucial tools for the detection and observation of these bacteria needed to be developed, particularly because they cannot be isolated. Initially, N-15 labeled ammonium and nitrite was the method of choice for the detection of anammox activity. However, the elucidation of the *Candidatus* 'Kuenenia stuttgartiensis' genome was of groundbreaking importance in this respect (Strous et al., 2006). The genome was deciphered from a 74% anammox-dominant community genome due to the difficulty in isolating this organism, this feat being achieved by the implementation of a metagenomic approach. The genome sequence was used for *in silico* reproduction of the anammox metabolic pathway and its intermediates. It also led to the design of primers aimed at amplifying taxonomic informative 16S rDNA gene sequences, which can be compared using phylogenetic analyses. In addition, it led to the design of fluorescent probes for spatial analysis using fluorescent *in situ* hybridization (FISH). Upon these developments, FISH became the 'gold standard' for anammox detection (Schmid et al., 2005). Anammox bacteria are microscopically identified as between 0.3 and 1 μm in diameter (Van Niftrik et al., 2004) and are characteristically doughnut shaped. However, numerous reports of autofluorescence have undermined the reliability of FISH (Amann et al., 1995; Moter and Gobel, 2000; Coskuner et al., 2001). Real Time PCR (qRT-

PCR) is quickly becoming the most popular quantitative tool of choice (Tsushima et al., 2007; Pynaert et al., 2003).

2.6 Ecology and Biogeochemical Contributions

The above-mentioned developments, particularly the sequencing of the *Candidatus* Kuenenia genome, have greatly facilitated anammox research. During the last decade anammox bacteria were found to be more metabolically versatile than initially assumed. Also, they were detected in more diverse environments than expected. Although they have been discovered to be widely present in nitrogen loaded wastewater environments, their role in the ocean has been particularly underestimated.

Oceanic environment. Anammox bacteria are now thought to contribute significantly to oceanic nitrogen cycling and are therefore a major source of global dinitrogen gas (Thamdrup and Dalsgaard, 2002). Initially, denitrifiers were thought to be the only important sink for fixed inorganic nitrogen in the ocean. However, in 2003, anammox bacteria were discovered for the first time in a natural ecological environment (Kuypers et al., 2003). They were discovered in the Black Sea, the world's largest anoxic basin, and, by the detection of ladderane lipids, were shown to be just below the oxic zone, consuming the ammonium diffusing up from the anoxic deep-sea regions. Already the potential contribution of anammox to the global biogeochemical cycle was envisioned, and later confirmed (Thamdrup and Dalsgaard, 2002). Since this discovery, anammox bacteria were evidenced in a broad geographical range of anoxic marine habitats, particularly in the sediments and anoxic water columns (Dalsgaard et al., 2003; Dalsgaard et al., 2005; Jaeschke et al., 2007). Trimmer et al. (2004) attributed the regulation of anammox bacteria in these sediments to the availability of nitrite and nitrate, as well as to the relative size of the anammox population. In a similar study, Engstrom et al. (2005) observed that the relative importance of anammox to nitrogen removal in sediments was inversely proportional to the remineralized solute production, benthic oxygen consumption and amount of chlorophyll a in the surface sediment, again emphasizing that competition for nitrite is the limiting factor. It was also found that anammox bacteria are the largest contributor to nitrogen removal in the oxygen minimum zone (OMZ) in one of the world's most productive oceanic regions, the Benguela upwelling system of the South Atlantic Ocean (Kuypers et al., 2005). *Candidatus* 'Scalindua' is the most common anammox genus in these oxygen-limited oceanic habitats (Humbert et al., 2010), and lower depths were found to harbor a greater *Planctomycete* diversity than the upper, more oxidized regions (Kirkpatrick et al., 2006). Studies have linked anammox with *Crenarchaeal* and bacterial nitrification in the Black Sea

(Lam et al., 2007). Nitrification in the suboxic zone by Marine Group I *Crenarchaeota* and aerobic ammonium oxidizers (γ-*Proteobacteria*) provided a direct source of nitrite for anammox, present in the same zone, thus coupling anammox neatly into the global nitrogen cycle.

In addition to the deep oceanic zones, anammox have since been found in a variety of aquatic habitats, including marshes and wetlands (both tidal and freshwater) and mangroves and subtropical freshwater river sediments (Meyer et al., 2005; Koop-Jacobsen and Giblin, 2009), as well as in Arctic sea ice and marine sediments (Rysgaard et al., 2004; Rysgaard et al., 2004). Although anammox bacteria were shown to contribute to nitrogen removal in constructed wetlands used to treat, for instance, livestock effluent (Dong et al., 2010); anammox activity in natural freshwater and saline wetlands is minimal (Koop-Jacobsen and Giblin, 2009). The contribution of anammox to nitrogen cycling in the sediments of marine tidal marshes reaches a maximum of 3%, and is even lower in freshwater marshes. In addition, an increase in nitrogen loading did not stimulate an increase in anammox contribution to nitrogen cycling. Similarly, an increase in wastewater nitrogen did not stimulate anammox activity in oceanic anammox enrichment cultures either (Kawagoshi et al., 2010).

Arctic. Although the presence of anammox was confirmed in Arctic marine sediments and ice, the contribution of anammox to nitrogen removal (in comparison to the denitrifiers) in the ice was also low, with a higher relative contribution in the sediments (Rysgaard et al., 2004a; Rysgaard et al., 2004b). Within the ice, despite algal activity, oxygen consumption by denitrifiers is significant, creating an oxygen-limited environment in which anammox bacteria can grow. The contribution of anammox to nitrogen removal in this ice habitat is less than 5%, whereas in the associated Arctic sediments it reaches as high as 35%, the rest being attributed to the heterotrophic denitrifiers. The temperature optimum of the ice-bound anammox bacteria is approximately 12°C, in stark contrast to that of anammox bacteria enriched from wastewater habitats, with limits of 20°C and 43°C, and an optimum of 30°C (Strous et al., 1999). This is an indication of how effectively anammox have adapted to natural ecological habitats, as well as to wastewater sludge.

Terrestrial habitats. Although aquatic habitats for anammox bacteria were most extensively studied, terrestrial habitats have also been discovered, even demonstrating a greater diversity than aquatic habitats (Humbert et al., 2010; Dong et al., 2010). Humbert and colleagues (2010) reported the presence of anammox bacteria in marshes, lakeshores, a contaminated porous aquifer, permafrost soil, agricultural soil, and interestingly, in association with nitrogen-fixing plants. Again, the ecological

specificity of anammox was demonstrated by the fact that they were found in a limited number of niches, characterized by specific depths and soil types. The genera *Candidatus* 'Kuenenia' and 'Brocadia' were those most commonly reported as terrestrial inhabitants.

2.7 Conclusion

Despite their relatively recent discovery, all aspects of anammox bacteria, from industrial applications, to metabolism, physiology and biochemistry, as well as ecological occurrences; have been thoroughly explored. However, questions still surround this slow-growing organism and its unique metabolism. The extensive knowledge that has been developed renders this technology ideal for implementation.

3. MATERIALS AND METHODS

3.1 Primary enrichment reactors

Small-scale primary enrichment reactors were employed to screen for the presence of anammox bacteria in various South African habitats, particularly those in which anammox bacterial concentrations are too low to detect with molecular techniques. Each small-scale reactor was inoculated with a sample from a different location, in some cases representing various microhabitats (i.e. biofilms) within the same reactor type of a WWTW. For this purpose, sixteen 1L cylindrical (diameter, 7 cm; height, 40 cm) gas-lift reactors were set up in a 30°C incubation room. Eight of these reactors were inoculated with samples taken from wastewater treatment and sludge handling works, anaerobic digesters, or anaerobic zones in natural aqueous habitats. The reactors were fed with a constant inflow of enrichment medium (0.0500 L/24 hr) and flushed with nitrogen gas (AFROX) to maintain anaerobic conditions due to oxygen inhibition of anammox activity (Van de Graaf et al., 1996). Anaerobic conditions in the reactor head spaces were monitored using Anaerotest® strips (Anaerotest®, Merck KGaA, Darmstadt, Germany). The pH was manually maintained between 7.5 and 8.5 with the addition of concentrated hydrochloric acid (Strous et al., 1999). The last eight reactors were to be inoculated six months later, in order to account for seasonal variation.

3.2 Secondary enrichment reactors

Secondary enrichment reactors were designed with larger volumes with the aim of producing sufficient biomass volumes for physiological batch tests and further industrial applications. Therefore, four larger laboratory scale gas-lift reactors (Fig 3) were inoculated with composite samples, either originating from wastewater of WWTW or from the oceanic environment. Reactor 1 (R1), with a 5:1 height to diameter ratio and a 4.5 L working volume, was inoculated in June 2008 from a combined sludge sample. Reactor 2 (R2), with a 10:1 height to diameter ratio (theoretically providing better diffusion of sparging gas into the culture fluid than a 5:1 ratio) and a working volume of 17 L, was inoculated in February 2009 to account for seasonal changes in the microbial consortium of the WWTW. Reactors 3 and 4, with similar height to diameter ratios and working volumes to R2, were inoculated from combined sediment samples collected from the anaerobic zones of St. Helena bay. R3 was incubated at 30°C, the optimal temperature for the anammox reaction (Van der Star et al., 2007), whereas R4 was incubated at 8°C. R4 was operated at lower temperatures to allow for the possibility that anammox bacteria obtained from the ocean depths may have evolved to remove nitrogen more effectively at temperatures closer to that of their natural environment. All four larger reactors were fed enrichment media at 0.50 L/24 hr and also flushed with gas, initially nitrogen, but later argon. Anaerobic conditions were also monitored with Anaerotest®strips (Anaerotest®, Merck KGaA, Darmstadt, Germany) and the pH maintained between 7.5 and 8.5 with the addition of concentrated hydrochloric acid. Biomass settling was evident in all the secondary enrichment reactors and, in order to increase the even exposure of the biomass to nutrients, pumps were implemented in March 2010 in all four reactors, effectively circulating the media upwards.

3.3 Bioprospecting: Sampling and collection

As discussed in the literature review, previous research indicates that anammox bacteria are most commonly found in anaerobic habitats exposed to nitrite and ammonium. The most commonly reported industrial anammox niches are anaerobic biofilm zones in wastewater treatment works, particularly anaerobic digesters. The most commonly reported natural environments for anammox bacteria include the anaerobic zones of oceanic mud sediments. Therefore, similar environments were identified in South Africa to sample from.

In July 2008 eight samples were collected from wastewater treatment and sludge handling works in the Cape Town area; including Athlone, Cape Flats, Macassar and Zandvliet. Activated sludge samples were all collected from the anoxic zone of treatment plants and partly scraped off the reactor walls to include possible biofilm growths. Sludge from anaerobic digesters was collected at the Cape Flats works (directly from the mesophilic digester) as well as from the Athlone works (from mixed sludge in the heat exchanger). Table 2 describes the precise sampling locations at each WWTW. Samples were collected using a specially designed sampler (a 500 ml stainless steel cup connected to a 1.5 m long handle). Each sample (ca. 200 ml) was decanted into an autoclaved 1 L glass jar, transported at ambient temperature and, upon arrival in the laboratory, transferred to a 1 L primary enrichment reactor. An identical sampling procedure was carried out on 4 February 2009 (Table 3) and the combined samples (ca. 1L) were used to inoculate R2. On 14 February 2009, samples were taken from five sampling stations in the anaerobic sediment zone of St. Helena Bay (and the Great Berg River estuary), off the South African west coast. The stations included St H11 (S32 40 21.7, E18 01 25.3, Depth: 28.0 m), St H24 (S32 35 59.3, E18 07 27.1, Depth: 32.0 m), 227 (S32 30 31.6, E18 05 39.3, Depth: 15.0 m), St H43a (S32 29 32.3, E18 06 57.6, Depth: 61.0 m) and the 228 (S32 44 38.5, E18 00 45.9, Depth: 8.0 m). Sampling was done by coring (300 mm cores) using an Ocean Instruments MC-200 multi-corer operated from the boat Wave Rider, and either kept in the acrylic tube of the corer or transferred to airtight plastic bags. Sediment samples were stored at 4°C, after 24 hours of storage similar aliquots of the samples were combined and the resulting ca. 1L composite sample was transferred to a reactor. The remainder of each sample was stored at 4°C. Table 4 highlights the most significant sampling and enrichment differences between the four reactors.

Table 2 Sampling sites at Western Cape WWTW, July 2008.

WWTW	**Sample origin**
Stellenbosch	Anaerobic digester mixed liquor
Stellenbosch	Activated sludge scraped off inside reactor wall, aerobic compartment
Franschhoek	Activated sludge scraped off inside reactor wall, anoxic compartment
Cape Flats	Anaerobic digester mixed liquor

Table 3 Sampling sites at Western Cape WWTW, February 2009.

WWTW	Sample origin
Athlone	Activated sludge scraped off inside reactor wall, aerobic compartment
Athlone	Anaerobic digester mixed liquor
Cape Flats	Activated sludge scraped off inside reactor wall, anoxic compartment
Cape Flats	Anaerobic digester mixed liquor
Macassar	Activated sludge scraped off inside reactor wall, anoxic compartment

Table 4 The most significant differences between the four large lab-scale experimental reactors

	Reactor 1	Reactor 2	Reactor 3	Reactor 4
Sample Origin	Combined WWTW: Biofilm from heat exchangers; anaerobic digester	Combined WWTW: Biofilm from heat exchangers; anaerobic digester	Combined ocean sediment mud samples	Combined ocean sediment mud samples
Date sampled	July 2008	February 2009	February 2009	February 2009
Sample transport: temperature and time	Ambient, 4 hrs	Ambient, 4 hrs	4°C, 24 hrs	4°C, 24 hrs
Reactor temperature	30°C	30°C	30°C	8°C
Reactor media	Distilled water	Distilled water	Sea water	Sea water

3.4 Enrichment and acclimatization

Enrichment of anammox bacteria was conducted by feeding the reactors with a synthetic medium of which the composition was adapted from literature (Jetten et al., 2005). This medium mimicked the composition of ammonium-rich wastewater and contained – compared to the anammox stoichiometry – a limiting amount of nitrite (Table 5) and a series of trace elements (Table 6). The primary enrichment reactors, as well as R1 and R2, received media prepared with distilled water. Reactors 3 and 4 received media prepared with filtered sea water, in order to maintain a natural pH and osmotic balance without synthetic additions. The feed flow-rate was 0.050 L/day into each of the 1 L reactors and 0.50 L/day into the four larger reactors. The strategy of enrichment involved increasing the ammonium and nitrite concentrations (nitrogen loading rate) in the influent medium after the anammox activity has been shown to have increased sufficiently to tolerate increased nitrite levels (Dapena-Mora et al., 2004). Complete consumption of the limiting substrate, nitrite, along with production of nitrate would have indicated a sufficient increase in anammox activity to warrant an increased nitrogen loading rate by eliminating the need for activation energy to reinitiate metabolic activity (Strous et al., 1999). These incremental increases in nitrogen loading rate are important since high nitrite levels are toxic to anammox bacteria. A fine balance must thus be maintained between nitrite impacting as a nutrient and as an inhibitor.

Table 5 Composition of anammox enrichment medium

Compound	Concentration
$KHCO_3$	1.25 g/L
NaH_2PO_4	0.05 g/L
$CaCl_2.2H_2O$	0.3 g/L
$MgSO_4.7H_2O$	0.2 g/L
$FeSO_4$	0.006 g/L
EDTA	0.006 g/L
KNO_3	0.2g/L
$NaNO_2$	0.12 g/L
$(NH_4)_2SO4$	0.15 g/L
Trace element solution	1.25 mL/L

It must be noted that in all the reactors, the composition of the influent medium was adjusted to include high nitrate (150 mg/L) concentrations for the first three months of reactor operation. This prevented heterotrophic denitrification via nitrite (nitrate serves as nitrite buffer), while a high organic load remained from the original sludge composition. At the same time the relatively high nitrate concentrations prevented biological sulphate reduction. Trace amounts of the anammox metabolic intermediates hydrazine and hydroxylamine (1.4 mgN/L and 0.7 mgN/L respectively) were also initially added to the reactors (Strous et al., 1999). This was done to "kickstart" the anammox process. Subsequently, all reactors received periodic additions of trace amounts of hydrazine and hydroxylamine, for stimulation of metabolic activity. These were added approximately once every two months, or when stoichiometry indicated a drop in nitrogen removal and activity needed to be reinitiated.

Table 6 Composition of trace element solution

Compound	**Concentration (g/L)**
H_3BO_3	0.5
$CuSO_4.5H_2O$	0.04
KI	0.1
$FeCl_3.6H_2O$	0.2
$MnSO_4.H_2O$	0.4
$Na_2MoO_4.H_2O$	0.2
$ZnSO_4.7H_2O$	0.4
NaCl	1
$CoSO_4$	0.1
$CaCl_2$	0.1
$AlK(SO_4)_2.12H_2O$	0.01

From April 2010 until the end of the experimental period a vitamin supplement was added to the feed of R1. Consequently, the additional vitamins and their concentrations (µg/L) in the feed of this reactor were as follows: Biotine, 1.00; calcium pantothenate, 200.00; folic acid, 1.00; inositol, 1000.00; *p*-aminobenzoic acid, 100.00; pyridoxine hydrochloride, 200.00; riboflavin, 100.00 and thiamine, 500.00. The reactors were incubated at 30°C (except for R4, incubated at 8°C), while the pH was maintained between 7.5 and 8.5 (Strous et al., 1999).

3.5 Stoichiometric monitoring of the reactors

Anammox metabolic activity involves the consumption of ammonium and nitrite along with the production of nitrate and dinitrogen gas (Van de Graaf et al., 1996). The presence of these bacteria within reactors can therefore be detected using periodic colorimetric analysis of nitrate, ammonium (Kartal et al., 2007) and nitrite (Egli et al., 2002) in the reactor effluents.

The test for nitrate involves the addition of 10 µL of saturated sulfamic acid to a 40 µL reactor effluent sample in order to remove the nitrite. A total of 0.2 mL 5% salicylic acid in 98% sulphuric acid and 2 mL 4 M NaOH (4ºC) is added to the sample, followed by spectrophotometric analysis (SmartSpec Plus, Biorad) at 420 nm after 30 minutes. Effluent ammonium concentration is determined by the addition of 760 µL of a solution, containing 0.54% ortho-pthalaldehyde, 0.05% β-mercaptoethanol and 10% ethanol in 400 mM phosphate buffer (pH 7.3), to a 40 µL effluent sample. The absorbance is measured at 420 nm after a 30 minute reaction. Spectrophotometric analysis of the effluent sample to determine nitrite concentrations involves the addition of 950 µL of a reagent containing 1% sulfanilic acid and 0.05% N-naphthylethylenediamine in 1 M H_3PO_4. Spectrophotometric analysis at 540 nm follows a 5 minute reaction. All reagents were obtained from Sigma-Aldrich (Steinham, Germany) and nitrogen removal rates (1) were monitored using the absorbance results of the colorimetric analysis, with negative removal rates implying production in the case of nitrate.

$$R = Q(C_{in} - C_{react}) - V/(t_1-t_0)(C_{r1}-C_{r0}) \quad (1)$$

Where R is nitrogen removal rate (mmol/day), Q is flow rate (L/24 h), C_{in} is influent concentration (mM), C_{react} is reactor concentration (mM), V is reactor volume (L), t_1-t_0 (h) is time elapsed between two consecutive analysis points and $C_{r1} - C_{r0}$ is the difference in concentration between the same consecutive analysis points.

3.6 Screening and imaging of reactor populations

DNA extraction. The ZR Fecal DNA kit (Zymo research, USA) was used for the first gDNA extraction after enrichment, according to the protocol specified by the manufacturers. For the second DNA extraction, the Fungal/Bacterial DNA kit (Zymo Research, USA) was used, again according to manufacturer instructions. Since 2009, GeneJET Genomic DNA Purification Kit (Fermentas Life

Sciences, Inqaba Biotec, South Africa) was used for DNA extraction, because it was found to be more effective than the Zymo merchandise. For pyrosequencing, DNA extractions were performed using the CTAB method and genomic DNA was obtained from reactor 1 (R1), reactor 3 (R3) and reactor 4 (R4) at concentrations of 30.81 ng/µL, 26.07 ng/µL and 25.13 ng/µL, respectively.

PCR amplification of relevant gene sequences. The polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene, which is a taxonomically relevant gene employed in identification and classification of bacteria at species level. Twelve primer combinations (Table 7), obtained from literature, were selected to detect every possible known anammox species (Inqaba Biotec, South Africa). However, anammox organisms display significant genus level rRNA diversity (Schmid et al., 2003) and it is therefore accepted that many species remain undiscovered due to the limitations of the probes and primers, which are all designed to amplify taxonomic genes. Therefore, primers (Table 7) were designed (Primer Designer, Version 1.01) to amplify the planctomycete KSU-1 hzoA gene (accession number: AB257585) encoding the hydrazine-oxidizing enzyme. This enzyme only occurs in anammox bacteria, as these organisms are unique in their natural inclusion of hydrazine in their metabolism (Strous et al., 2006; Karllson et al., 2009). They are only able to tolerate its toxicity due to the unique ladderane lipids that surround the anammoxosome, the hub of their catabolic activity (Van Niftrik et al., 2004). It must be noted that nitrifiers all contain a hydroxylamine-oxidizing enzyme. However, according to Shimamura et al., 2006, the hydrazine-oxidising enzyme has no affinity for hydroxylamine, and alignment of the gene sequence of the two enzymes showed low homology levels. A web-based BLAST search aligned only anammox bacteria with this enzyme sequence. The primers were also only significantly (E value < 15 and E value < 10 for Anam1f and Anam2r respectively) aligned with anammox species. Primer design was adjusted to prevent high GC content and dimer formation. Similar primers have since been designed and published by Quan et al. (2008).

The 50 µL PCR reaction contained 200 µM of each deoxynucleotide triphosphate, 0.25 µM of each primer, 2 mM $MgCl_2$, 2.5 U Taq DNA polymerase (Fermentas Life Sciences, EU) and approximately 100 ng of template DNA (adapted from Amano et al., 2007). The PCR was initiated with a 4 min denaturation at 94°C and concluded with a final 7 min elongation at 72°C. The reaction comprised thirty cycles, each with a 30 s denaturation at 94°C, a 30 s annealing at 56°C and a 60 s elongation at 70°C. Amplification of the functional gene was carried out according to the same protocol, with the annealing temperature adjusted to 60°C after optimization. The reactions were performed in a 2527 Thermal Cycler (Applied Biosystems, Singapore). PCR products were visualized under UV light in a

0.8% agarose gel. Bovine Serum Albumin was added (2.5 μL at 2 mg/mL) in order to overcome inhibition by wastewater contaminants. PCR was performed approximately every 90 days to monitor the presence of various anammox species (Table 7). Negative controls were included in all instances. Where multiple PCR products were obtained from one primer set or streaking of the PCR product occurred, PCR amplicons were extracted directly from the gel using the DNA Extraction kit (Fermentas Life Sciences, EU), according to manufacturer instructions.

Gene sequencing and phylogenetic analyses. An ABI PRISM (model 3100) genetic sequencer was used for all sequencing and the online Basic Local Alignment Search Tool (BLAST) was used to detect homology with known anammox species on the NCBI (National Centre for Biotechnology Information) website database (http://www.ncbi.nlm.nih.gov/BLAST/). Amplified 16S rDNA sequences were manually aligned and edited (Clustal, Version 2.0.11) against existing sequences which were selected from the BLAST results and subsequently obtained from GenBank, as well as existing sequences on the NCBI database. Sequences that aligned 100% with their respective target species were imported into PAUP (Version. 4.0b10), and a heuristic search strategy was used for Maximum Parsimony analysis. The quality of the branching patterns was assessed by bootstrap resampling of the data sets with 1000 replications

Table 7 Primers specific for the amplification of the 16S rDNA region of a range of anammox species, as well as primers designed for the detection of the functional gene encoding the hydrazine-oxidizing enzyme.

Trivial name	**Specificity**	**Sequence 5'-3'**
Anam1f	All organisms capable of anammox (hypothesis)	GTTGCGTAATGTTAATCACGG[x]
Anam2r	All organisms capable of anammox (hypothesis)	CCTCTTAGTGATGAGCGCC[x]
Pla46f	*Planctomycetales*	GGATTAGGCATGCAAGTC[y]
Amx368f	All anammox except *Anammoxoglobus propionicus*	TTCGCAATGCCCGAAAGG[y]
Amx60f	All anammox except *A. propionicus* and *Scalindua wagneri*	AGGGTGAGTAATGCATWGATWACCT[y]
Amx1480r	All anammox bacteria	TACGACTTAGTCCTCCTCAC[y]
Amx820r	*Brocadia anammoxidans, B. fulgida, Kuenenia Stuttgartiensis*	AAAACCCCTCTACTTAGTGCCC[y]
1037r	Universal bacterial primer	CGACAAGGAATTTCGCTAC[y]
BS820r	*S. wagneri* and *S. sorokinii*	TAATTCCCTCTACTTAGTGCCC[y]

[x]Designed in this study.
[y]Amano et al., 2007.

It must be noted that the diverse consortia in R1 and R2 seem to have led to non-specific primer binding and inconclusive sequences. The Promega pGEM-T Vector System (Inqaba Biotec) was thus used for the cloning and transformation of the gene sequences before re-amplification, in order to overcome this problem. The sequences were ligated into the pGEM vector and cloned and transformed into competent *E. coli* cells, according to manufacturer instructions. Plasmids were isolated from single colonies using the GeneJET Plasmid Miniprep kit (Inqaba Bitoec, South Africa) and PCR products were again amplified from the plasmids and subsequently sequenced and phylogenetically analyzed as described above.

Spatial analysis of biofilms using FISH. Microscope slides were inserted into R1 at the start of experimentation to allow for biofilm formation on the slides. Fluorescent *in situ* hybridization (FISH) was used to analyze these biofilms, as well as samples scraped directly from each reactor. Planctomycetes, including anammox bacteria, were probed according to Amann et al., 1995. To prepare fluorescent probes for FISH the Amx820 and Pla46 primers (Inqaba, South Africa; Table 7) were labeled with Alexa FLOUR 488 and Alexa FLOUR 594 fluorescent dyes respectively (Invitrogen, Adcock Ingram Scientific Group). Alexa FLOUR 488 excites at 488 nm and absorbs at 495 nm, therefore successfully hybridized anammox species are identified by green fluorescence under the FITC filter. In contrast, AlexaFLOUR 594 excites at 590 nm and emits at 617 nm, and therefore the planctomycetes are detected by red fluorescence using the Texas Red filter.

Using the above-mentioned labeled probes the FISH protocol of Amann et al., 1995 was adjusted to include formamide to increase the stringency, due to the interruption of hydrogen bonds, during the hybridization steps. A formamide concentration of 20% (20% formamide hybridization buffer/159 mM NaCl wash buffer) produced the most effective stringency (Schmid *et al*., 2000). The hybridization temperature was 48°C. Incubation in 50 mL buffer was described as a wash step by Amann et al., but for our procedure, the wash buffer was adjusted to 0.07 M NaCl for stringency, along with the addition of 1% EDTA.

After the hybridization and washing steps the slides were viewed directly without mounting in glycerol. A negative control, without the addition of any probe, was included for every FISH analysis. For image acquisitions, a Nikon Eclipse fluorescent microscope, equipped with a Nikon DS-Fi1 camera and DFT, FITC, as well as Texas Red filters, was used in conjunction with Nikon NIS Elements Imaging Software, Version 2.32 F, for data capturing and analysis.

It must be noted that FISH analyses of the anammox populations in Reactors 1 and 2 was hampered by autofluorescence of the particulate matter in the wastewater. However, much less autofluorescence was observed in the ocean sediment samples. Consequently, an attempt at photobleaching was made to decrease autofluorescence in samples taken from Reactors 1 and 2. Photobleaching involves the photochemical destruction of a fluorophore by over-exposure to the stimulatory wavelength specific to the molecule (Eggeling et al., 1998). Due to the unavailability of the equipment necessary to stimulate the fluorescent probes at their excitation wavelengths, sunlight was attempted as an alternative.

Pyrosequencing. A PCR reaction was performed on all the extracted DNA samples from reactors R1, R3 and R4. For bacteria, the primers 27F.1 and 1492R (DeSantis *et al.,* 2007) (Table 8) were used in the amplification reaction. These primers are degenerate and amplify a greater diversity of microbes. Each 50 µl PCR reaction contained 5 µl of 10 x Taq Buffer (supplied with the Taq polymerase), 2 mM $MgCl_2$ (supplied with the Taq Polymerase), 0.25 mM dNTPs, 0.1 µM of each primer, 1.5 U Super-Therm DNA Polymerase (Southern Cross), 1 µl of extracted DNA and NFW up to the final reaction volume. The PCR was performed as follows: an initial denaturation step at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min. A final extension was included at 72°C for 5 min that was then cooled to 4°C. Amplification was verified by running the samples on a 1% agarose gel at 90 V for 30 minutes. For fungal taxa, the primer pair ITS1F and ITS4 was used in the initial PCR reaction (Table 8). These reactions were performed in 50 µl volumes and contained the following: 5 µl of 10 x Taq Buffer including 15 mM $MgCl_2$, 0.25 mM dNTPs, 0.2 µM of each primer, 5 U Super-therm Gold DNA Polymerase (Southern Cross), as well as 5 µl extracted DNA and NFW to 50 µl. The PCR cycle was as follows: initial denaturation at 96°C for 1 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 50°C for 3 min and extension at 72°C for 2 min. A final extension step at 72°C for 5 min was included and then cooling down to 4°C. Amplification was confirmed by running the product on a 1% agarose gel.

The entire PCR reaction was loaded on a 1% agarose gel, followed by excision of the correct band size (approximately 1500 bp). The GeneJET™ gel extraction kit (Fermentas) was employed to recover the DNA from the gel slices. During cleaning the DNA binds to the membrane in a spin-column which is then eluted.

The DNA was subsequently re-amplified with sets of primers containing the appropriate adaptor and barcode sequences necessary for 454 pyrosequencing (Table 9a & b). For the bacteria, each 50 µl PCR reaction contained 5 µl of 10 x Taq Buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.1 µM of each primer, 1.5 U Super-Therm DNA Polymerase (Southern Cross), 1 µl of extracted DNA and NFW up to the final reaction volume. The PCR cycle was performed as follows: an initial denaturation step at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C (Table 9a) for 1 min and extension at 72°C for 1 min. A final extension at 72°C for 5 min was included, that was then cooled to 4°C. Amplification was verified by running the samples on a 1% agarose gel as described above. For the fungi, PCR reactions were performed in 50 µl volumes and contained the following: 5 µl of 10 x Taq Buffer including 15 mM $MgCl_2$, 0.25 mM dNTPs, 0.2 µM of each primer, 5 U Super-therm Gold DNA Polymerase (Southern Cross), as well as 5 µl extracted DNA and NFW to 50 µl. The PCR cycle was as follows: initial denaturation at 96°C for 1 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 52°C (Table 9b) for 3 min and extension at 72°C for 2 min. A final extension step at 72°C for 5 min was included and then cooling down to 4°C. Amplification was confirmed by running the product on a 1% agarose gel.

Table 8. Primer numbers and sequences used in the PCR reactions.

Primer nr	***Primer sequence***
27F.1	5’ AGRGTTTGATCMTGGCTCAG 3’
1492R	5’ GGTTACCTTGTTACGACTT 3’
A2.1	5' CGTATCGCCTCCCTCGCGCCATCAGACGCTCGACAGTGCCAGCMGCNGCGG 3'
A2.2	5' CGTATCGCCTCCCTCGCGCCATCAGCGTGTCTCTAGTGCCAGCMGCNGCGG 3'
A2.3	5' CGTATCGCCTCCCTCGCGCCATCAGCTCGCGTGTCGTGCCAGCMGCNGCGG 3'
B2	5' CTATGCGCCTTGCCAGCCCGCTCAGACGAGCTGACGACARCCATG 3'
ITS1F	5’CTTGGTCATTTAGAGGAAGTAA3’
ITS4	5’TCCTCCGCTTATTGATATGC3’

Table 8. (Continued).

Primer nr	*Primer sequence*
ITS1F-04	5'CGTATCGCCTCCCTCGCGCCATCAGAGCACTGTAGCTTGGTCATTTAGAGGAAGTAA3'
ITS1F-06	5'CGTATCGCCTCCCTCGCGCCATCAGATATCGCGAGCTTGGTCATTTAGAGGAAGTAA3'
ITS4-Btag	5'CTATGCGCCTTGCCAGCCCGCTCAGTCCTCCGCTTATTGATATGC 3'

Table 9a. The primer pairs, annealing temperatures and regions amplified for the bacterial species present in the reactors.

Sample and Reactor nr	Primer pair	Region amplified	Annealing temperature
R1	A2.1 & B2		
R3	A2.2 & B2	V4-7	56°C
R4	A2.3 & B2		

Table 9b. The primer pairs, annealing temperatures and regions amplified for the fungal species present in the reactors.

Sample and Reactor nr	Primer pair	Region amplified	Annealing temperature
R1	ITS1F-04 & ITS4Btag	ITS1/5.8S/ITS2	52°C
R4	ITS1F-06 & ITS4Btag		

The entire PCR product was run on a 1% agarose gel as described above. The correct band size (500-600 bp) was excised from the gel and subsequently purified as described above. The DNA concentrations were quantified using a Nanodrop. The various DNA samples were pooled in equal concentrations (300 ng/sample) to a total concentration of 1500 ng DNA (R1V4-7=R3V4-7=R4V4-7=R1ITS=R4ITS). The combined sample was subsequently sent for pyrosequencing using the GS-FLX-titanium series and results for the bacterial taxa were analyzed by means of the RDP Pyro-pipeline on the RDP-database (http://rdp.cme.msu.edu/). To obtain an indication of the fungal metagenome of each reactor 100 sequences were randomly selected from the list of sequences

originally amplified with primer pair ITS1F and ITS4, and identified using a BLAST search on the NCBI database (http://www.ncbi.nlm.nih.gov/).

4. RESULTS AND DISCUSSION

4.1 Enrichment for anammox bacteria

During maintenance of the primary reactors, a decision was made to terminate the reactors, as oxygen leakages led to significant diffusion impacts due to the small volumes, and the focus changed to the large laboratory scale reactors (Reactors 1-4). Three reactor sizes were evaluated in this research for anammox enrichment: 1 L, 5 L and 17 L reactors. One litre reactors were deemed inappropriate as the small volumes resulted in the entire reactor experiencing the effects of small oxygen leakages due to diffusion. Most success was obtained with the 5 L reactor, but it was inoculated the earliest and the success may have been due to a longer enrichment period. However, the 5 L reactor seemed adequate to enrich for anammox biomass and required less hydrazine and hydroxylamine than the larger reactors to attain similar concentrations of these supplements. A 5 L reactor seems to be the appropriate choice to enrich for anammox biomass from small samples containing low concentrations of anammox bacteria. Larger volume reactors may be desirable if seeding with large samples from a previously enriched culture.

In terms of maintaining an anaerobic environment, anaerobic monitoring of the reactor head spaces showed that Technical Grade dinitrogen gas (reported by AFROX to be 99.5% pure) was not sufficient to keep the reactor completely anaerobic. It was thus replaced with Ultra-High Pure (UHP) nitrogen gas (99.997% purity) on 12 December 2008 to prevent oxygen contamination. As earlier alluded to, oxygen is a strong inhibitor of anammox activity (Van de Graaf et al., 1996), therefore it is suspected that this contamination, as well as oxygen leakages that occurred during reactor set-up, had a significant impact on the reactor consortium composition, particularly that of R1. As the enrichment progressed, and anammox biomass was not enriched at a satisfactory rate, it was suspected that the use of nitrogen gas (a product of the anammox metabolism) could be influencing the enrichment. Hence, nitrogen was replaced by argon, effective from 01 April 2010. As discussed in the stoichiometric analysis of the reactors, an improvement in nitrogen removal, particularly in R1, followed the implementation of argon gas for reactor flushing. Since nitrogen gas is a major by-product of anammox metabolism, flushing with nitrogen gas may influence the metabolic rate of the organisms. Therefore, our results indicate that argon gas is preferable for use in maintaining anaerobic conditions. With respect to flushing the

reactors, literature makes no mention of partial pressure in anammox reactors, however all reactors were described as being completely and continuously flushed and are nevertheless successful. Therefore, it seems that partial pressure should not be an inhibiting factor in anammox enrichment.

Although difficult to confirm, the addition of hydrazine and hydroxylamine also seemed to play a role in anammox enrichment. It appeared effective in kick-starting anammox metabolism, particularly after toxicity events where nitrogen removal dropped, since every toxicity event was followed by the addition of these metabolic intermediates, and nitrogen removal was always reestablished. In addition, as also discussed in the stoichiometric analysis, adding a vitamin solution to the reactors seemed to have a significant effect on the nitrogen removal capacity of R1, and it is recommended that these growth factors are used during the enrichment of anammox bacteria.

Pump systems were implemented in all four secondary reactors, in order to keep the biomass in suspension and well-exposed to the nutrients, and to prevent an accumulation of nutrients in any areas of the reactors. Shortly after the introduction of the pump system, nitrogen removal in Reactor 1 improved significantly. However, no change was demonstrated in the nitrogen removal capacity of the other three reactors, although all designs were adapted to include pumps. Therefore, it is unlikely that the pump system had a positive impact on anammox activity. In addition, the pumps resulted in biomass washout, which is a significant problem with such slow-growing organisms. There is great debate in literature concerning the impact of shear stress on anammox activity. Arrojo et al. (2005) report a high tolerance to shear stress in Anammox bacteria, however, a threshold limit was described in sequencing batch reactors.

In summary, enrichment for anammox bacteria can be carried out as described in this work, with the greatest focus on eliminating any oxygen contamination whatsoever. In addition, argon gas is preferable over nitrogen gas, and vitamins seem to improve the nitrogen removal capacity of the consortium. Mixing of the biomass helps to expose the bacteria to the influent nitrogen. However, shear stress interrupts biofilm formation and leads to biomass washout, so very slow mixing is advised.

4.2 Screening of the reactors: Stoichiometry

Nitrite, ammonium and nitrate concentrations in all the reactors were monitored in order to ascertain whether ammonium and nitrite were consumed and nitrate produced according to the traditional anammox stoichiometric ratio (Van der Star et al., 2007):

$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 2.03\ H_2O + 0.066\ CH_2O_{0.5}N_{0.15}$ (biomass)

The ratio of which to take note is the ratio of nitrite consumption to ammonium consumption (*ca*. 1:1.3). Deviations from this ratio indicate the contribution of other nitrogen removal processes in the reactors. Incremental increases in ammonium and nitrite (anammox metabolic substrates) concentrations in the reactor feed only ensued complete consumption of nitrite as indicated by stoichiometric analysis, since nitrite is toxic to anammox bacteria above a 2 mM threshold (Strous et al., 1999).

Therefore, stoichiometric analysis served two purposes: to determine whether anammox activity was dominant in the reactor, and to monitor the nitrite concentrations in order to prevent toxicity and allow for incremental increases of the nitrogen load. The stoichiometric analysis of the primary and secondary enrichment reactors were significantly different, and are discussed separately.

Primary enrichment reactors. Of the sixteen 1 L reactors built for primary enrichment, eight were inoculated and monitored using the stoichiometric techniques. Constant sampling for stoichiometry, however, led to a severe oxygen impact upon enrichment, evidenced in the stoichiometric progress of these reactors. The severity of the oxygen impact was due to the small volume of the reactors. The rapid oxygen diffusion in such small volumes made enrichment and screening in these 1L reactors impractical. Therefore, operation of the reactors was terminated and the focus turned to the larger enrichment reactors.

Secondary enrichment reactors. Figures 4 to 11 describe the progression of the removal of the nitrogen load of all four reactors. In all four reactors, nitrate production remained effectively zero (data not included), indicating low anammox catabolism, confirmed by a lack of substantial biomass production. However, the ammonium removal capacities of both Reactors 1 and 2 remained relatively high throughout the enrichment procedure (Fig 5 and 7). This is in contrast to Reactors 3 and 4, which displayed high reactor ammonium concentrations throughout enrichment (Fig 9 and 11). It therefore seemed that the reactor consortia originating from the ocean displayed a consistently less effective ammonium removal rate than those originating from the WWTW.

Since R2 was exposed to the enrichment process as long as R3 and R4, and still removed significantly more ammonium than the oceanic reactors from start-up, it is unlikely that the greater ammonium removal capacity in the WWTW reactors is due to adaptation during the enrichment procedure. This effective ammonium removal capacity is likely due to previous adaptations of consortium activity, since WWTW samples are exposed to high levels of ammonium in their original habitat, whereas ammonium is scarce in the ocean.

The influent nitrogen loads were determined by the reactor levels of nitrite (Fig 4, 6, 8 and 10), and were dropped if reactor nitrite concentrations reached toxic levels, and therefore influent nitrogen loads fluctuated independently in the four reactors throughout the enrichment procedure. The nitrite removal capacity of Reactor 1 remained inconsistent and nitrite concentrations regularly reached levels very close to the toxicity threshold (Fig 4a and 4b). However, a consistent and complete removal was observed for approximately 3 months during April until June 2010 (Marked X on Fig 4a and 4b), albeit at very low influent nitrite concentrations. This is the only time during the enrichment procedure that this complete consumption was observed. A number of changes were implemented in the set-up and running of the reactors just prior to this positive response. Nitrogen gas, used to maintain anaerobic conditions, was replaced with argon gas. In addition, pumps were implemented to improve the mixing capability of the reactors, as the gas-lift mechanism was observed to be ineffective in overcoming the settling tendency of the reactors. Lastly, a vitamin solution was added to the influent in R 1, in the hope that this might speed up the metabolism. All of these design and maintenance adaptation have a potential influence on the improvement of the nitrogen removal capacity of the reactors. In response to this increase in nitrogen removal capacity, the influent nitrogen load was increased slowly and incrementally. The reactor biomass responded, increasing its removal capacity in accordance, until July, when reactor nitrite concentrations increased to near-toxic levels. This could be attributed to biomass washout due to the new and effective stirring mechanism, or due to the influent nitrogen load being raised too quickly. In response to this, nitrite influent was stopped, and later raised to the enrichment starting concentration of 1.739 mmol again.

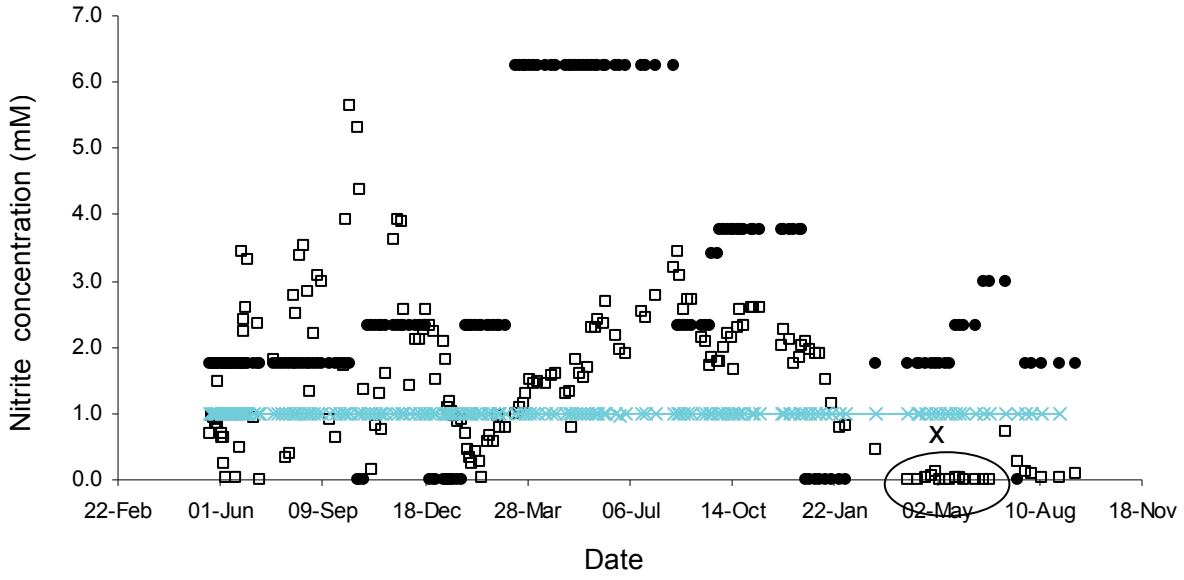


Fig 4a Nitrite fluctuations of R1 in terms of concentration. Concentrations were calculated from the average values of duplicate absorbance determinations. (●- Influent nitrite-N concentration; □ - Reactor nitrite-N concentration; Blue – Toxicity threshold; x – Period of complete nitrogen removal).

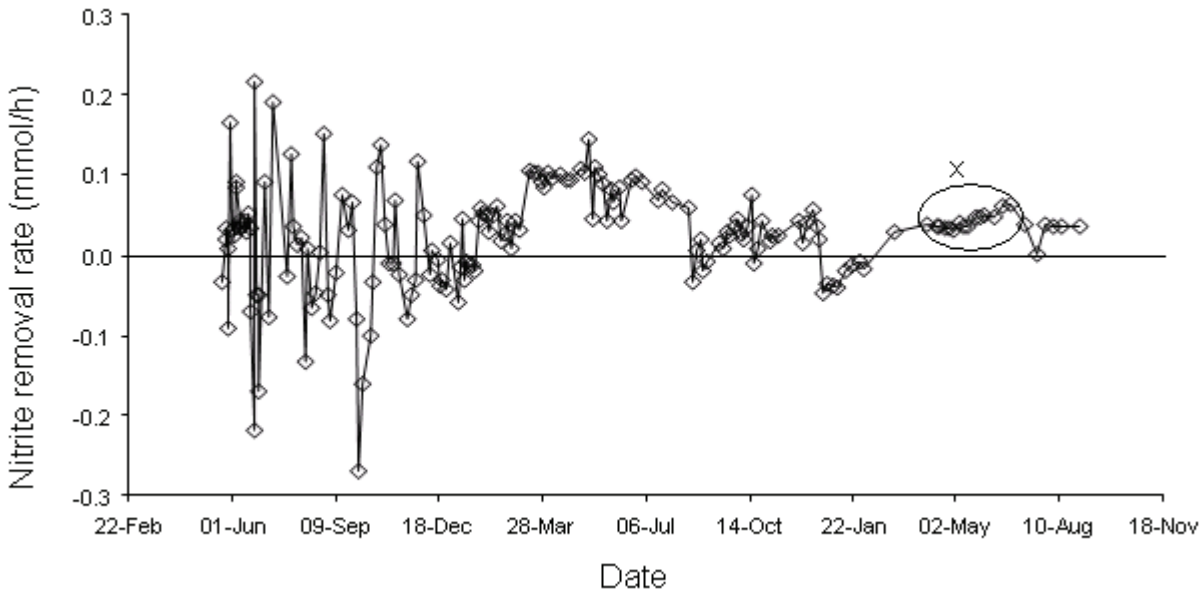


Fig 4b Nitrite fluctuations of R1 in terms of removal rate. Concentrations were calculated from the average values of duplicate absorbance determinations. (x – Period of consistent and complete nitrogen removal).

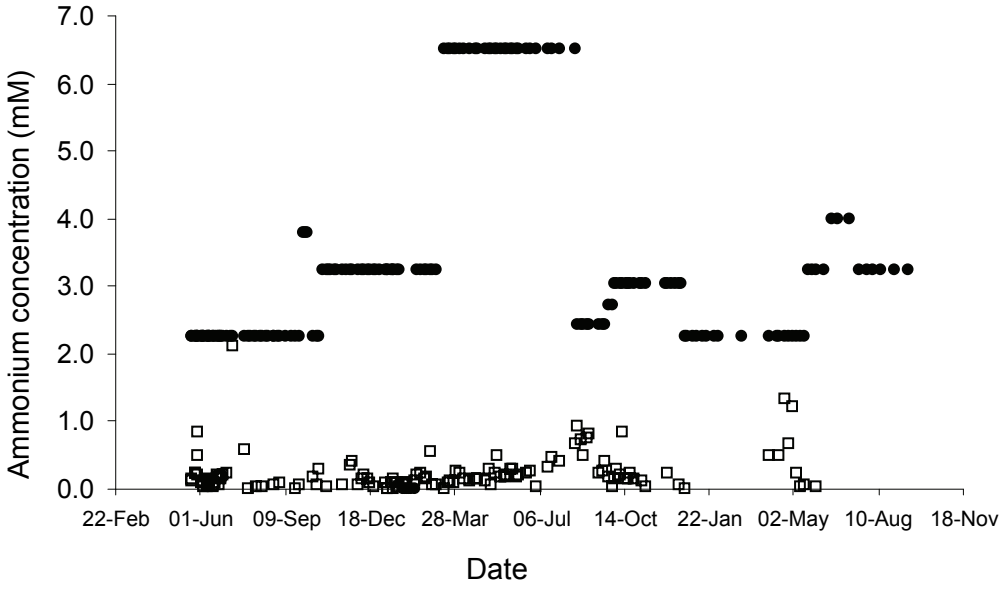


Fig 5a Ammonium fluctuations of R1 in terms of concentration. Concentrations were calculated from the average values of duplicate absorbance determinations. (●- Influent ammonium-N concentration; □ - Reactor ammonium-N concentration).

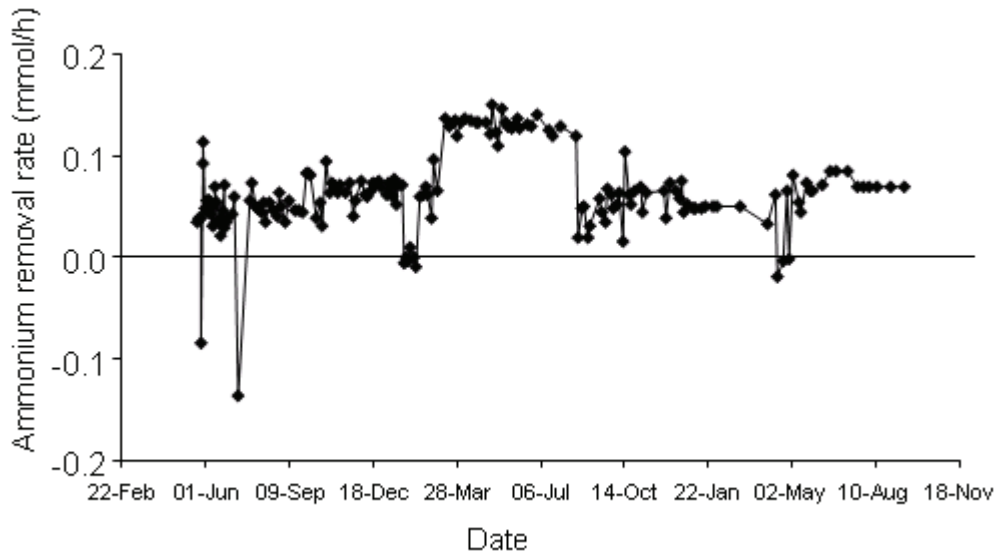


Fig 5b Ammonium fluctuations of R1 in terms of removal rate. Concentrations were calculated from the average values of duplicate absorbance determinations.

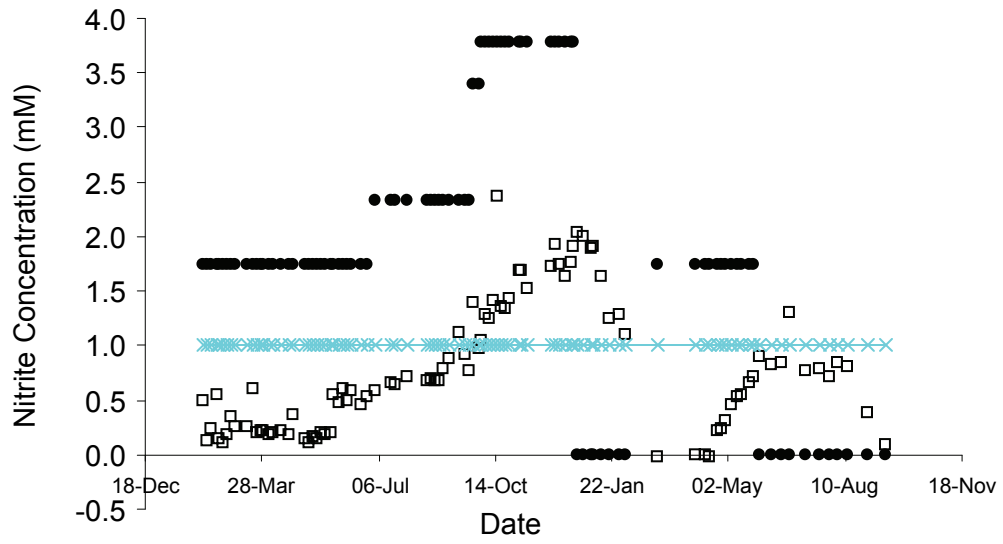


Fig 6a Nitrite fluctuations of R2 in terms of concentration. Concentrations were calculated from the average values of duplicate absorbance determinations. (●- Influent nitrite-N concentration; □ - Reactor nitrite-N concentration; Blue – Toxicity threshold).

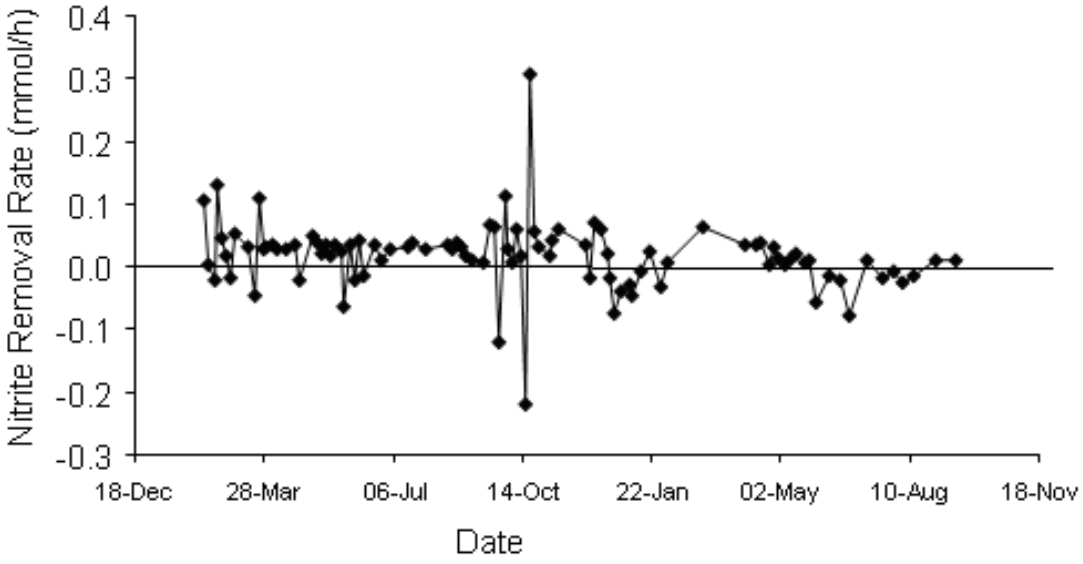


Fig 6b Nitrite fluctuations of R2 in terms of removal rate. Concentrations were calculated from the average values of duplicate absorbance determinations.

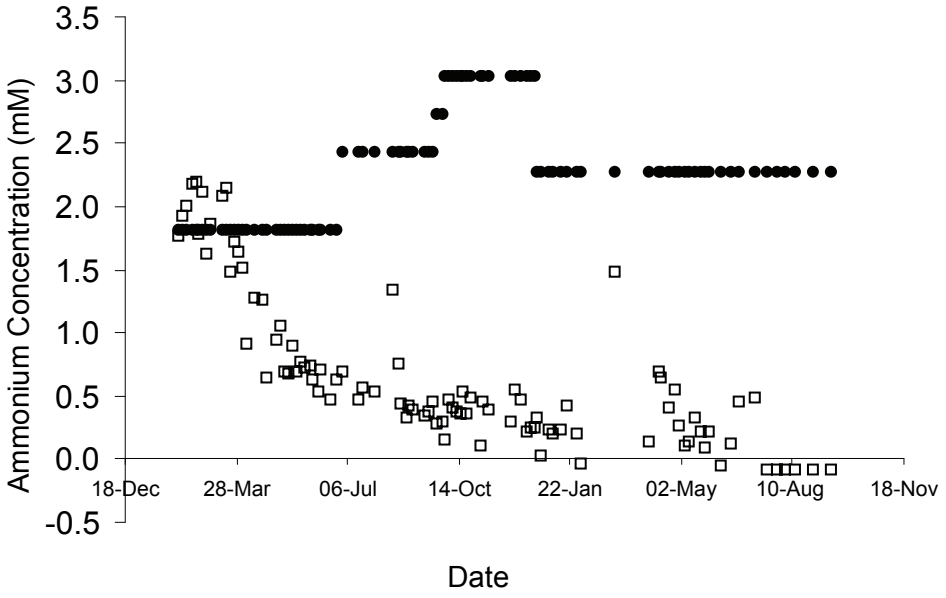


Fig 7a Ammonium fluctuations of R2 in terms of concentration. Concentrations were calculated from the average values of duplicate absorbance determinations. (●- Influent ammonium-N concentration; □ - Reactor ammonium-N concentration).

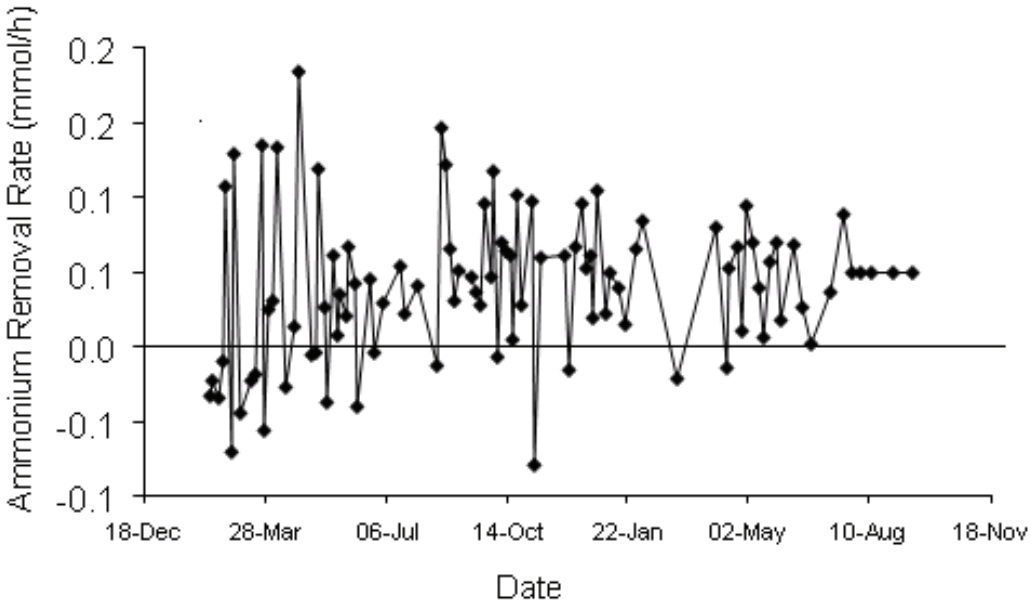


Fig 7b Ammonium fluctuations of R2 in terms of removal rate. Concentrations were calculated from the average values of duplicate absorbance determinations.

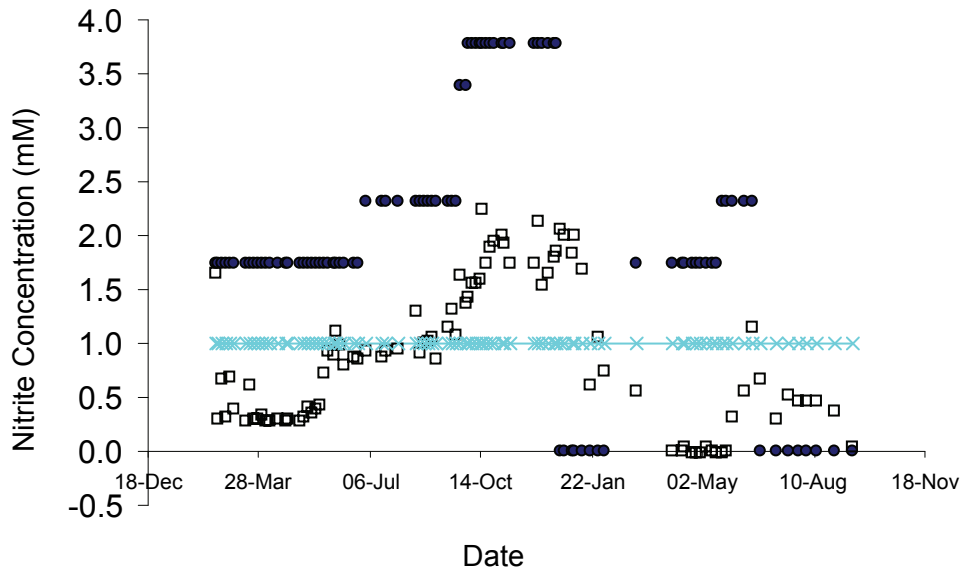


Fig 8a Nitrite fluctuations of R3 in terms of concentration. Concentrations were calculated from the average values of duplicate absorbance determinations. (●- Influent nitrite-N concentration; □ - Reactor nitrite-N concentration; Blue – Toxicity threshold).

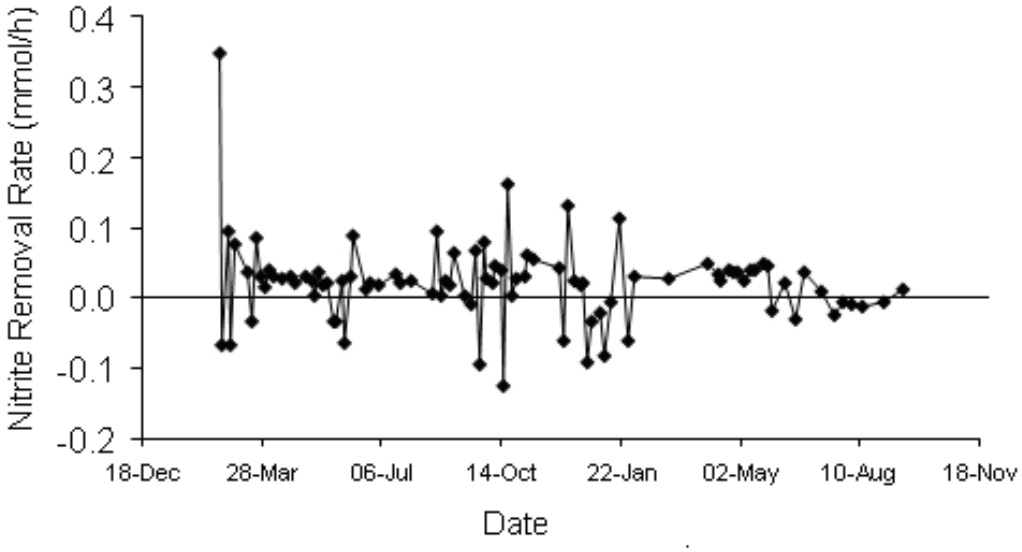


Fig 8b Nitrite fluctuations of R3 in terms of removal rate. Concentrations were calculated from the average values of duplicate absorbance determinations.

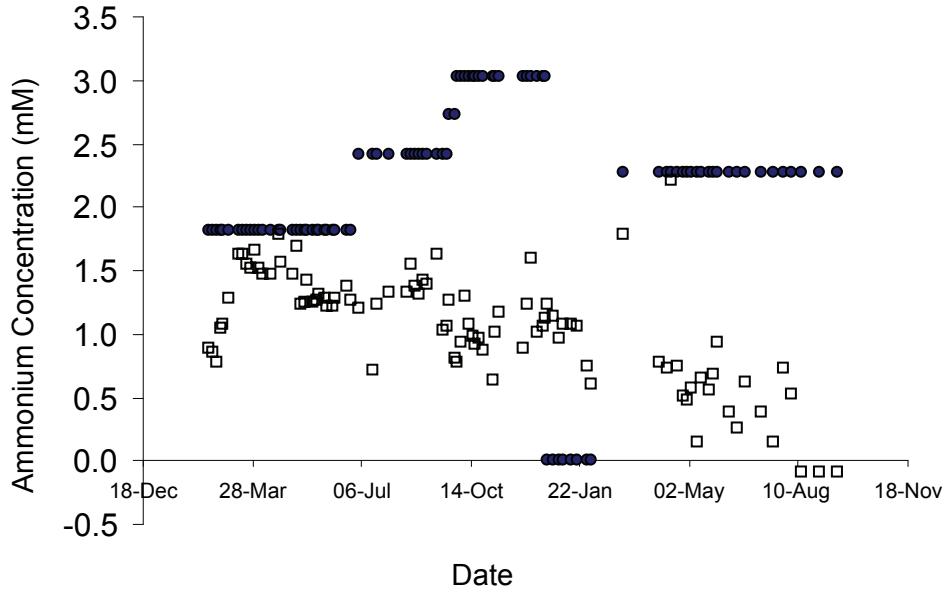


Fig 9a Ammonium fluctuations of R3 in terms of concentration. Concentrations were calculated from the average values of duplicate absorbance determinations. (●- Influent ammonium-N concentration; □- Reactor ammonium-N concentration).

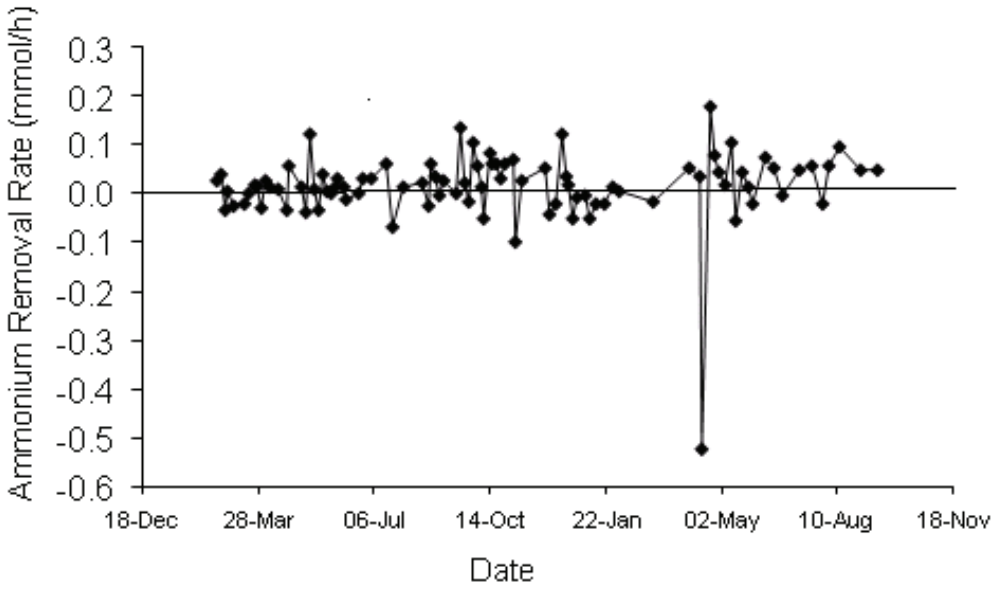


Fig 9b Ammonium fluctuations of R3 in terms of removal rate. Concentrations were calculated from the average values of duplicate absorbance determinations.

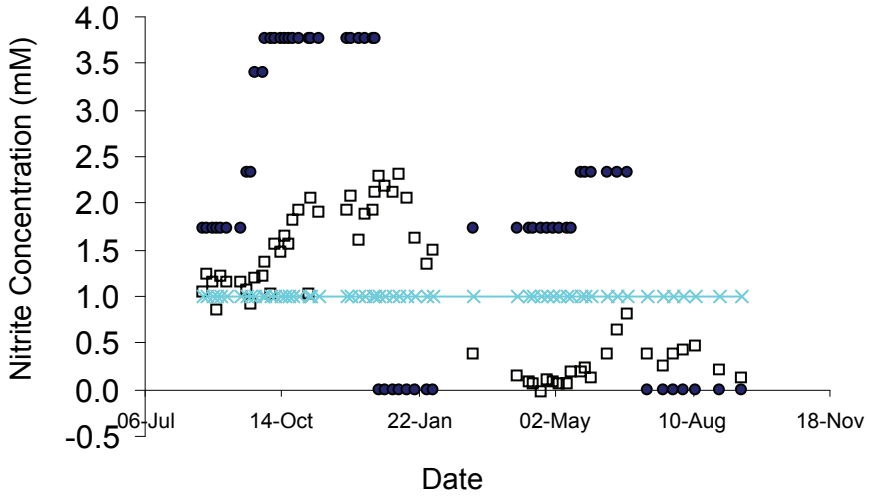


Fig 10a Nitrite fluctuations of R4 in terms of concentration. Concentrations were calculated from the average values of duplicate absorbance determinations. (● - Influent nitrite-N concentration; □ - Reactor nitrite-N concentration; Blue – Toxicity threshold).

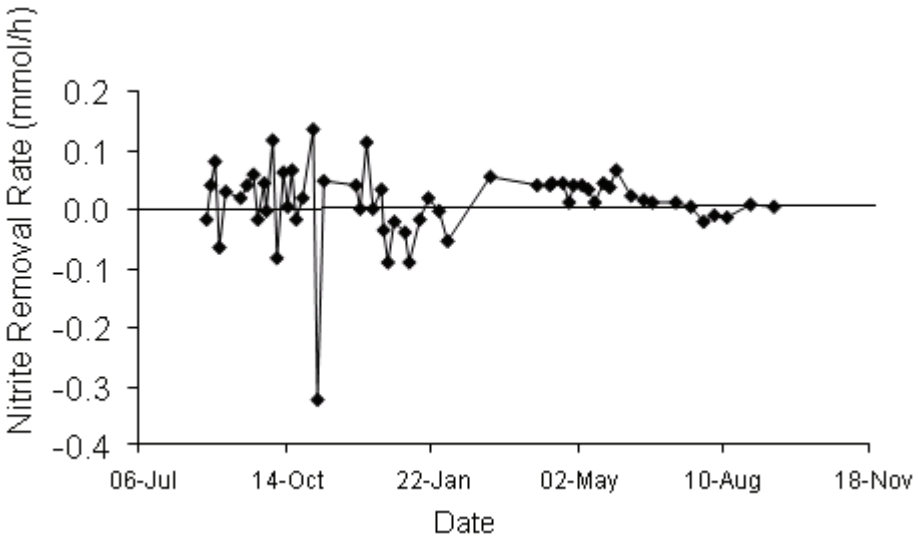


Fig 10b Nitrite fluctuations of R4 in terms of removal rate. Concentrations were calculated from the average values of duplicate absorbance determinations.

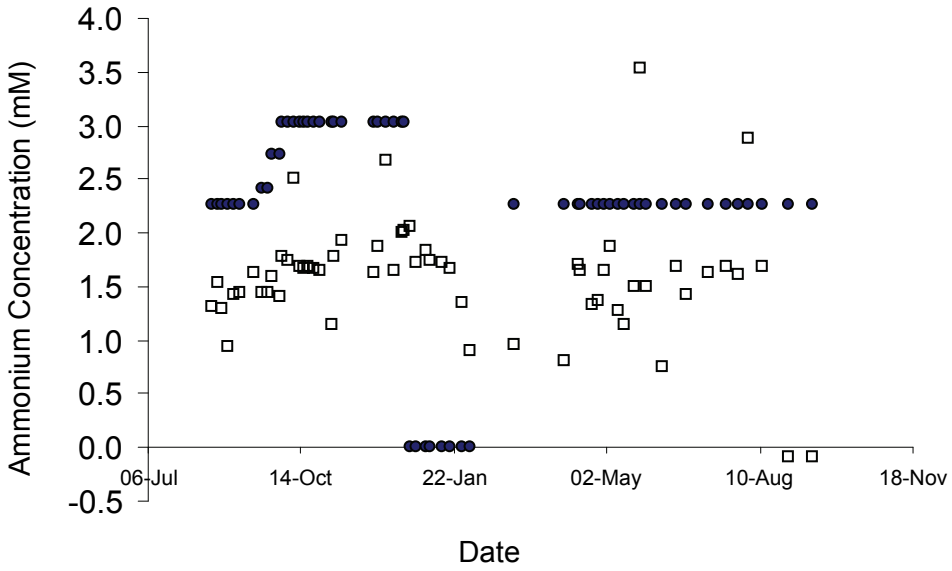


Fig 11a Ammonium fluctuations of R4 in terms of concentration. Concentrations were calculated from the average values of duplicate absorbance determinations. (● - Influent ammonium-N concentration; □ - Reactor ammonium-N concentration).

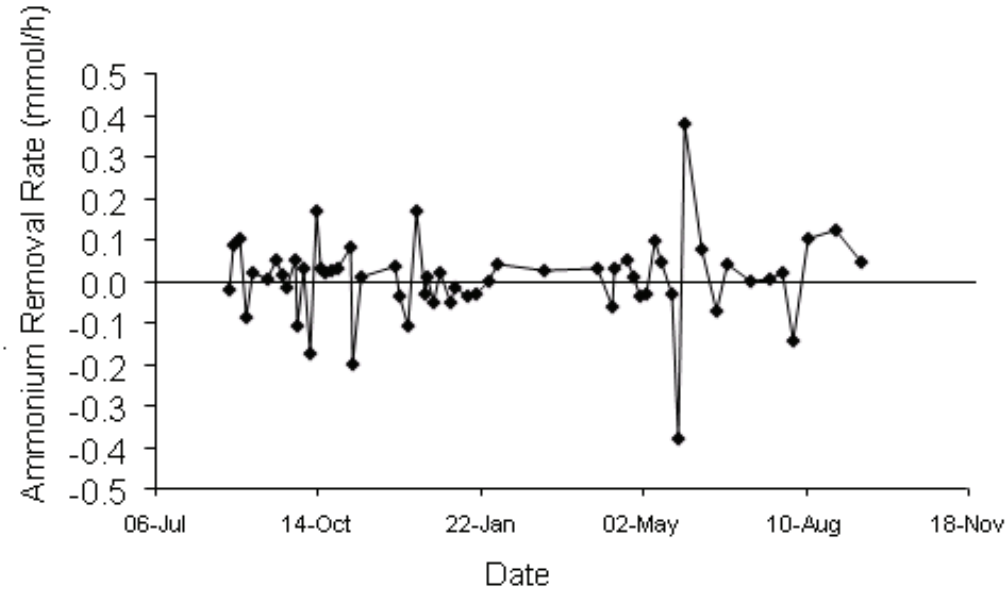


Fig 11b Ammonium fluctuations of R4 in terms of removal rate. Concentrations were calculated from the average values of duplicate absorbance determinations.

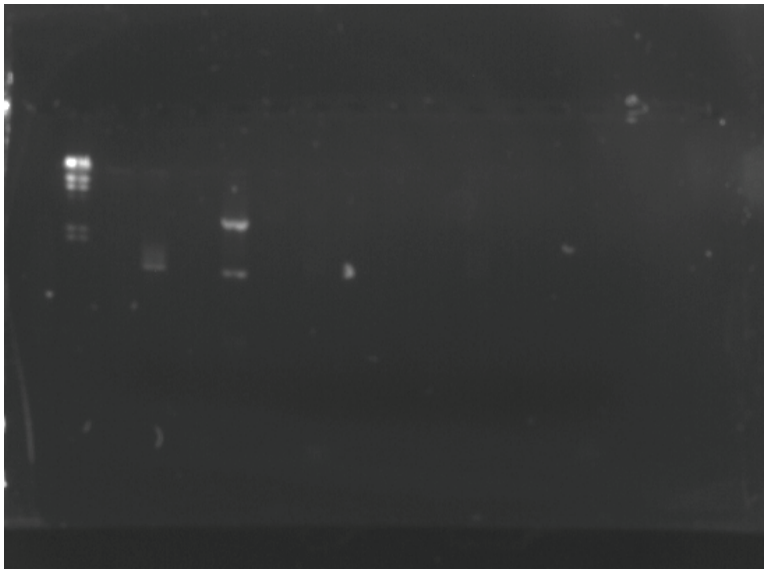
Reactors 2, 3 and 4 never responded well to increases in the nitrogen load. The reactor nitrite concentrations quickly reached toxic levels and influent nitrogen load had to be dropped as a result. At the lowest enrichment concentrations of influent nitrite, however, nitrite was consistently removed. The fact that an increase in nitrite inhibited the nitrogen removal in all four reactors is an indication that anammox was involved in the nitrogen removal rate of all the reactors. The reactor that demonstrated the best nitrogen removal capacity was Reactor 1; whereas Reactors 2, 3 and 4 demonstrated a much lower nitrogen removal capacity. However, this may be due to the vitamins added to Reactor 1 to stimulate activity. It must nevertheless be emphasized that the nitrogen removal rates discussed here, even that of Reactor 1 at its optimum, remained at very low, laboratory scale levels. It seems that anammox populations were present in the reactors, but were being limited by competition in the reactor consortia, rather than responding to enrichment. This was confirmed by stoichiometric ratio of Reactor 1 during the period of optimal nitrogen removal. During the month of June, the removal ratio of ammonium:nitrite was 1.2:1, the inverse of theoretical anammox stoichiometry. This ratio reflects the involvement of consortia activity in nitrogen removal.

4.3 Screening of the reactors: PCR amplification of taxonomic informative gene sequences

Twelve primer combinations obtained from literature were used in an attempt to amplify the 16S ribosomal RNA gene region of all known anammox species, any of which may have been present in the reactors. In addition, a set of primers were designed in order to attempt to amplify a functional gene encoding a unique protein involved in anammox metabolism. These were used in the hope of identifying novel species. It must be noted that genes that aren't expressed may not be amplified due to the structural constraints of DNA conformation, and therefore this functional gene is likely to be amplified only if anammox activity is detected with the stoichiometric techniques.

An analysis of the 16S rRNA gene sequences, amplified by the above-mentioned primer combinations, of R1 was attempted in October 2008. Figure 12 depicts the only two successful primer combinations (highlighted in bold). The Amx368f-1037r primer combination amplifies a 3726 bp region of the *Kuenenia stuttgartiensis* genome (the only anammox species whose genome is fully sequenced (Strous et al., 2006).

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13



F**ig 12** Gel electrophoresis of the 16S rDNA taxonomic gene fragments amplified (October 2008) from DNA extracted from the reactor by biomass washout. Lane 1, λ-HindIII DNA marker; Lane 2, Amx368f-Amx820r, **Lane 3, an approximately 1500 bp fragment amplified by primers AMX368f and AMX1480r;** Lane 4, Amx 368f-BS820r; **Lane 5, an approximately 3500 bp fragment amplified by primers AMX368f and 1037r, as well as a smaller 1500 bp amplicon;** Lane 6, Amx60f-Amx1480r; Lane 7, Amx60f-Amx820r; Lane 8, Amx60f-BS820r; Lane 9, Amx60f-1037r; Lane 10, Pla46f-Amx820r; Lane 11, Pla46f-Amx1480r; Lane 12, Pla46-BS820r; Lane 13, Pla46-1037r (Negative controls on a separate gel, data not shown).

This correlates with the primer combination and size of the larger fragment in lane 5, Fig 12. However, sequencing and tentative identification showed that the universal reverse primer detected a region of the *Delftia acidovorans* 16S rRNA gene. *D. acidovorans* is a denitrifier, indicating that there was a consortium of nitrogen-dependent microbes in the reactor.

The primer combination AMX 368f-AMX1480r amplifies a 1143 base pair region of the *Kuenenia stuttgartiensis* genome and is expected to amplify a similar size region in all anammox bacteria. The band in lane 3 (Fig 12) shows that this primer combination amplified an approximately 1500 bp region from the total DNA extracted from the sludge. After sequencing, the region was tentatively identified, using a BLAST homology search, as an unidentified marine organism. Lane 8 in Fig 13 indicates that our new functional primer set was successful, hinting that the unidentified organism is possibly a novel anammox species. However, subsequent sequencing and repetitions of PCR analyses did not produce any conclusive results, probably due the enrichment of diverse populations, stimulated by oxygen contamination during start-up. After a period of enrichment under completely anaerobic conditions,

anammox was expected to predominate again, but as discussed later, consortia competition seemed to inhibit this.

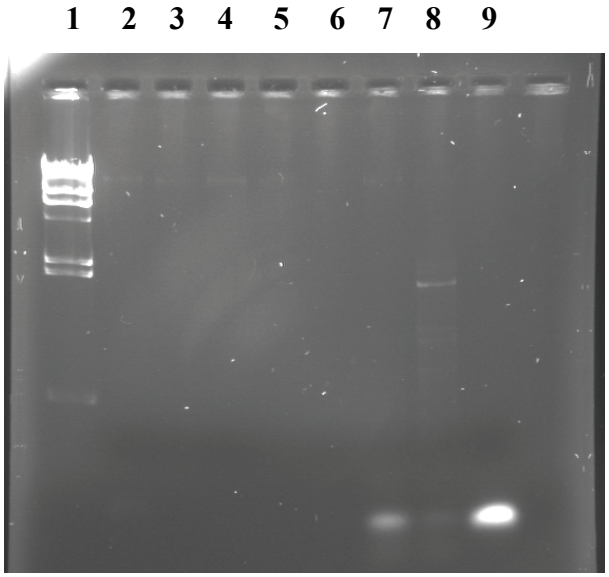


Fig 13 Gel electrophoresis of DNA amplified (October 2008) with novel primers designed to amplify the functional gene encoding the hydrazine oxidising enzyme. Lane1, λ-HindIII DNA marker; Lane 7, Anam1f-Anam2r, gDNA extracted from reactor biomass washout; Lane 8, Anam1f-Anam2r, gDNA extracted from sedimental biofilm; Lane 9, negative control.

Preliminary testing of Reactor R2 using molecular methods, in February 2009 directly after inoculation, showed no positive PCR results. This was expected, since anammox bacteria do not predominate in sewage due to their slow growth rate. However, PCR analysis of the mud zone in the St. Helena bay seabed in April 2009 revealed the presence of a number of presumptive anammox species (Fig 14). All of the PCR products showed in Fig 14 were subsequently sequenced. NCBI BLAST homology searches were used to tentatively identify the PCR products as representatives of uncultured planctomycete clones, with the exception of lanes 4 and 8, Gel 2. These two primer combinations amplified sequences with high homology to the 16S rRNA gene sequence of *Candidatus* "Scalindua". All sequences grouped within the anammox cluster (Fig 15, indicated as "OCEAN CLONES, Sediment"). The results therefore clearly indicate that anammox bacteria are present in the samples taken from the anoxic mud zone of the seabed in St Helena bay.

Gel 1: Lane 1 2 3 4 5 6 7 8 **Gel 2: Lane 1 2 3 4 5 6 7 8**

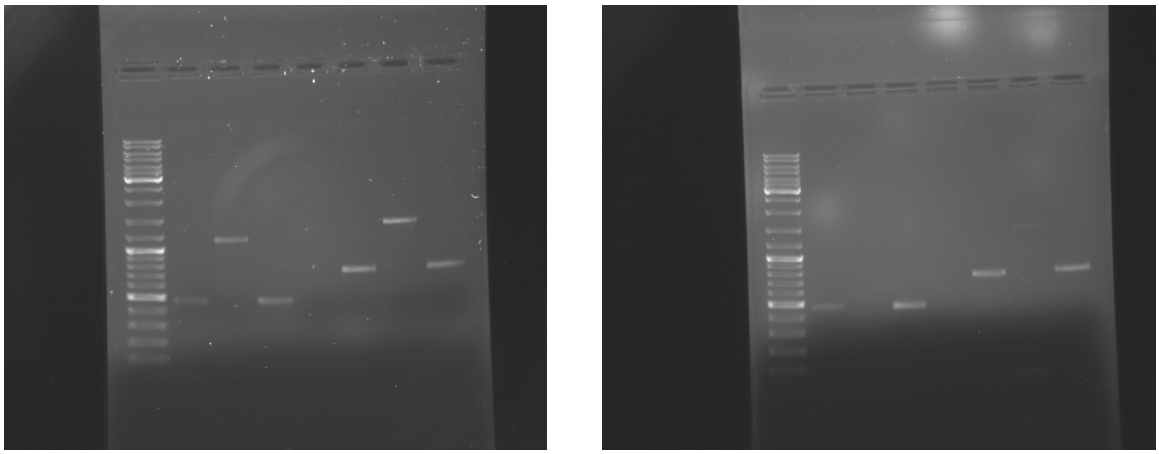


Fig 14 Gel electrophoresis of the 16S rDNA taxonomic gene fragments amplified from DNA extracted from two oceanic samples (43a and 24), taken at coordinates S32 29 32.3 E18 06 57.6 and S32 35 59.3 E18 07 27.1. The PCR products amplified from sample 43a (Gel 1): Lane 1, O'GeneRuler 1kb DNA ladder (Fermentas); Lane 2, Amx368f-BS820r, Lane 3, Amx368f-Amx1480r**;** Lane 4, Amx 368f-Amx820r; Lane 5, Amx368f-1037r; Lane 6, Amx60f-BS820r; Lane 7, Amx60f-1480r; Lane 8, Amx60f-Amx820r. Gel 2: PCR products amplified from sample 24, with the same primer combinations per lane.

Since inoculation, total genomic DNA was periodically extracted from all four secondary enrichment reactors and analyzed using the PCR detection technique. No positive results were obtained in Reactor 2 at all, as well as initially in Reactors 1, 3 and 4; most probably since anammox concentrations within the secondary enrichment reactors were below the detection limit of the PCR analyses, due to low anammox levels in wastewater and dilutions of the oceanic sediment samples. However, on 28 May 2010, PCR screening of both R3 and R4 indicated positive results (Fig 16 and 17), which correlated in size with the bands previously identified during screening of the mud samples (Fig 14). These amplified genes were sequenced, aligned and the organisms identified as *Candidatus* "Scalindua" and a closely related uncultured marine anammox species (Fig 15, indicated as "OCEAN CLONES, Reactor").

The results therefore indicate that anammox populations grew slowly within Reactors 3 and 4 and that these populations were at least being maintained. Stoichiometrically, the reactors did not perform according to theoretical anammox metabolic ratios, and nitrogen removal was not increasing acceptably for industrial applications. However, the fact that the removal of nitrogen dropped so significantly after an increase in nitrogen concentrations dangerously close to toxicity, lends to the

theory that there was an anammox population in the reactor that was being enriched, however slowly. This is confirmed by the PCR analysis of these reactors.

PCR screening of the total genomic DNA extracted from R1 on 28 May 2010 also indicated the presence of anammox bacteria. The gene sequences amplified with the Amx368-1037 primer combination were gel-extracted, cloned and sequenced (Fig 17, Lanes 2 and 3).

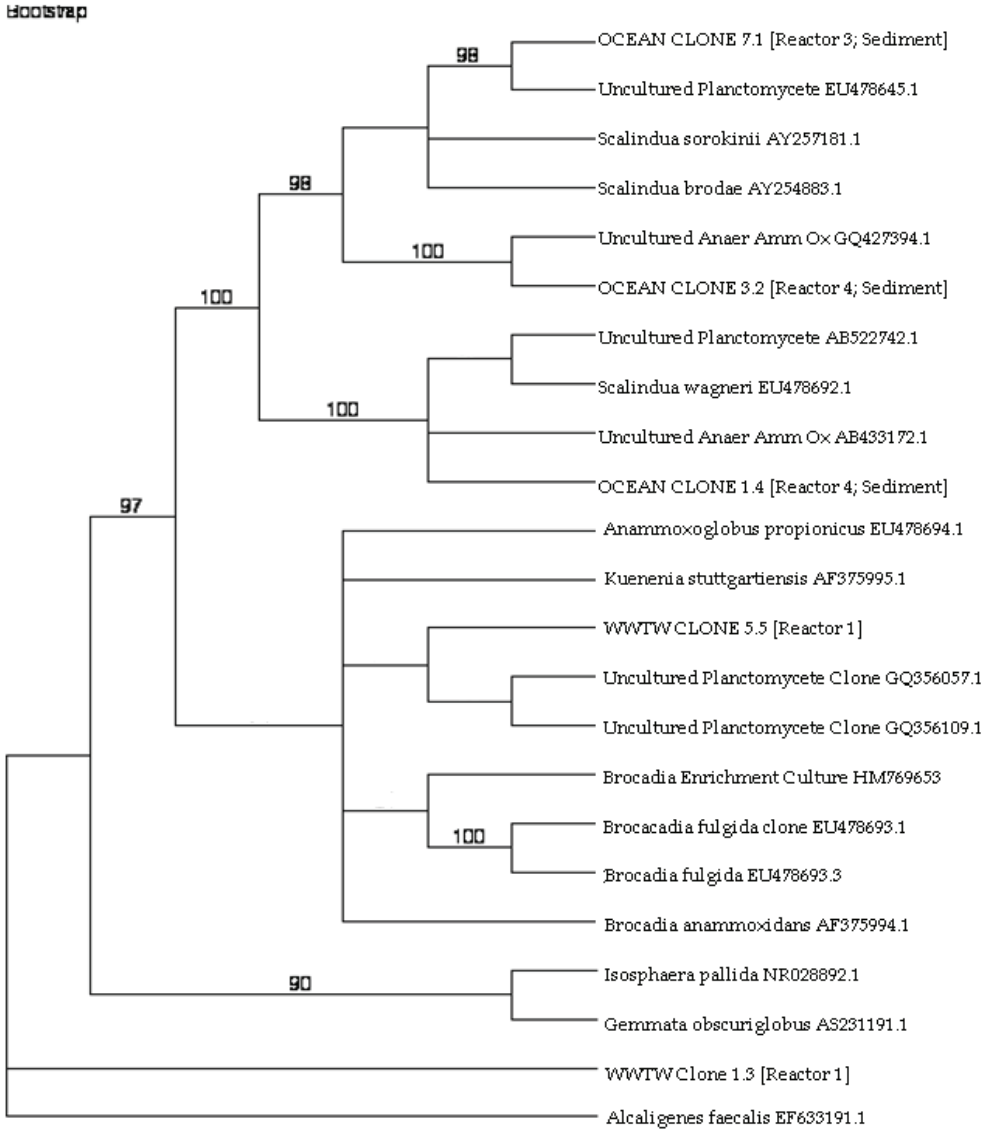


Fig 15 Dendrogram representing the relationships amongst *Candidatus* 'Kuenenia stuttgartiensis' and closely related species with *Alcaligenes faecalis* as an outgroup. The tree is based upon 16S rDNA gene sequence data and was constructed using the Maximum Parsimony method. The nodal numbers represent percentages from 1000 bootstrap samplings, only those over 80% are included. The capitalized strains represent those amplified in this study, and the Genbank accession numbers of the nearest related strains, according to a BLAST homology search are included. Capitalised branch labels represent PCR products cloned in this study.

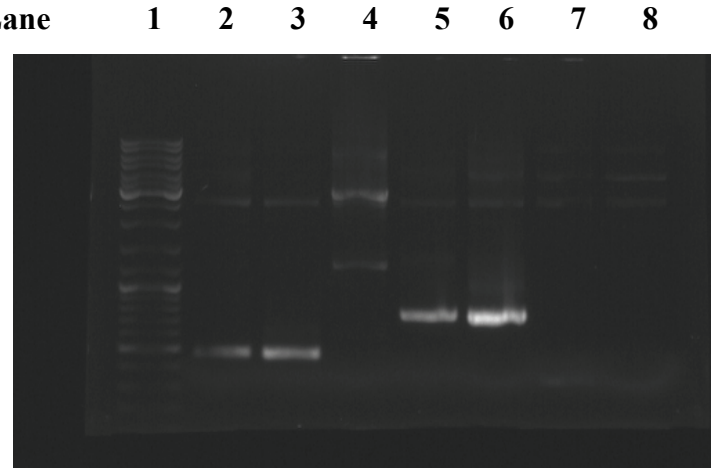


Fig 16 Gel electrophoresis of the 16S rDNA taxonomic gene fragments amplified (May 2010) from DNA extracted from Reactor 3. Lane 1, O'GeneRuler 1kb DNA ladder (Fermentas); Lane 2, Amx368f-Amx820r, Lane 3, Amx368f-Bs820r; Lane 4, Amx368f-Amx1480r; Lane 5, Amx60f-Amx820r; Lane 6, Amx60f-Bs820r; Lanes 7 & 8, Negative controls (others on a separate gel, data not included).

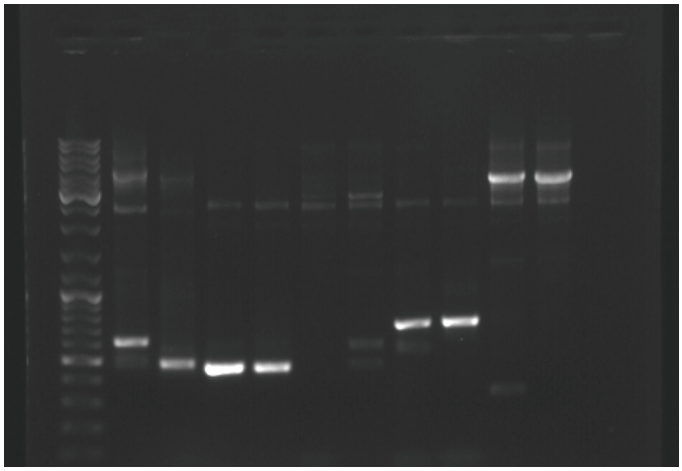


Fig 17 Gel electrophoresis of the 16S rDNA taxonomic gene fragments amplified (May 2010) from DNA extracted from Reactors 1 (lanes 2 and 3) and 4. Lane 1, O'GeneRuler 1kb DNA ladder (Fermentas); Lane 2, Amx368f-1037r (gel-excised), Lane 3, Amx368f-1037r (gel-excised); Lane 4, Amx368f-Amx820r; Lane 5, Amx368-Bs820; Lane 6, Amx368f-Amx1480r; Lane 7, Amx368f-1037r; Lane 8, Amx60f-Amx820r; Lane 9, Amx60f-Bs820r; Lane 10, Amx60f-Amx1480r; Lane 11, Pla60f-Amx1480r; Lanes 12 & 13, Negative controls (others on a separate gel, data not included).

One sequence (WWTW Clone 5.5) showed notable homology with sequences representing known freshwater anammox genera (Fig 15). However, many clones were sequenced before this positive result was obtained. WWTW Clone 1.3 was included in the phylogenetic tree to demonstrate that most sequenced clones originating from R1 fell outside the anammox cluster. Yet again this supports our hypothesis, suggested after the first round of sequence detection, which stated that a diverse consortium existed within this reactor, perhaps due to oxygen contamination during the reactor set-up. This consortium seems to have stabilized within the reactor, and, according to stoichiometric analysis, is removing nitrogen more effectively than the populations within any of the other reactors. Such stable nitrogen removal populations, including an anammox component, have been reported in upflow reactors (Qiao et al., 2009; Li et al., 2008).

4.4 Screening of the reactors: Spatial analysis of biofilms using FISH

Initially, FISH analyses of the anammox populations in Reactors 1 and 2 were notably hampered by autofluorescence. Consequently, an attempt at photobleaching was made to decrease this fluorescence in samples taken from the reactors. Since the solar spectrum (Geuymard, 2003) includes the wavelengths needed to excite Alexa FLOUR 488 and 594, and there was significant autofluorescence in those ranges, it was decided to utilize sunlight to photobleach the samples.

To obtain an indication of the exposure time needed to adequately bleach the samples using sunlight, three samples were put through the same procedure as a control sample and the slides were viewed and photographed on day 0. They were exposed to direct sunlight for approximately ten hours and viewed and photographed again. This procedure was followed for four consecutive days, with the aim of notably reducing the autofluorescence before conducting FISH. However, after photobleaching, some autofluorescence was still evident (Fig 18) and since DNA degradation takes place whilst exposed to the solar spectrum, photobleaching should be conducted with caution.

After photobleaching samples from R3 and R4 for 24 hours it was found that the DNA was still intact for sufficient hybridization. Thus, all samples were subsequently photobleached for 24 hours before FISH analyses. However, some autofluorescence still occurred in the samples from Reactors 1 and 2 and any hybridization that did take place could not be detected at a higher intensity than the background fluorescence. It should also be noted that the FISH probes used were constructed by labelling the two most common anammox primers (Pla46 and Amx820), neither of which was

successfully amplified from the genomic DNA in Reactors 1 and 2 using PCR. We therefore concluded that the anammox species for which these probes were designed seemed to be absent in Reactors 1 and 2.

Initial FISH analysis of Reactors 3 and 4 depicted less autofluorescence than the wastewater, but nevertheless enough to interrupt any conclusions based on fluorescent results. However, continued operation of the reactors led to a decrease in autofluorescence, probably due to washing out of impurities from the reactors. In August 2010, a FISH analysis of Reactors 3 and 4 indicated low concentrations of anammox biomass within these reactors, clustered together in small, sparse clumps (Fig 19 and 20). Both the Amx820 and Pla46 probes fluoresced upon microscopic visualization, therefore revealing true anammox bacteria. In addition, the fluorescent cells are ca. 1 μm in diameter, which falls within the size range of anammox bacteria reported in literature – on average between 0.3 and 1 μm in diameter (Strous et al., 1999). However, as mentioned, the bacteria were sparse, and therefore likely to be present in low concentrations, supporting the conclusions drawn from the stoichiometric and PCR analyses conducted on the microbial consortia in these reactors. Thus, anammox bacteria were present in the reactors and were proliferating at a slow rate.

In summary, stoichiometric screening of the reactors, as well as the PCR amplification, cloning and FISH detection techniques described in this work are all effective for the detection of anammox bacteria. However, an improvement to FISH analyses that was introduced in this study is photobleaching, a simple and effective method to decrease the characteristic autofluorescence of particulate matter in wastewater.

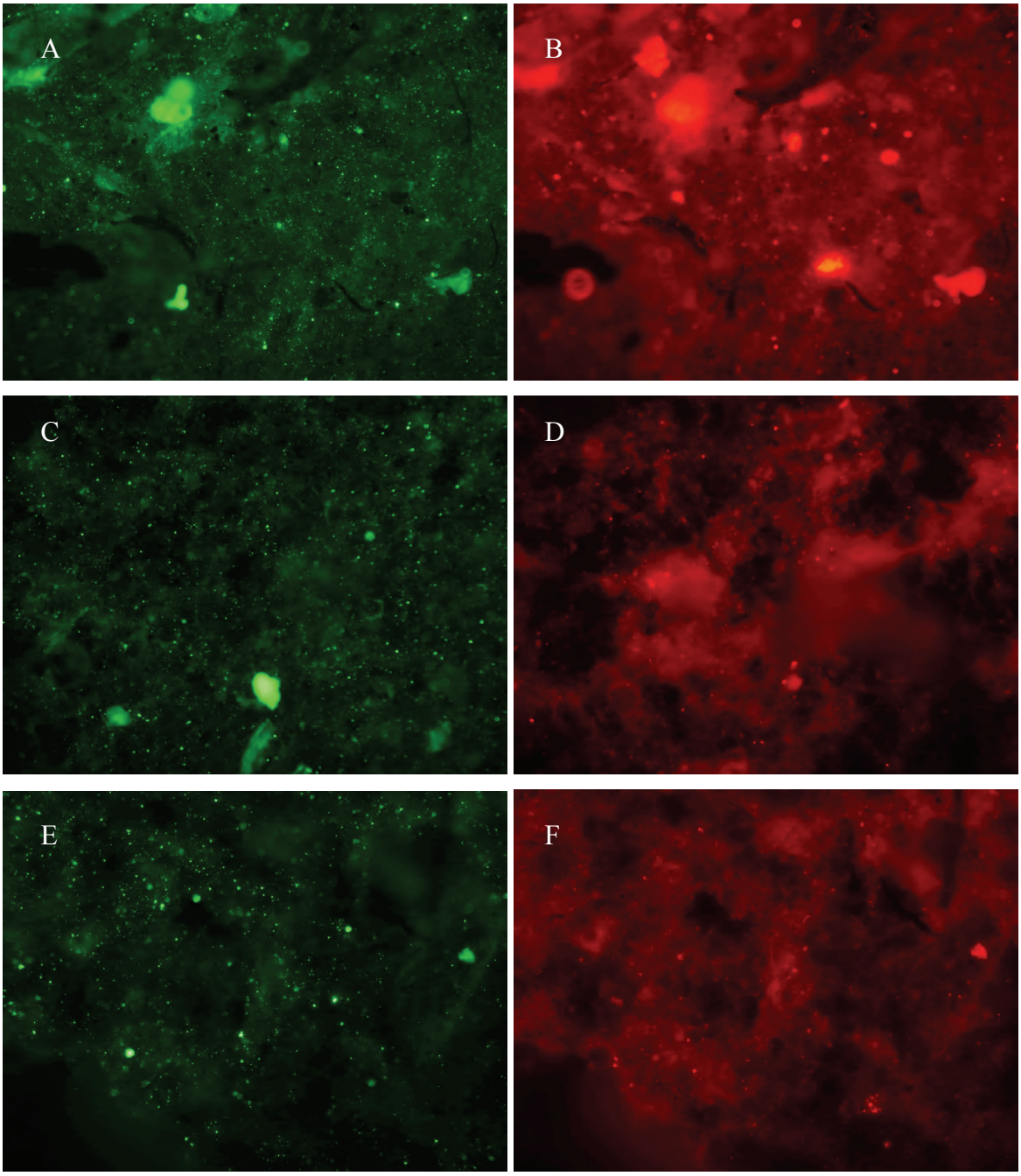
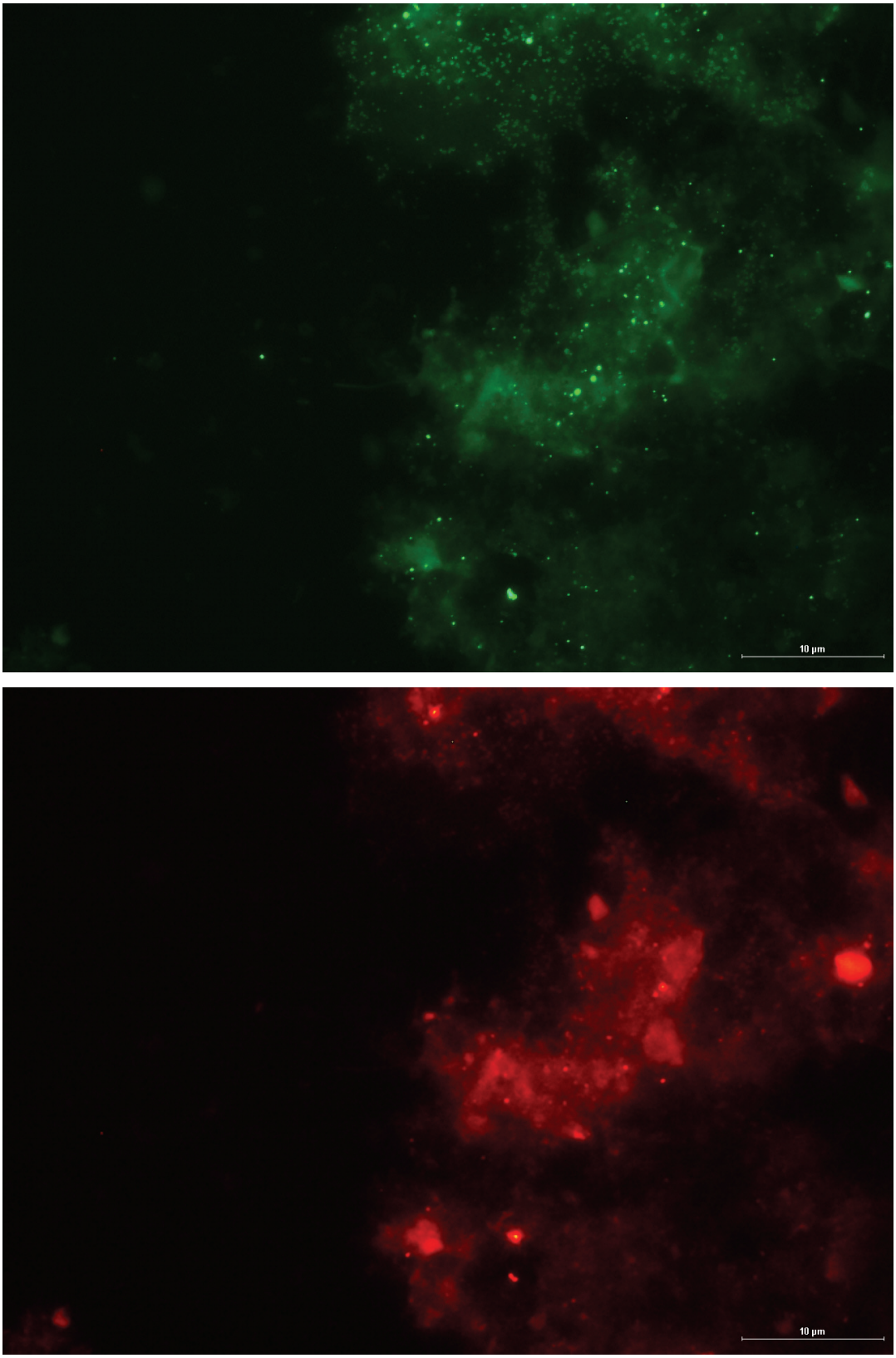
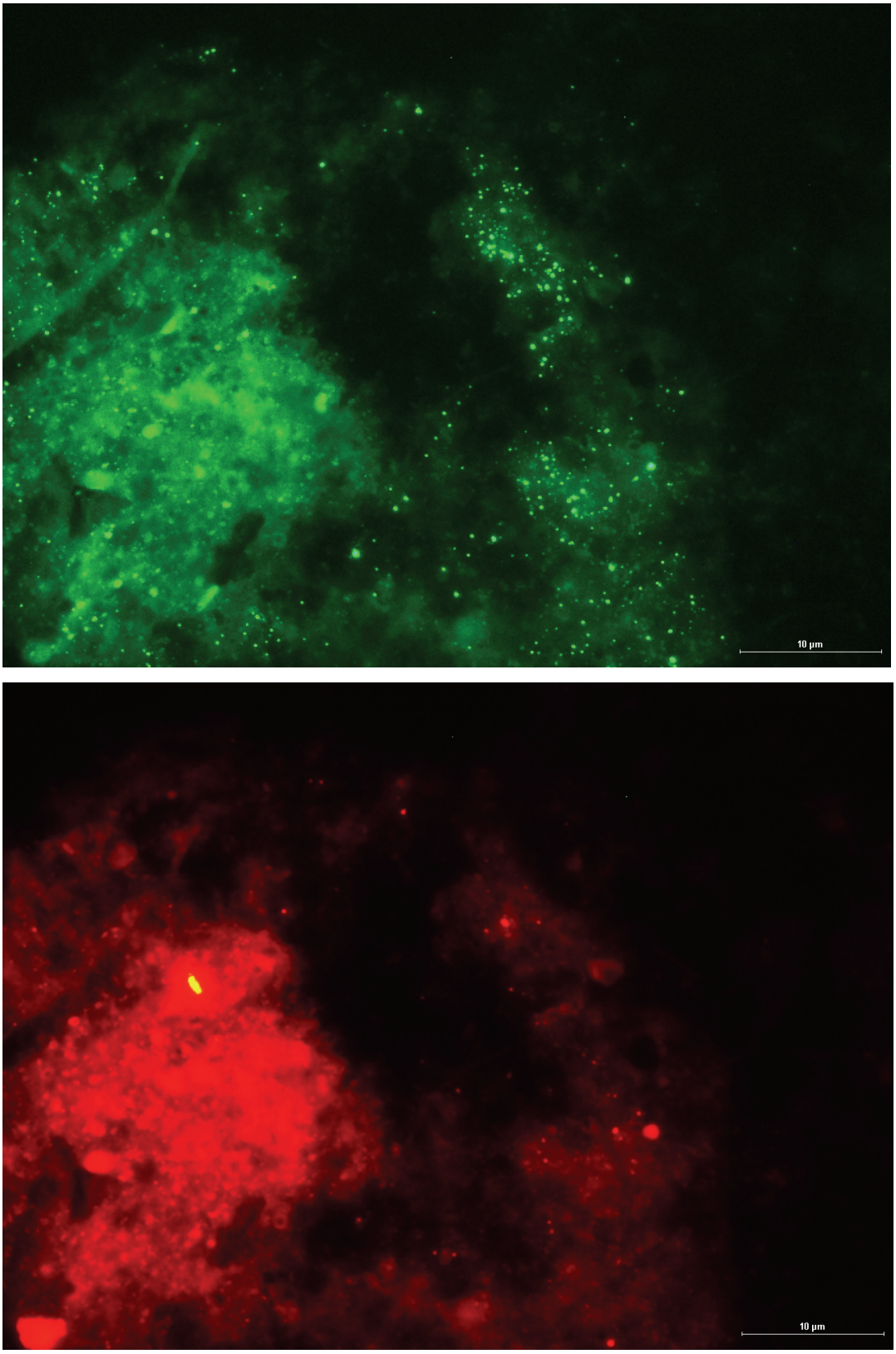


Fig 18 The decrease in autofluorescence of a control sample removed from R1 and bleached for four consecutive days by exposure to the solar spectrum (sunlight). A and B, imaging of the sample directly upon removal from the reactor under the FITC and Texas Red filters respectively (analysed on day 0); C and D, imaging of the same sample after approximately 10 h photobleaching (analysed on day 1); E and F, imaging of the sample after approximately 30 h photobleaching (analysed on day 4).



F**ig 19** Probe fluorescence after hybridization with samples from Reactor 3 under: Top, FITC and Bottom, Texas Red filters. The Top micrograph, represents the Amx820 probe and the Bottom one the Pla46 probe, both images are viewed under 1000x magnification. The bar represents 10µm. Fluorescent detection of both probes reveals anammox bacteria.



F**ig 20** Probe fluorescence after hybridization with samples from Reactor 4 under: Top, FITC and Bottom, Texas Red filters. The Top micrograph represents the Amx820 probe and Bottom one the Pla46 probe, both images are viewed under 1000x magnification. The bar represents 10µm. Fluorescent detection of both probes reveals anammox bacteria.

4.5 Screening of the reactors: Analyses of diversity via Pyrosequencing

To estimate microbial diversity and elucidate potential competition within the reactor consortia, that seemed to be limiting anammox growth, pyrosequencing was used to analyze microbial populations within the microbial consortia of the reactors. The results showed that Planctomycetes, containing anammox bacteria, represented about 8% total bacterial population in Reactor 1 (Fig. 21A). However, the Planctomycetes in Reactors 3 and 4 represented less than 1% of the total bacterial population in these reactors (Fig. 21B and C). The results therefore supported the data obtained for the stoichiometric and microscopic analyses of the reactors, which indicated that low concentrations of anammox bacteria were present within the microbial consortium of Reactor 1, and that even lower concentrations of anammox bacteria were present within the microbial consortia of Reactors 3 and 4.

The dominant bacterial phylum in Reactor 1 was Proteobacteria, comprising 51% of the total bacterial sequences found in the metagenome of the bacterial community (Fig 21A). The dominant class within the Proteobacteria was Betaproteobacteria, comprising 40% of the total bacterial sequences in the metagenome. The dominant genera within this class were *Diaphorobacter, Hydrogenophaga* and *Thauera*, respectively comprising 5%, 4% and 1% of the total bacterial sequences in the metagenome. These genera are all known to harbour nitrifiers and/or denitrifiers (Etchebehere et al., 2001; Khardenavis et al. 2007; Willems et al. 1989) capable of competing with anammox bacteria for the ammonium and nitrite in the enrichment medium (Table 5). What is remarkable is that the sludge in Reactor 1 was collected in January/February 2007 and cultivated for nearly four years when the pyro-sequencing was done. Had there indeed been nitrifiers present among the bacterial consortium, as suggested by the results of the pyrosequencing, it would point towards an oxygen leak in the system. Similar results were obtained for Reactor 3; *Bacillus*, the dominant bacterial genus in Reactor 3 is known to harbour species utilizing nitrite for anaerobic respiration (Nakano and Zuber, 1998). This genus, a member of the Firmicutes (Fig 21B), comprised 39% of the total bacterial sequences found in the metagenome of the reactor.

Similar to the microbial consortium in Reactor 1, the dominant bacterial phylum in Reactor 4 was Proteobacteria, comprising 87% of the total bacterial sequences found in the metagenome of the bacterial community (Fig 21C). The dominant class within the Proteobacteria was however Gammaproteobacteria, comprising 57% of the total bacterial sequences in the metagenome. The dominant genus within this class was *Marinobacter*, comprising 12% of the total bacterial sequences in

the metagenome. Representatives of this genus, known to harbour denitrifiers (Yoshie et al., 2006), were found to be capable of anaerobic ammonium oxidation, mediated by Mn-oxides occurring in marine sediments (Javanaud et al., 2011). The latter process could have happened in both Reactors 3 and 4 since *Marinobacter* was also detected in Reactor 3 (representing 2% of bacterial sequences in the metagenome), and both reactors received marine sediment as part of the inoculum and were fed with enrichment medium prepared with seawater. These results confirm that the anammox bacteria in the reactors were competing for nitrogen with a wide diversity of bacterial taxa.

Analyses of the fungal metagenome in the reactors using pyrosequencing revealed no fungi in Reactor 3, however fungal taxa were found in Reactors 1 and 4. A random sample comprising 100 sequences from a total of 1113 obtained for Reactor 1 revealed that 96% of the sequences represented protistan eukaryotes related to choanoflagellates. The latter are known predators of bacteria (Pernthaler, 2005) indicating that bacterial blooms, as a result of the enrichment process in the reactor, would have been compromised by the presence of these protists. Only a small portion of the sequences represented dikaryomycotan fungal taxa; ascomycetous yeasts and the filamentous fungus *Toxicocladosporium veloxum,* respectively comprised 3% and 1% of the total sequences in the fungal metagenome. In contrast to the results obtained for Reactor 1, analyses of 100 random sequences from a total of 2827 obtained for Reactor 4 yielded only one sequence representing a choanoflagellate. The rest represented ascomycetous yeasts (37%), basidiomycetous yeasts (11%), filamentous fungi (12%) and unidentified fungal taxa (39%). It must be noted that potentially these fungi were also able compete with the anammox bacteria for the nitrite and ammonium in the enrichment medium. Marine fungi from sediment were reported to be involved in denitrification processes (Shoun et al., 1992; Cathrine and Raghukumar, 2009), and some authors found that many denitrifying fungi can produce dinitrogen gas (N_2) or N_2O molecules through a process where nitrogen atoms from nitrite and other nitrogen compounds are combined during denitrifying conditions. They defined this process as 'codenitrification' explaining that cosubstrates such as azide and NH_4^+ are denitrified by the system when induced by nitrite or nitrate. However, these nitrogen compounds are not able to induce denitrification by themselves (Shoun et al., 1992; Takaya, 2002; Hayatsu et al., 2008).

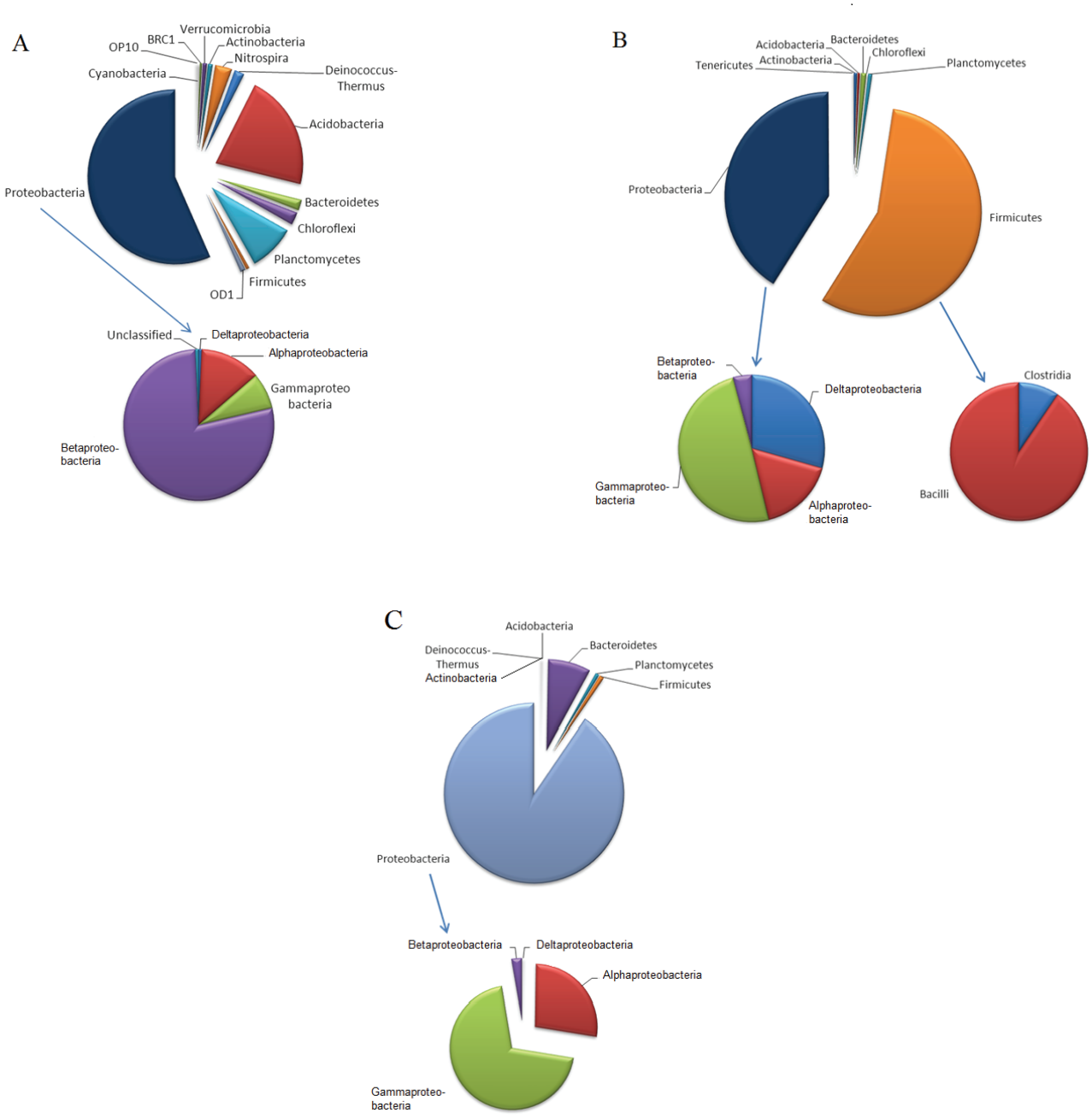


Fig 21 Pie graphs illustrating the ratios of the phyla and classes of the bacterial populations in (A) Reactor 1, (B) Reactor 3, and (C) Reactor 4, as determined using pyrosequencing.

4.6 Conclusions and Recommendations

Using a combination of molecular and enrichment techniques anammox bacteria were found in a number of anaerobic environments in South Africa. Direct molecular screening showed the presence of

anammox bacteria in oceanic sediment originating from the anaerobic zones of St. Helena Bay, off the West Coast of South Africa. In addition, further enrichment demonstrated the growth of these bacteria at both 8°C and 30°C at equally slow rates. Anammox bacteria were not directly detected in wastewater; however, after enrichment of a wastewater inoculum, low concentrations were demonstrated in a reactor. Small volume reactors were shown to be ineffective due to oxygen leakage, and 5 L reactors were found to be the most effective for anammox enrichment. Therefore, anammox bacteria were demonstrated in both natural and artificial environments, but enrichment for these bacteria was too slow for industrial purposes. Pyrosequencing of the metagenome indicated that the slow enrichment for anammox bacteria could be ascribed to the presence of bacterial competitors and protistan predators of bacteria within the microbial consortia of the reactors.

It is recommended that further attempts to obtain sufficient quantities of anammox biomass for experimental and industrial purposes should involve maintaining reactors similar to those described in this study. Since anammox activity is cell-density dependent, 5 L, or larger, reactors inoculated with high concentrations of anammox bacteria would be the most effective starting point, resulting in robust cultures that should recover easily if samples are removed, allowing for further characterization studies and industrial applications. This study was approached according to Beyerink's mantra: "Everything is everywhere. The environment selects." However, inocula for enrichment ought to be sourced with more care, and specifically from existing wastewater treatment processes that demonstrate stable nitrogen removal under abnormal conditions. Processes that demonstrate good nitrogen removal, against the odds, are likely to harbour anammox like organisms in larger quantities.

If decided to proceed with the enrichment of anammox biomass originating from habitats in South Africa, using laboratory scale anaerobic gas-lift reactors similar to those used in this study, it is recommended that:

- 5 L, or larger, gas-lift reactors be used;
- argon gas be used to maintain anaerobic conditions in the reactors;
- stoichiometry be used to monitor reactor performance;
- the effect of vitamin cocktails be tested to increase anammox biomass production;
- instead of FISH, PCR amplification of taxonomic informative gene sequences be used to detect anammox bacteria in the microbial consortia within the reactors. However, if FISH is to be used

the samples on the slides should be photobleached for 24 hours before probe hybridization is attempted;

- inocula for the reactors be obtained from habitats that may harbour aerobic nitrifiers in close proximity of ammonium produced via anaerobic microbial degradation of complex nitrogen compounds. Typically, such habitats are biofilms, containing both aerobic and anaerobic microhabitats in close proximity from each other, on the rock media of trickling filters used by WWTW in South Africa.

The results of the present study also present an alternative to using near pure anammox biomass for industrial purposes. This alternative may encompass purposeful selection for a mixed microbial consortium, containing anammox bacteria, nitrifiers and denitrifiers, similar to the consortium obtained in Reactor 1. This mixed microbial consortium may then be used to remove inorganic nitrogen from wastewater. The experimentation described above may bring anammox technology one step closer to being implemented for the benefit of the South African wastewater treatment industry.

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APPENDIX A

CAPACITY BUILDING REPORT AND CONFERENCE PRESENTATIONS

CAPACITY BUILDING

The project not only resulted in an increased capacity in the Department of Microbiology at Stellenbosch University to screen for anammox bacteria, it also increased the capacity of post-graduate training at Stellenbosch University. Using the project as part of her studies Ms. Wendy Stone passed her Honours degree *cum laude*. Since then, she successfully completed her MSc study. Also, Mr. Ferdinand Postma, an Honours student at Stellenbosch University, assisted and was trained in anammox detection and maintenance techniques. Another student, Ms. Darnielle Delport, was offered a student-assistantship. During university recesses, she worked as a laboratory assistant in the Department of Microbiology at Stellenbosch University, mainly to gain laboratory experience.

During the final quarter of 2008 Grade 11 learners, from selected schools around Stellenbosch, who showed the best marks for Science during that year, were invited to visit the facilities of the Department of Microbiology at Stellenbosch University. During a full-day visit the learners were briefed on the challenges our society faces regarding water scarcity and pollution. The learners were also given the opportunity to obtain some first-hand experience with the basic methods used in Microbiology. For example, they were taught how to work with a microscope, aseptically prepare dilution series and spread plates, as well as separate DNA fragments using gel electrophoresis.

During the first semester of 2009 the marks obtained for Science by the above-mentioned learners, during the final exam of the previous year, were evaluated by Prof A. Botha and Dr. Jac Wilsenach, whereafter bursaries were allocated to the learners. The bursaries, which ranged from R500 to R1500, were presented to each learner at one of the regular assemblies of his/her school. During this presentation all the learners of each school were briefed on the commitment of the CSIR, Stellenbosch University and The Water Research Commission to solve the water crisis in South Africa by encouraging potential engineers and other scientists to study science.

The schools and their learners that were involved with the project were:
Kayamandi High School; Mr Vusumzi Ncaphayi
Kylemore High School; Mr Logan Jooste; Ms Gloriaan Hendricks, Mr Marvin Moses
Lückhoff Senior Secondary School; Mr Johnwin Meyer, Ms Ashlyn Meyer, Ms Lauren Meyer.
Stellenbosch High School; Ms Jarien du Preez, Ms Euodia Vermeulen, Mr Johan van der Walt.

CONFERENCE PRESENTATIONS

Preliminary results of the project were presented by one of the students working on the project, Ms Stone, at two different conferences:

1. Cape Biotechnology Forum, Lord Charles Hotel, Somerset West, South Africa, 30 November-2 December 2008. Title: FISHing for Anammox: Bioprospecting for indigenous bacteria capable of anaerobic ammonium oxidation. W Stone*, J. Wilsenach, M. Mouton and A. Botha.

2. Small Wastewater Treatment Works Division 2nd Conference, East London, South Africa, 23-25 November 2010. Title: Bioprospecting for anammox bacteria in South Africa. W Stone*, J. Wilsenach, M. Mouton and A. Botha.

APPENDIX B

ORIGINAL STOICHIOMETRIC DATA

REACTOR 1 4.5

Date	Time	T	Q	Δt	Influent Concentration (mM)			Abs ave (Reactor) [A]			Conc ave (Reactor) [mM]			Removal (Reactor) [mmol/hr]			toxic N
	hh:mm	h	l/d	h	Nitrite	Nitrate	Ammonium	Nitrite	Nitrate	Ammonium	Nitrite	Nitrate	Ammonium	Nitrite	Nitrate	Ammonium	mmol NO2
22-May	09:00	0	0.5	0	1.739	0.000	2.270	0.189	0.010	0.032	0.703	0.000	0.109	undefined	undefined	undefined	1
23-May	09:00	24	0.5	24	1.739	0.000	2.270	0.253	0.008	0.040	0.966	0.000	0.158	-0.033	0.000	0.035	1
26-May	09:00	96	0.5	72	1.739	0.000	2.270	0.242	0.000	0.050	0.921	0.000	0.219	0.020	0.000	0.039	1
27-May	09:00	120	0.5	24	1.739	0.000	2.270	0.223	0.001	0.054	0.843	0.000	0.244	0.033	0.000	0.038	1
28-May	09:00	144	0.5	24	1.739	0.000	2.270	0.236	0.002	0.152	0.896	0.000	0.848	0.008	0.000	-0.084	1
29-May	13:00	172	0.5	28	1.739	0.000	2.270	0.260	0.000	0.097	1.492	0.000	0.506	-0.091	0.000	0.092	1
30-May	09:00	192	0.5	20	1.739	0.000	2.270	0.222	0.010	0.046	0.836	0.000	0.195	0.166	0.000	0.113	1
02-Jun	09:00	264	0.5	72	1.739	0.000	2.270	0.184	0.002	0.035	0.682	0.000	0.127	0.032	0.000	0.049	1
03-Jun	09:00	288	0.5	24	1.739	0.000	2.270	0.170	0.000	0.027	0.625	0.000	0.078	0.034	0.000	0.055	1
04-Jun	09:00	312	0.5	24	1.739	0.000	2.270	0.170	0.000	0.027	0.625	0.000	0.078	0.023	0.000	0.046	1
05-Jun	13:00	340	0.5	28	1.739	0.000	2.270	0.079	0.008	0.018	0.249	0.000	0.022	0.091	0.000	0.056	1
06-Jun	09:00	360	0.5	20	1.739	0.000	2.270	0.026	0.012	0.012	0.032	0.000	-0.015	0.085	0.000	0.056	1
09-Jun	09:00	432	0.5	72	1.739	0.000	2.270	0.002	0.005	0.021	-0.067	0.000	0.038	0.044	0.000	0.043	1
10-Jun	09:00	456	0.5	24	1.739	0.000	2.270	0.006	0.019	0.033	-0.051	0.000	0.115	0.034	0.000	0.030	1
11-Jun	09:00	480	0.5	24	1.739	0.000	2.270	0.005	0.033	0.029	-0.055	0.000	0.087	0.038	0.000	0.051	1
12-Jun	13:00	508	0.5	28	1.739	0.000	2.270	0.006	0.000	0.040	-0.051	0.000	0.158	0.037	0.000	0.033	1
13-Jun	09:00	528	0.5	20	1.739	0.000	2.270	0.000	0.001	0.024	-0.073	0.000	0.056	0.043	0.000	0.069	1
16-Jun	11:00	602	0.5	74	1.739	0.000	2.270	0.023	0.000	0.008	0.021	0.000	-0.039	0.030	0.000	0.054	1
17-Jun	11:00	626	0.5	24	1.739	0.000	2.270	0.005	0.000	0.019	-0.055	0.000	0.025	0.052	0.000	0.035	1
19-Jun	11:00	674	0.5	48	1.739	0.000	2.270	0.012	0.000	0.030	-0.024	0.000	0.096	0.034	0.000	0.039	1
20-Jun	11:00	698	0.5	24	1.739	0.000	2.270	0.137	0.005	0.049	0.487	0.000	0.213	-0.070	0.000	0.021	1
23-Jun	11:00	770	0.5	72	1.739	0.000	2.270	0.855	0.009	0.044	3.437	0.000	0.179	-0.220	0.000	0.046	1
24-Jun	11:00	794	0.5	24	1.739	0.000	2.270	0.560	0.006	0.022	2.226	0.000	0.047	0.217	0.000	0.071	1
25-Jun	11:00	818	0.5	24	1.739	0.000	2.270	0.607	0.002	0.036	2.417	0.000	0.133	-0.050	0.000	0.028	1
26-Jun	11:00	842	0.5	24	1.739	0.000	2.270	0.648	-0.006	0.041	2.588	0.000	0.164	-0.050	0.000	0.038	1
27-Jun	11:00	866	0.5	24	1.739	0.000	2.270	0.826	-0.002	0.048	3.318	0.000	0.207	-0.170	0.000	0.035	1
03-Jul	11:00	1010	0.5	144	1.739	0.000	2.270	0.249	0.029	0.051	0.947	0.000	0.226	0.091	0.000	0.042	1
07-Jul	14:00	1109	0.5	99	1.739	0.000	2.270	0.594	0.026	0.009	2.366	0.000	-0.036	-0.078	0.000	0.060	1
10-Jul	11:00	1178	0.5	69	1.739	0.000	2.270	0.019	0.010	0.356	0.005	0.000	2.103	0.190	0.000	-0.136	1
24-Jul	11:00	1514	0.5	336	1.739	0.000	2.270	0.462	0.016	0.106	1.824	0.000	0.565	-0.026	0.000	0.056	1
28-Jul	11:00	1610	0.5	96	1.739	0.000	2.270	-0.001	0.006	0.015	-0.077	0.000	0.001	0.127	0.000	0.074	1
31-Jul	11:00	1682	0.5	72	1.739	0.000	2.270	0.004	0.018	0.011	-0.057	0.000	-0.021	0.036	0.000	0.049	1
04-Aug	11:00	1778	0.5	96	1.739	0.000	2.270	0.095	-0.002	0.011	0.317	0.000	-0.024	0.012	0.000	0.048	1
07-Aug	11:00	1850	0.5	72	1.739	0.000	2.270	0.117	-0.004	0.018	0.407	0.000	0.022	0.022	0.000	0.044	1
11-Aug	11:00	1946	0.5	96	1.739	0.000	2.270	0.695	0.000	0.000	2.781	0.000	-0.089	-0.133	0.000	0.054	1
13-Aug	11:00	1994	0.5	48	1.739	0.000	2.270	0.631	-0.002	0.021	2.518	0.000	0.038	0.008	0.000	0.035	1
18-Aug	11:00	2114	0.5	120	1.739	0.000	2.270	0.840	0.135	0.001	3.376	0.684	-0.086	-0.066	-0.040	0.054	1
21-Aug	11:00	2186	0.5	72	1.739	0.000	2.270	0.879	0.078	0.007	3.534	0.000	-0.049	-0.047	0.043	0.046	1
26-Aug	11:00	2306	0.5	120	1.739	0.000	2.270	0.709	0.015	0.026	2.838	0.000	0.072	0.003	0.000	0.041	1
28-Aug	11:00	2354	0.5	48	1.739	0.000	2.270	0.339	0.010	0.000	1.319	0.000	-0.089	0.151	0.000	0.064	1
01-Sep	11:00	2450	0.5	96	1.739	0.000	2.270	0.553	0.039	0.000	2.197	0.000	-0.089	-0.051	0.000	0.049	1
04-Sep	12:00	2523	0.5	73	1.739	0.000	2.270	0.771	0.047	0.027	3.093	0.000	0.078	-0.083	0.000	0.035	1
09-Sep	14:00	2645	0.5	122	1.739	0.000	2.270	0.744	0.020	0.001	2.982	0.000	-0.083	-0.022	0.000	0.055	1
16-Sep	09:00	2808	0.5	163	1.739	0.000	2.270	0.236	0.136	0.008	0.896	0.690	-0.039	0.075	-0.033	0.047	1
22-Sep	12:30	2956	0.5	148	1.739	0.000	2.270	0.170	0.003	0.015	0.623	0.000	0.001	0.032	0.021	0.046	1
26-Sep	12:30	3052	0.5	96	1.739	0.000	2.270	0.014	-0.002	0.022	-0.018	0.000	0.047	0.067	0.000	0.044	1
30-Sep	13:15	3148	0.5	97	1.739	0.000	3.780	0.436	0.003	0.011	1.715	0.000	-0.024	-0.080	0.000	0.083	1
02-Oct	09:30	3193	0.5	44	1.739	0.000	3.780	0.972	0.000	0.006	3.916	0.000	-0.052	-0.269	0.000	0.083	1
06-Oct	10:00	3289	0.5	97	1.739	0.000	3.780	1.393	0.000	0.006	5.646	0.000	-0.052	-0.162	0.000	0.080	1
13-Oct	10:30	3458	0.5	169	0.000	0.000	2.270	1.311	-0.002	0.041	5.308	0.000	0.161	-0.102	0.000	0.038	1
16-Oct	09:45	3529	0.5	71	0.000	0.000	2.270	1.085	0.006	0.023	4.382	0.000	0.053	-0.033	0.000	0.053	1
20-Oct	12:30	3628	0.5	99	0.000	0.000	2.270	0.347	0.025	0.063	1.350	0.000	0.300	0.110	0.000	0.030	1
23-Oct	11:00	3698	0.5	71	2.320	0.000	3.230	0.007	0.000	0.000	-0.044	0.000	-0.089	0.138	0.000	0.094	1
28-Oct	13:00	3820	0.5	122	2.320	0.000	3.230	0.052	0.000	0.019	0.140	0.000	0.025	0.039	0.000	0.063	1
31-Oct	12:00	3891	0.5	71	2.320	0.000	3.230	0.215	0.262	0.007	0.808	1.423	-0.046	-0.011	-0.120	0.073	1
03-Nov	13:00	3964	0.5	73	2.320	0.000	3.230	0.337	0.120	0.001	1.311	0.596	-0.086	-0.010	0.039	0.072	1
06-Nov	11:00	4034	0.5	70	2.320	0.000	3.230	0.202	0.060	0.012	0.754	0.000	-0.015	0.068	0.038	0.063	1
10-Nov	13:30	4133	0.5	99	2.320	0.000	3.230	0.407	0.139	0.005	1.598	0.707	-0.058	-0.024	-0.047	0.070	1
17-Nov	13:00	4300	0.5	168	2.320	0.000	3.230	0.897	0.096	0.022	3.610	0.456	0.047	-0.081	-0.003	0.063	1

21-Nov	09:00	4392	0.5	92	2.320	0.000	3.230	0.975	0.014	0.007	3.930	0.000	-0.046	-0.049	0.022	0.073	1
25-Nov	11:00	4490	0.5	98	2.320	0.000	3.230	0.966	0.014	0.073	3.893	0.000	0.358	-0.031	0.000	0.041	1
27-Nov	11:00	4538	0.5	48	2.320	0.000	3.230	0.646	0.023	0.079	2.579	0.000	0.398	0.118	0.000	0.055	1
04-Dec	03:00	4710	0.5	172	2.320	0.000	3.230	0.364	0.026	0.026	1.419	0.000	0.068	0.049	0.000	0.074	1
09-Dec	09:00	4824	0.5	114	2.320	0.000	3.230	0.536	0.046	0.039	2.126	0.000	0.152	-0.024	0.000	0.061	1
12-Dec	08:00	4895	0.5	71	2.320	0.000	3.230	0.531	0.012	0.048	2.105	0.000	0.204	0.006	0.000	0.060	1
17-Dec	02:00	5021	0.5	126	2.320	0.000	3.230	0.572	0.019	0.039	2.273	0.000	0.152	-0.005	0.000	0.066	1
19-Dec	08:00	5063	0.5	42	2.320	0.000	3.230	0.645	0.052	0.027	2.575	0.000	0.078	-0.038	0.000	0.074	1
22-Dec	08:00	5135	0.5	72	0.000	0.000	3.230	0.582	0.033	0.018	2.317	0.000	0.019	-0.032	0.000	0.071	1
26-Dec	08:00	5231	0.5	96	0.000	0.000	3.230	0.565	0.023	0.000	2.245	0.000	-0.089	-0.043	0.000	0.074	1
29-Dec	08:00	5303	0.5	72	0.000	0.000	3.230	0.386	0.020	0.000	1.510	0.000	-0.089	0.014	0.000	0.069	1
05-Jan	08:00	5471	0.5	168	0.000	0.000	3.230	0.523	0.026	0.028	2.072	0.000	0.084	-0.058	0.000	0.061	1
07-Jan	10:00	5521	0.5	50	0.000	0.000	3.230	0.461	0.035	0.016	1.818	0.000	0.010	-0.015	0.000	0.074	1
09-Jan	10:00	5569	0.5	48	0.000	0.000	3.230	0.284	0.045	0.017	1.093	0.000	0.013	0.045	0.000	0.067	1
12-Jan	09:00	5640	0.5	71	0.000	0.000	3.230	0.307	0.013	0.030	1.185	0.000	0.096	-0.031	0.000	0.060	1
14-Jan	10:00	5689	0.5	49	0.000	0.000	3.230	0.271	0.000	0.014	1.038	0.000	-0.006	-0.008	0.000	0.077	1
16-Jan	13:00	5740	0.5	51	0.000	0.000	3.230	0.251	0.107	0.038	0.957	0.517	0.142	-0.013	-0.056	0.051	1
19-Jan	09:30	5809	0.5	69	0.000	0.000	3.230	0.228	0.068	0.027	0.863	0.000	0.075	-0.012	0.034	0.070	1
22-Jan	10:00	5881	0.5	73	0.000	0.000	3.230	0.235	0.025	0.018	0.892	0.000	0.019	-0.020	0.000	0.070	1
26-Jan	11:00	5978	0.5	97	2.320	0.000	0.000	0.184	0.071	0.029	0.682	0.000	0.090	0.044	0.000	-0.005	1
28-Jan	10:00	6025	0.5	47	2.320	0.000	0.000	0.130	0.036	0.026	0.461	0.000	0.068	0.060	0.000	0.001	1
30-Jan	10:00	6073	0.5	48	2.320	0.000	0.000	0.100	0.033	0.030	0.338	0.000	0.093	0.053	0.000	-0.004	1
02-Feb	13:00	6148	0.5	75	2.320	0.000	0.000	0.078	0.032	0.006	0.245	0.000	-0.055	0.049	0.000	0.010	1
05-Feb	13:00	6220	0.5	72	2.320	0.000	0.000	0.123	0.103	0.009	0.430	0.494	-0.036	0.028	-0.041	0.000	1
09-Feb	10:00	6313	0.5	93	2.320	0.000	0.000	0.084	0.149	0.035	0.270	0.763	0.127	0.050	-0.029	-0.011	1
12-Feb	11:00	6386	0.5	73	2.320	0.000	3.230	0.027	0.087	0.046	0.036	0.000	0.192	0.062	0.047	0.059	1
17-Feb	11:00	6506	0.5	120	2.320	0.000	3.230	0.155	0.126	0.050	0.563	0.631	0.219	0.017	-0.037	0.062	1
20-Feb	11:00	6578	0.5	72	2.320	0.000	3.230	0.183	0.089	0.039	0.678	0.415	0.152	0.027	0.005	0.068	1
23-Feb	11:00	6650	0.5	72	2.320	0.000	3.230	0.156	0.053	0.044	0.567	0.000	0.179	0.043	0.026	0.062	1
27-Feb	11:00	6746	0.5	96	2.320	0.000	3.230	0.255	0.095	0.105	0.974	0.447	0.555	0.009	-0.030	0.038	1
02-Mar	11:00	6818	0.5	72	2.320	0.000	3.230	0.209	0.132	0.025	0.783	0.666	0.062	0.044	-0.028	0.097	1
06-Mar	11:00	6914	0.5	96	2.320	0.000	3.230	0.212	0.094	0.025	0.797	0.444	0.065	0.031	0.001	0.066	1
16-Mar	11:00	7154	0.5	240	6.232	0.000	6.510	0.258	0.120	0.016	0.984	0.596	0.010	0.106	-0.015	0.136	1
20-Mar	11:00	7250	0.5	96	6.232	0.000	6.510	0.281	0.165	0.031	1.081	0.859	0.099	0.103	-0.030	0.129	1
24-Mar	11:00	7346	0.5	96	6.232	0.000	6.510	0.299	0.054	0.033	1.153	0.000	0.115	0.102	0.040	0.133	1
27-Mar	11:00	7418	0.5	72	6.232	0.000	6.510	0.337	0.046	0.030	1.311	0.000	0.096	0.093	0.000	0.135	1
30-Mar	11:00	7490	0.5	72	6.232	0.000	6.510	0.388	0.072	0.058	1.520	0.000	0.266	0.085	0.000	0.119	1
03-Apr	13:00	7588	0.5	98	6.232	0.000	6.510	0.367	0.087	0.0515	1.434	0.000	0.229	0.104	0.000	0.133	1
07-Apr	13:00	7684	0.5	96	6.232	0.000	6.510	0.381	0.061	0.0395	1.491	0.000	0.155	0.096	0.000	0.136	1
14-Apr	13:00	7852	0.5	168	6.232	0.000	6.510	0.374	0.092	0.0335	1.463	0.432	0.118	0.100	-0.021	0.134	1
21-Apr	13:00	8020	0.5	168	6.232	0.000	6.510	0.3965	0.093	0.037	1.555	0.438	0.139	0.095	-0.009	0.132	1
24-Apr	11:00	8092	0.5	72	6.232	0.000	6.510	0.410	0.135	0.037	1.610	0.684	0.139	0.093	-0.030	0.133	1
04-May	11:00	8332	0.5	240	6.232	0.000	6.510	0.337	0.103	0.034	1.311	0.497	0.118	0.108	-0.007	0.134	1
08-May	11:00	8428	0.5	96	6.232	0.000	6.510	0.339	0.092	0.063	1.317	0.430	0.300	0.102	-0.006	0.121	1
11-May	11:00	8500	0.5	72	6.232	0.000	6.510	0.213	0.109	0.024	0.799	0.529	0.056	0.146	-0.017	0.150	1
15-May	11:00	8596	0.5	96	6.232	0.000	6.510	0.4605	0.1195	0.0515	1.818	0.593	0.229	0.044	-0.015	0.123	1
18-May	11:00	8668	0.5	72	6.232	0.000	6.510	0.4085	0.1555	0.0945	1.604	0.804	0.494	0.110	-0.030	0.109	1
22-May	11:00	8764	0.5	96	6.232	0.000	6.510	0.395	0.0785	0.0445	1.549	0.000	0.186	0.100	0.038	0.146	1
26-May	11:00	8860	0.5	96	6.232	0.000	6.510	0.428	0.106	0.041	1.684	0.514	0.164	0.088	-0.035	0.133	1
29-May	11:00	8932	0.5	72	6.232	0.000	6.510	0.3905	0.172	0.0465	2.295	0.900	0.198	0.044	-0.043	0.129	1
03-Jun	11:00	9052	0.5	120	6.232	0.000	6.510	0.3885	0.1385	0.06	2.283	0.704	0.281	0.083	-0.007	0.127	1
05-Jun	11:00	9100	0.5	48	6.232	0.000	6.510	0.412	0.082	0.06	2.428	0.000	0.281	0.066	0.066	0.130	1
10-Jun	11:00	9220	0.5	120	6.232	0.000	6.510	0.3975	0.0865	0.042	2.339	0.000	0.170	0.084	0.000	0.136	1
12-Jun	11:00	9268	0.5	48	6.232	0.000	6.510	0.4515	0.104	0.0495	2.671	0.503	0.216	0.043	-0.058	0.127	1
22-Jun	11:00	9508	0.5	240	6.232	0.000	6.510	0.369	0.0325	0.0505	2.163	0.000	0.223	0.094	0.009	0.131	1
26-Jun	11:00	9604	0.5	96	6.232	0.000	6.510	0.3355	0.1135	0.055	1.957	0.558	0.250	0.099	-0.038	0.129	1
03-Jul	11:00	9772	0.5	168	6.232	0.000	6.510	0.325	0.078	0.019	1.892	0.000	0.028	0.092	0.015	0.141	1
17-Jul	11:00	10108	0.5	336	6.232	0.000	6.510	0.4285	0.112	0.0645	2.529	0.549	0.309	0.069	-0.019	0.125	1
21-Jul	09:00	10204	0.5	96	6.232	0.000	6.510	0.4135	0.06	0.0875	2.437	0.000	0.451	0.083	0.026	0.120	1
31-Jul	09:00	10444	0.5	240	6.232	0.000	6.510	0.4705	0.082	0.0795	2.788	0.000	0.401	0.065	0.000	0.128	1
17-Aug	09:00	10852	0.5	408	6.232	0.000	6.510	0.538	0.032	0.12	3.204	0.000	0.651	0.059	0.000	0.119	1
21-Aug	09:00	10948	0.5	96	2.320	0.000	2.420	0.574	0.095	0.1625	3.426	0.450	0.913	-0.033	-0.030	0.019	1
24-Aug	09:00	11020	0.5	72	2.320	0.000	2.420	0.5195	0.095	0.1305	3.090	0.450	0.716	0.005	-0.009	0.048	1
28-Aug	09:00	11116	0.5	96	2.320	0.000	2.420	0.432	0.0815	0.0955	2.551	0.000	0.500	0.020	0.021	0.050	1

31-Aug	09:00	11188	0.5	72	2.320	0.000	2.420	0.459	0.0845	0.136	2.717	0.000	0.750	-0.019	0.000	0.019	1
04-Sep	09:00	11284	0.5	96	2.320	0.000	2.420	0.4595	0.099	0.1475	2.720	0.473	0.821	-0.008	-0.032	0.030	1
14-Sep	09:00	11524	0.5	240	2.320	0.000	2.420	0.3645	0.0735	0.0505	2.135	0.000	0.223	0.015	0.009	0.057	1
18-Sep	09:00	11620	0.5	96	2.320	0.000	2.420	0.3555	0.0635	0.0555	2.080	0.000	0.253	0.008	0.000	0.044	1
22-Sep	09:00	11716	0.5	96	2.320	0.000	2.420	0.2985	0.0945	0.08	1.729	0.447	0.404	0.029	-0.030	0.035	1
25-Sep	09:00	11788	0.5	72	3.396	0.000	2.727	0.319	0.053	0.0445	1.855	0.000	0.186	0.024	0.028	0.067	1
30-Sep	09:00	11908	0.5	120	3.396	0.000	2.727	0.309	0.05	0.0185	1.793	0.000	0.025	0.036	0.000	0.062	1
02-Oct	09:00	11956	0.5	48	3.774	0.000	3.030	0.305	0.049	0.0385	1.769	0.000	0.149	0.044	0.000	0.048	1
06-Oct	09:00	12052	0.5	96	3.774	0.000	3.030	0.3415	0.0225	0.06	1.994	0.000	0.281	0.027	0.000	0.051	1
09-Oct	09:00	12124	0.5	72	3.774	0.000	3.030	0.375	0	0.047	2.200	0.000	0.201	0.020	0.000	0.064	1
13-Oct	09:00	12220	0.5	96	3.774	0.000	3.030	0.3675	0.0635	0.1505	2.154	0.000	0.839	0.036	0.000	0.016	1
16-Oct	09:00	12292	0.5	72	3.774	0.000	3.030	0.2865	0.031	0.0375	1.655	0.000	0.142	0.075	0.000	0.104	1
19-Oct	09:00	12364	0.5	72	3.774	0.000	3.030	0.392	0.0675	0.0365	2.305	0.000	0.136	-0.010	0.000	0.061	1
22-Oct	09:00	12436	0.5	72	3.774	0.000	3.030	0.4365	0.0555	0.054	2.579	0.000	0.244	0.008	0.000	0.051	1
26-Oct	09:00	12532	0.5	96	3.774	0.000	3.030	0.3935	0.063	0.038	2.314	0.000	0.145	0.043	0.000	0.065	1
03-Nov	09:00	12724	0.5	192	3.774	0.000	3.030	0.44	0.0375	0.003	2.600	0.000	-0.070	0.018	0.000	0.070	1
05-Nov	09:00	12772	0.5	48	3.774	0.000	3.030	0.441	0.0275	0.031	2.606	0.000	0.102	0.024	0.000	0.045	1
10-Nov	09:00	13012	0.5	240	3.774	0.000	3.030	0.44	0.031	0.019	2.600	0.000	0.028	0.025	0.000	0.064	1
01-Dec	09:00	13516	0.5	504	3.774	0.000	3.030	0.345	0.1315	0.0005	2.015	0.663	-0.086	0.042	-0.020	0.066	1
04-Dec	09:00	13588	0.5	72	3.774	0.000	3.030	0.3865	0.1255	0.0505	2.271	0.628	0.223	0.015	-0.011	0.039	1
09-Dec	09:00	13708	0.5	120	3.774	0.000	3.030	0.3595	0.112	0.009	2.104	0.549	-0.033	0.041	-0.008	0.073	1
14-Dec	09:00	13828	0.5	120	3.774	0.000	3.030	0.3015	0.081	0.004	1.747	0.000	-0.064	0.056	0.021	0.066	1
19-Dec	09:00	13948	0.5	120	3.774	0.000	3.030	0.318	0.136	0.0235	1.849	0.690	0.056	0.036	-0.040	0.057	1
21-Dec	09:00	13996	0.5	48	3.774	0.000	3.030	0.346	0.112	0.005	2.021	0.549	-0.058	0.020	0.002	0.075	1
24-Dec	09:00	14068	0.5	72	0.000	0.000	2.270	0.355	0.101	0.0145	2.077	0.485	0.001	-0.047	-0.006	0.044	1
28-Dec	09:00	14164	0.5	96	0.000	0.000	2.270	0.338	0.0985	0.003	1.972	0.470	-0.070	-0.036	-0.009	0.052	1
04-Jan	09:00	14332	0.5	168	0.000	0.000	2.270	0.325	0.048	0.0075	1.892	0.000	-0.043	-0.037	0.013	0.047	1
07-Jan	09:00	14404	0.5	72	0.000	0.000	2.270	0.3255	0.0495	0.0065	1.895	0.000	-0.049	-0.040	0.000	0.049	1
14-Jan	09:00	14572	0.5	168	0.000	0.000	2.270	0.261	0.006	0.050	1.495	0.000	-0.052	-0.020	0.000	0.048	1
20-Jan	09:00	14716	0.5	144	0.000	0.000	2.270	0.205	0.000	0.027	1.150	0.000	-0.089	-0.013	0.000	0.050	1
28-Jan	09:00	14908	0.5	192	0.000	0.000	2.270	0.1455	0.000	0.024	0.786	0.000	-0.089	-0.008	0.000	0.049	1
02-Feb	09:00	15028	0.5	120	0.000	0.000	2.270	0.149	0.000	0.005	0.808	0.000	-0.089	-0.018	0.000	0.049	1
03-Mar	09:00	15772	0.5	744	1.739	0.000	2.270	0.457	0.000	0.164	0.451	0.853	-0.089	0.029	-0.023	0.049	1
04-Apr	09:00	16492	0.5	720	1.739	0.000	2.270	0.0155	0.0965	0.3285	-0.002	1.815	0.506	0.039	-0.044	0.033	1
13-Apr	10:30	16710	0.5	218	1.739	0.000	2.270	0.0055	0.000	0.064	-0.013	0.000	-0.089	0.037	0.038	0.061	1
15-Apr	10:30	16758	0.5	48	1.739	0.000	2.270	0.001	0.097	0.061	-0.017	0.000	0.506	0.037	0.000	-0.019	1
22-Apr	09:00	16924	0.5	167	1.739	0.000	2.270	0.049	0.232	0.0635	0.032	0.000	1.342	0.034	0.000	-0.003	1
26-Apr	10:30	17022	0.5	98	1.739	0.000	2.270	0.0635	0.122	0.056	0.047	0.000	0.663	0.035	0.000	0.065	1
30-Apr	15:00	17122	0.5	101	1.739	0.000	2.270	0.132	0.21	0.0125	0.117	0.000	1.206	0.031	0.000	-0.002	1
05-May	09:00	17236	0.5	114	1.739	0.000	2.270	0.03	0.052	0.016	0.013	0.000	0.232	0.040	0.000	0.081	1
10-May	12:00	17359	0.5	123	1.739	0.000	2.270	0.02	0.0185	0.0545	0.002	0.000	0.025	0.037	0.000	0.054	1
14-May	12:00	17455	0.5	96	1.739	0.000	2.270	0.0205	0.0235	0.0665	0.003	0.000	0.056	0.036	0.000	0.045	1
20-May	12:00	17599	0.5	144	2.320	0.000	3.230	0.0385	0	0.1215	0.021	0.605	-0.089	0.047	-0.032	0.074	1
24-May	15:00	17698	0.5	99	2.320	0.000	3.230	0.053	0.0105	0.1085	0.036	0.529	-0.024	0.047	-0.008	0.065	1
28-May	14:00	17793	0.5	95	2.320	0.000	3.230	0.0095	0.018	0.177	-0.009	0.929	0.022	0.051	-0.038	0.065	1
08-Jun	11:00	18054	0.5	261	2.320	0.000	3.230	0.0075	0	0.018	-0.011	0.000	-0.089	0.049	0.016	0.071	1
16-Jun	11:00	18246	0.5	192	3.000	0.000	4.000	0.024	0	0.1575	0.006	0.815	-0.089	0.062	-0.036	0.085	1
23-Jun	09:00	18411	0.5	165	3.000	0.000	4.000	0.017	0	0.1015	-0.001	0.488	-0.089	0.063	-0.001	0.085	1
08-Jul	09:00	18771	0.5	360	3.000	0.000	4.000	0.712	0	0.039	0.713	0.000	-0.089	0.039	0.006	0.085	1
19-Jul	09:00	19035	0.5	264	0.000	0.000	3.230	0.281	0	0	0.270	0.000	-0.089	0.002	0.000	0.069	1
27-Jul	09:00	19227	0.5	192	1.739	0.000	3.230	0.128	0	0	0.113	0.000	-0.089	0.038	0.000	0.069	1
03-Aug	09:00	19395	0.5	168	1.739	0.000	3.230	0.102	0	0	0.086	0.000	-0.089	0.035	0.000	0.069	1
12-Aug	09:00	19611	0.5	216	1.739	0.000	3.230	0.043	0	0.000	0.026	0.000	-0.089	0.037	0.000	0.069	1
29-Aug	09:00	20019	0.5	408	1.739	0.000	3.230	0.058	0	0.000	0.041	0.000	-0.089	0.035	0.000	0.069	1
14-Sep	09:00	20403	0.5	384	1.739	0.000	3.230	0.096	0	0.028	0.080	0.000	-0.089				

REACTOR 2 18.5

Date	Time	T	Q	Δt	Influent Conc. (mM)			Abs Ave (A)			Conc. Ave (mM)			Removal (mmol/hr)			toxic N
	hh:mm	h	l/d	hr	Nitrite	Nitrate	Amm	Nitrite	Nitrate	Amm	Nitrite	Nitrate	Amm	Nitrite	Nitrate	Amm	mmol NO2
05-Feb	13:00	0	0.5	0	1.739	1.765	1.816	0.258	0.039	0.299	0.492	0.000	1.755	undefined	undefined	undefined	1
09-Feb	10:00	93	0.5	93	1.739	1.765	1.816	0.080	0.031	0.325	0.127	0.000	1.915	0.106	0.037	-0.034	1
12-Feb	11:00	166	0.5	73	1.739	1.765	1.816	0.132	0.006	0.338	0.234	0.000	1.992	0.004	0.037	-0.023	1
17-Feb	11:00	286	0.5	120	1.739	1.765	1.816	0.281	0.025	0.367	0.539	0.000	2.171	-0.022	0.037	-0.035	1
20-Feb	11:00	358	0.5	72	1.739	1.765	1.816	0.092	0.076	0.369	0.151	0.000	2.183	0.133	0.037	-0.011	1
23-Feb	11:00	430	0.5	72	1.739	0.000	1.816	0.069	0.027	0.302	0.105	0.000	1.770	0.046	0.000	0.107	1
27-Feb	11:00	526	0.5	96	1.739	0.000	1.816	0.103	0.060	0.357	0.175	0.000	2.109	0.019	0.000	-0.071	1
02-Mar	11:00	598	0.5	72	1.739	0.000	1.816	0.190	0.119	0.278	0.354	0.590	1.622	-0.017	-0.164	0.129	1
06-Mar	11:00	694	0.5	96	1.739	0.000	1.816	0.137	0.062	0.315	0.245	0.000	1.853	0.052	0.114	-0.045	1
16-Mar	11:00	934	0.5	240	1.739	0.000	1.816	0.137	0.120	0.352	0.245	0.596	2.078	0.031	-0.058	-0.023	1
20-Mar	11:00	1030	0.5	96	1.739	0.000	1.816	0.313	0.052	0.362	0.606	0.000	2.143	-0.046	0.115	-0.019	1
24-Mar	11:00	1126	0.5	96	1.739	0.000	1.816	0.118	0.007	0.254	0.206	0.000	1.477	0.109	0.000	0.135	1
27-Mar	11:00	1198	0.5	72	1.739	0.000	1.816	0.127	0.025	0.292	0.223	0.000	1.708	0.027	0.000	-0.057	1
30-Mar	11:00	1270	0.5	72	1.739	0.000	1.816	0.123	0.000	0.279	0.215	0.000	1.628	0.034	0.000	0.025	1
03-Apr	13:00	1368	0.5	98	1.739	0.000	1.816	0.109	0.000	0.2585	0.187	0.000	1.505	0.038	0.000	0.030	1
07-Apr	13:00	1464	0.5	96	1.739	0.000	1.816	0.1135	0.000	0.1625	0.196	0.000	0.913	0.030	0.000	0.133	1
14-Apr	13:00	1632	0.5	168	1.739	0.000	1.816	0.1265	0.000	0.22	0.223	0.000	1.268	0.029	0.000	-0.028	1
21-Apr	13:00	1800	0.5	168	1.739	0.000	1.816	0.1075	0.0065	0.2185	0.184	0.000	1.258	0.037	0.000	0.013	1
24-Apr	11:00	1872	0.5	72	1.739	0.000	1.816	0.1985	0.0015	0.118	0.371	0.000	0.639	-0.020	0.000	0.184	1
04-May	11:00	2112	0.5	240	1.739	0.000	1.816	0.0865	0.009	0.1675	0.141	0.000	0.944	0.051	0.000	-0.005	1
08-May	11:00	2208	0.5	96	1.739	0.000	1.816	0.0735	0.001	0.185	0.114	0.000	1.052	0.039	0.000	-0.005	1
11-May	11:00	2280	0.5	72	1.739	0.000	1.816	0.096	0.003	0.1245	0.161	0.000	0.679	0.021	0.000	0.120	1
15-May	11:00	2376	0.5	96	1.739	0.000	1.816	0.0875	0	0.1225	0.143	0.000	0.666	0.037	0.000	0.026	1
18-May	11:00	2448	0.5	72	1.739	0.000	1.816	0.113	0.014	0.1585	0.195	0.000	0.888	0.019	0.000	-0.038	1
22-May	11:00	2544	0.5	96	1.739	0.000	1.816	0.1045	0.014	0.1265	0.178	0.000	0.691	0.036	0.000	0.061	1
26-May	11:00	2640	0.5	96	1.739	0.000	1.816	0.118	0.002	0.139	0.206	0.000	0.768	0.027	0.000	0.007	1
29-May	11:00	2712	0.5	72	1.739	0.000	1.816	0.106	0.0315	0.1315	0.543	0.000	0.722	-0.062	0.000	0.035	1
03-Jun	11:00	2832	0.5	120	1.739	0.000	1.816	0.095	0	0.134	0.475	0.000	0.737	0.037	0.000	0.020	1
05-Jun	11:00	2880	0.5	48	1.739	0.000	1.816	0.1145	0	0.1165	0.596	0.000	0.629	-0.022	0.000	0.066	1
10-Jun	11:00	3000	0.5	120	1.739	0.000	1.816	0.0975	0	0.1005	0.491	0.000	0.531	0.042	0.000	0.042	1
12-Jun	11:00	3048	0.5	48	1.739	0.000	1.816	0.1135	0.013	0.1275	0.589	0.000	0.697	-0.014	0.000	-0.041	1
22-Jun	11:00	3288	0.5	240	1.739	0.000	1.816	0.0925	0	0.091	0.460	0.000	0.472	0.037	0.000	0.045	1
26-Jun	11:00	3384	0.5	96	1.739	0.000	1.816	0.103	0.0095	0.1155	0.525	0.000	0.623	0.013	0.000	-0.004	1
03-Jul	11:00	3552	0.5	168	2.32	0.000	2.42	0.1135	0.0135	0.1255	0.589	0.000	0.685	0.029	0.000	0.029	1
17-Jul	11:00	3888	0.5	336	2.32	0.000	2.42	0.1245	0	0.0895	0.657	0.000	0.463	0.031	0.000	0.053	1
21-Jul	09:00	3984	0.5	96	2.32	0.000	2.42	0.122	0	0.104	0.642	0.000	0.552	0.038	0.000	0.022	1
31-Jul	09:00	4224	0.5	240	2.32	0.000	2.42	0.134	0	0.1005	0.716	0.000	0.531	0.028	0.000	0.041	1
17-Aug	09:00	4632	0.5	408	2.32	0.000	2.42	0.126	0	0.2295	0.666	0.000	1.326	0.037	0.000	-0.013	1
21-Aug	09:00	4728	0.5	96	2.32	0.000	2.42	0.13	0	0.1355	0.691	0.000	0.747	0.029	0.000	0.147	1
24-Aug	09:00	4800	0.5	72	2.32	0.000	2.42	0.126	0	0.085	0.666	0.000	0.435	0.041	0.000	0.121	1
28-Aug	09:00	4896	0.5	96	2.32	0.000	2.42	0.1275	0	0.0665	0.676	0.000	0.321	0.032	0.000	0.066	1
31-Aug	09:00	4968	0.5	168	2.32	0.000	2.42	0.1465	0	0.0825	0.793	0.000	0.420	0.019	0.000	0.031	1
04-Sep	09:00	5040	0.5	72	2.32	0.000	2.42	0.159	0	0.0775	0.870	0.000	0.389	0.010	0.000	0.050	1
14-Sep	09:00	5280	0.5	240	2.32	0.000	2.42	0.198	0	0.07	1.110	0.000	0.343	0.007	0.000	0.047	1
18-Sep	09:00	5376	0.5	96	2.32	0.000	2.42	0.1665	0	0.075	0.916	0.000	0.374	0.067	0.000	0.037	1
22-Sep	09:00	5472	0.5	96	2.32	0.000	2.42	0.141	0	0.087	0.759	0.000	0.448	0.063	0.000	0.027	1
25-Sep	09:00	5544	0.5	72	3.396	0.000	2.727	0.243	0.0115	0.0585	1.387	0.000	0.272	-0.120	0.000	0.096	1
30-Sep	09:00	5664	0.5	120	3.396	0.000	2.727	0.176	0.0205	0.0625	0.974	0.000	0.297	0.114	0.000	0.047	1
02-Oct	09:00	5712	0.5	48	3.774	0.000	3.030	0.1875	0	0.0385	1.045	0.000	0.149	0.030	0.000	0.117	1
06-Oct	09:00	5808	0.5	96	3.774	0.000	3.030	0.2245	0	0.0895	1.273	0.000	0.463	0.008	0.000	-0.007	1
09-Oct	09:00	5880	0.5	72	3.774	0.000	3.030	0.2195	0	0.08	1.242	0.000	0.404	0.061	0.000	0.070	1
13-Oct	09:00	5976	0.5	96	3.774	0.000	3.030	0.2465	0.0565	0.0735	1.409	0.000	0.364	0.017	0.000	0.063	1
16-Oct	09:00	6048	0.5	72	3.774	0.000	3.030	0.403	0.011	0.0705	2.372	0.000	0.346	-0.218	0.000	0.061	1
19-Oct	09:00	6120	0.5	72	3.774	0.000	3.030	0.239	0.052	0.1005	1.362	0.000	0.531	0.310	0.000	0.005	1
22-Oct	09:00	6192	0.5	72	3.774	0.000	3.030	0.2355	0.000	0.07125	1.341	0.000	0.350	0.056	0.000	0.102	1
26-Oct	09:00	6288	0.5	96	3.774	0.000	3.030	0.249	0.000	0.093	1.424	0.000	0.485	0.033	0.000	0.027	1
03-Nov	09:00	6480	0.5	192	3.774	0.000	3.030	0.291	0.000	0.0315	1.683	0.000	0.105	0.019	0.000	0.097	1
05-Nov	09:00	6528	0.5	48	3.774	0.000	3.030	0.2915	0.000	0.0875	1.686	0.000	0.451	0.042	0.000	-0.079	1
10-Nov	09:00	6768	0.5	240	3.774	0.000	3.030	0.266	0.000	0.0775	1.529	0.000	0.389	0.059	0.000	0.060	1

01-Dec	09:00	7272	0.5	504	3.774	0.000	3.030	0.296	0.030	0.0605	1.713	0.000	0.284	0.036	0.000	0.061	1
04-Dec	09:00	7344	0.5	72	3.774	0.000	3.030	0.331	0.000	0.103	1.929	0.000	0.546	-0.017	0.000	-0.016	1
09-Dec	09:00	7464	0.5	120	3.774	0.000	3.030	0.3015	0.000	0.089	1.747	0.000	0.460	0.070	0.000	0.067	1
14-Dec	09:00	7584	0.5	120	3.774	0.000	3.030	0.283	0.0245	0.0495	1.633	0.000	0.216	0.062	0.000	0.096	1
19-Dec	09:00	7704	0.5	120	3.774	0.000	3.030	0.303	0.1045	0.055	1.757	0.506	0.250	0.023	-0.088	0.053	1
21-Dec	09:00	7752	0.5	48	3.774	0.000	3.030	0.327	0.014	0.054	1.904	0.000	0.244	-0.018	0.195	0.060	1
24-Dec	09:00	7824	0.5	72	0.000	0.000	2.270	0.3475	0.027	0.0675	2.031	0.000	0.327	-0.075	0.000	0.019	1
28-Dec	09:00	7920	0.5	96	0.000	0.000	2.270	0.3435	0.034	0.019	2.006	0.000	0.028	-0.037	0.000	0.104	1
04-Jan	09:00	8088	0.5	168	0.000	0.000	2.270	0.324	0.0215	0.0505	1.886	0.000	0.223	-0.026	0.000	0.021	1
07-Jan	09:00	8160	0.5	72	0.000	0.000	2.270	0.327	0	0.0465	1.904	0.000	0.198	-0.044	0.000	0.050	1
14-Jan	09:00	8328	0.5	168	0.000	0.000	2.270	0.284	0.000	0.0515	1.639	0.000	0.229	-0.005	0.000	0.039	1
20-Jan	09:00	8472	0.5	144	0.000	0.000	2.270	0.22	0.000	0.0825	1.245	0.000	0.420	0.025	0.000	0.014	1
28-Jan	09:00	8664	0.5	192	0.000	0.000	2.270	0.226	0.000	0.046	1.282	0.000	0.195	-0.030	0.000	0.065	1
02-Feb	09:00	8784	0.5	120	0.000	0.000	2.270	0.195	0.000	0.0085	1.091	0.000	-0.036	0.007	0.000	0.084	1
03-Mar	09:00	9528	0.5	744	1.739	10.000	2.270	0	0.000	0.254	-0.018	0.000	1.477	0.064	0.208	-0.021	1
04-Apr	09:00	10248	0.5	720	1.739	10.000	2.270	0.012	0.000	0.035	-0.006	0.000	0.127	0.036	0.208	0.079	1
13-Apr	10:30	10466	0.5	218	1.739	10.000	2.270	0.014	0.000	0.1265	-0.004	0.000	0.691	0.036	0.208	-0.015	1
15-Apr	10:30	10514	0.5	48	1.739	10.000	2.270	0.0035	0.119	0.053	-0.015	0.000	0.645	0.041	0.208	0.052	1
22-Apr	09:00	10680	0.5	167	1.739	10.000	2.270	0.2355	0.079	0.024	0.223	0.000	0.398	0.005	0.208	0.066	1
26-Apr	10:30	10778	0.5	98	1.739	10.000	2.270	0.2395	0.1015	0.0355	0.228	0.000	0.537	0.031	0.208	0.010	1
30-Apr	15:00	10878	0.5	101	1.739	10.000	2.270	0.3255	0.0555	0.1535	0.316	0.792	0.253	0.013	0.046	0.094	1
05-May	09:00	10992	0.5	114	1.739	10.000	2.270	0.458	0.0315	0.0785	0.452	0.000	0.105	0.005	0.337	0.069	1
10-May	12:00	11115	0.5	123	1.739	10.000	2.270	0.5255	0.037	0.1345	0.521	0.681	0.139	0.015	0.092	0.039	1
14-May	12:00	11211	0.5	96	1.739	10.000	2.270	0.5485	0.0665	0.1375	0.545	0.698	0.321	0.020	0.190	0.006	1
20-May	12:00	11355	0.5	144	1.739	10.000	2.270	0.6555	0.0485	0.0115	0.655	0.000	0.210	0.008	0.298	0.057	1
24-May	15:00	11454	0.5	99	1.739	10.000	2.270	0.7065	0.0275	0.0915	0.707	0.430	0.081	0.012	0.119	0.070	1
28-May	14:00	11549	0.5	95	0	5.000	2.270	0.8865	0.049	0.481	0.892	2.706	0.213	-0.055	-0.396	0.017	1
08-Jun	11:00	11810	0.5	261	0	5.000	2.270	0.8245	0.004	0.368	0.828	2.046	-0.064	-0.013	0.108	0.068	1
16-Jun	11:00	12002	0.5	192	0	5.000	2.270	0.8415	0.0345	0.1545	0.846	0.798	0.124	-0.019	0.208	0.027	1
23-Jun	09:00	12168	0.5	166	0	5.000	2.270	1.2875	0.088	0.1295	1.303	0.652	0.454	-0.078	0.107	0.001	1
08-Jul	09:00	12528	0.5	360	0	0.000	2.270	0.7675	0.0925	0.0065	0.770	0.000	0.482	0.011	0.033	0.036	1
19-Jul	09:00	12792	0.5	264	0	0.000	2.270	0.7915	0	0.024	0.794	0.000	-0.089	-0.018	0.000	0.089	1
27-Jul	09:00	12984	0.5	192	0	0.000	2.270	0.7195	0	0.0125	0.720	0.000	-0.089	-0.008	0.000	0.049	1
03-Aug	09:00	13392	0.5	408	0	0.000	2.270	0.841	0	0.0175	0.845	0.000	-0.089	-0.023	0.000	0.049	1
12-Aug	09:00	13608	0.5	216	0	0.000	2.270	0.801	0	0	0.804	0.000	-0.089	-0.013	0.000	0.049	1
29-Aug	09:00	14016	0.5	408	0	0.000	2.270	0.3915	0	0	0.384	0.000	-0.089	0.011	0.000	0.049	1
14-Sep	09:00	14400	0.5	384	0	0.000	2.270	0.101	0	0	0.085	0.000	-0.089	0.013	0.000	0.049	1

REACTOR 3 17.0

Date	Time	T	Q	Δt	Influent Conc. (mM)			Abs Ave (A)			Conc. Ave (mM)			Removal (mmol/hr)			toxic N
	hh:mm	h	l/d	hr	Nitrite	Nitrate	Amm	Nitrite	Nitrate	Amm	Nitrite	Nitrate	Amm	Nitrite	Nitrate	Amm	mmol NO2
17-Feb	11:00	0	0.5	0	1.739	1.765	1.816	0.823	0.162	0.158	1.652	0.842	0.882	undefined	undefined	undefined	1
20-Feb	11:00	72	0.5	72	1.739	1.765	1.816	0.166	0.096	0.153	0.304	0.453	0.855	0.348	0.119	0.027	1
23-Feb	11:00	144	0.5	72	1.739	1.765	1.816	0.346	0.038	0.140	0.673	0.117	0.774	-0.065	0.114	0.041	1
27-Feb	11:00	240	0.5	96	1.739	0.000	1.816	0.167	0.025	0.184	0.306	0.041	1.043	0.095	0.013	-0.031	1
02-Mar	11:00	312	0.5	72	1.739	0.000	1.816	0.348	0.089	0.190	0.677	0.415	1.080	-0.065	-0.097	0.007	1
06-Mar	11:00	408	0.5	96	1.739	0.000	1.816	0.211	0.019	0.221	0.396	0.006	1.274	0.078	0.072	-0.023	1
16-Mar	11:00	648	0.5	240	1.739	0.000	1.816	0.157	0.072	0.278	0.286	0.316	1.625	0.038	-0.029	-0.021	1
20-Mar	11:00	744	0.5	96	1.739	0.000	1.816	0.315	0.091	0.279	0.609	0.427	1.631	-0.034	-0.029	0.003	1
24-Mar	11:00	840	0.5	96	1.739	0.000	1.816	0.163	0.020	0.267	0.298	0.009	1.554	0.085	0.074	0.019	1
27-Mar	11:00	912	0.5	72	1.739	0.000	1.816	0.159	0.004	0.261	0.289	-0.082	1.517	0.032	0.023	0.015	1
30-Mar	11:00	984	0.5	72	1.739	0.000	1.816	0.185	0.013	0.282	0.342	-0.029	1.650	0.016	-0.012	-0.028	1
03-Apr	13:00	1082	0.5	98	1.739	0.000	1.816	0.155	0.0205	0.261	0.282	0.015	1.520	0.041	-0.008	0.029	1
07-Apr	13:00	1178	0.5	96	1.739	0.000	1.816	0.1555	0.0125	0.253	0.283	-0.032	1.471	0.030	0.009	0.016	1
14-Apr	13:00	1346	0.5	168	1.739	0.000	1.816	0.158	0.033	0.2515	0.288	0.088	1.462	0.030	-0.014	0.008	1
21-Apr	13:00	1514	0.5	168	1.739	0.000	1.816	0.1515	0.018	0.304	0.274	0.000	1.785	0.032	0.009	-0.032	1
24-Apr	11:00	1586	0.5	72	1.739	0.000	1.816	0.165	0.033	0.267	0.302	0.085	1.557	0.023	-0.022	0.059	1
04-May	11:00	1826	0.5	240	1.739	0.000	1.816	0.155	0.005	0.253	0.282	-0.076	1.471	0.032	0.013	0.013	1
08-May	11:00	1922	0.5	96	1.739	0.000	1.816	0.168	0.002	0.289	0.307	-0.094	1.690	0.025	0.005	-0.036	1
11-May	11:00	1994	0.5	72	1.739	0.000	1.816	0.214	0.002	0.213	0.403	-0.094	1.224	0.005	0.002	0.122	1
15-May	11:00	2090	0.5	96	1.739	0.000	1.816	0.1895	0.000	0.2165	0.352	-0.105	1.246	0.038	0.004	0.008	1
18-May	11:00	2162	0.5	72	1.739	0.000	1.816	0.209	0.000	0.2445	0.393	-0.105	1.419	0.019	0.002	-0.032	1
22-May	11:00	2258	0.5	96	1.739	0.000	1.816	0.2235	0.000	0.2165	0.422	-0.105	1.246	0.022	0.002	0.042	1
26-May	11:00	2354	0.5	96	1.739	0.000	1.816	0.374	0.0305	0.2205	0.731	0.073	1.271	-0.034	-0.033	0.007	1
29-May	11:00	2426	0.5	72	1.739	0.000	1.816	0.1695	0.0215	0.226	0.934	0.020	1.305	-0.031	0.012	0.003	1
03-Jun	11:00	2546	0.5	120	1.739	0.000	1.816	0.1615	0.002	0.222	0.885	-0.094	1.280	0.025	0.018	0.015	1
05-Jun	11:00	2594	0.5	48	1.739	0.000	1.816	0.197	0.002	0.2125	1.104	-0.094	1.221	-0.064	0.002	0.033	1
10-Jun	11:00	2714	0.5	120	1.739	0.000	1.816	0.178	0.001	0.2125	0.987	-0.099	1.221	0.032	0.003	0.012	1
12-Jun	11:00	2762	0.5	48	1.739	0.000	1.816	0.146	0.003	0.222	0.790	-0.088	1.280	0.090	-0.002	-0.010	1
22-Jun	11:00	3002	0.5	240	1.739	0.000	1.816	0.159	0.003	0.2365	0.870	-0.091	1.369	0.012	0.002	0.003	1
26-Jun	11:00	3098	0.5	96	1.739	0.000	1.816	0.1555	0	0.219	0.848	-0.105	1.261	0.022	0.005	0.031	1
03-Jul	11:00	3266	0.5	168	2.320	0.000	2.420	0.1695	0.000	0.2095	0.934	-0.105	1.203	0.020	0.002	0.031	1
17-Jul	11:00	3602	0.5	336	2.320	0.000	2.420	0.16	0.000	0.1295	0.876	-0.105	0.710	0.033	0.002	0.061	1
21-Jul	09:00	3698	0.5	96	2.320	0.000	2.420	0.1675	0.000	0.214	0.922	-0.105	1.231	0.021	0.002	-0.067	1
31-Jul	09:00	3938	0.5	240	2.320	0.000	2.420	0.1725	0.000	0.2305	0.953	-0.105	1.332	0.026	0.002	0.015	1
17-Aug	09:00	4346	0.5	408	2.320	0.000	2.420	0.2285	0.000	0.2295	1.298	-0.105	1.326	0.007	0.002	0.023	1
21-Aug	09:00	4442	0.5	96	2.320	0.000	2.420	0.1665	0.000	0.2665	0.916	-0.105	1.554	0.097	0.002	-0.022	1
24-Aug	09:00	4514	0.5	72	2.320	0.000	2.420	0.1815	0.000	0.2375	1.008	-0.105	1.375	0.006	0.002	0.064	1
28-Aug	09:00	4610	0.5	96	2.320	0.000	2.420	0.1835	0.000	0.2265	1.021	-0.105	1.308	0.025	0.002	0.035	1
31-Aug	09:00	4682	0.5	72	2.320	0.000	2.420	0.188	0.000	0.244	1.048	-0.105	1.416	0.020	0.002	-0.005	1
04-Sep	09:00	4778	0.5	96	2.320	0.000	2.420	0.1575	0.000	0.2395	0.860	-0.105	1.388	0.064	0.002	0.026	1
14-Sep	09:00	5018	0.5	240	2.320	0.000	2.420	0.2035	0.000	0.2785	1.144	-0.105	1.628	0.004	0.002	-0.001	1
18-Sep	09:00	5114	0.5	96	2.32	0.000	2.42	0.23	0	0.181	1.307	-0.105	1.027	-0.008	0.002	0.135	1
22-Sep	09:00	5210	0.5	96	2.32	0.000	2.42	0.192	0	0.187	1.073	-0.105	1.064	0.067	0.002	0.022	1
25-Sep	09:00	5282	0.5	72	3.396	0.000	2.727	0.2825	0	0.22	1.630	-0.105	1.268	-0.095	0.002	-0.018	1
30-Sep	09:00	5402	0.5	120	3.396	0.000	2.727	0.2405	0.021	0.146	1.372	0.018	0.811	0.079	-0.018	0.105	1
02-Oct	09:00	5450	0.5	48	3.774	0.000	3.030	0.2505	0	0.1405	1.433	-0.105	0.777	0.027	0.046	0.059	1
06-Oct	09:00	5546	0.5	96	3.774	0.000	3.030	0.2715	0	0.166	1.562	-0.105	0.935	0.023	0.002	0.016	1
09-Oct	09:00	5618	0.5	72	3.774	0.000	3.030	0.2715	0	0.2255	1.562	-0.105	1.301	0.046	0.002	-0.051	1
13-Oct	09:00	5714	0.5	96	3.774	0.000	3.030	0.277	0	0.1875	1.596	-0.105	1.067	0.039	0.002	0.082	1
16-Oct	09:00	5786	0.5	72	3.774	0.000	3.030	0.383	0	0.173	2.249	-0.105	0.978	-0.122	0.002	0.064	1
19-Oct	09:00	5858	0.5	72	3.774	0.000	3.030	0.2995	0.000	0.162	1.735	-0.105	0.910	0.164	0.002	0.060	1
22-Oct	09:00	5930	0.5	72	3.774	0.000	3.030	0.3245	0.000	0.171	1.889	-0.105	0.965	0.003	0.002	0.030	1
26-Oct	09:00	6026	0.5	96	3.774	0.000	3.030	0.3345	0.000	0.1555	1.951	-0.105	0.870	0.027	0.002	0.062	1
03-Nov	09:00	6218	0.5	192	3.774	0.000	3.030	0.3425	0.000	0.116	2.000	-0.105	0.626	0.033	0.002	0.072	1
05-Nov	09:00	6266	0.5	48	3.774	0.000	3.030	0.332	0.000	0.1795	1.935	-0.105	1.018	0.061	0.002	-0.097	1
10-Nov	09:00	6506	0.5	240	3.774	0.000	3.030	0.3015	0.000	0.2035	1.747	-0.105	1.166	0.056	0.002	0.028	1
01-Dec	09:00	7010	0.5	504	3.774	0.000	3.030	0.2995	0.000	0.159	1.735	-0.105	0.891	0.043	0.002	0.054	1
04-Dec	09:00	7082	0.5	72	3.774	0.000	3.030	0.3635	0.000	0.213	2.129	-0.105	1.224	-0.059	0.002	-0.041	1
09-Dec	09:00	7202	0.5	120	3.774	0.000	3.030	0.2665	0.000	0.2725	1.532	-0.105	1.591	0.131	0.002	-0.022	1

14-Dec	09:00	7322	0.5	120	3.774	0.000	3.030	0.2865	0.000	0.179	1.655	-0.105	1.015	0.027	0.002	0.124	1
19-Dec	09:00	7442	0.5	120	3.774	0.000	3.030	0.3105	0.000	0.187	1.803	-0.105	1.064	0.020	0.002	0.034	1
21-Dec	09:00	7490	0.5	48	3.774	0.000	3.030	0.3185	0.000	0.1975	1.852	-0.105	1.129	0.023	0.002	0.017	1
24-Dec	09:00	7562	0.5	72	0.000	0.000	0.000	0.3505	0.000	0.2135	2.049	-0.105	1.227	-0.089	0.002	-0.049	1
28-Dec	09:00	7658	0.5	96	0.000	0.000	0.000	0.342	0.000	0.1995	1.997	-0.105	1.141	-0.032	0.002	-0.008	1
04-Jan	09:00	7826	0.5	168	0.000	0.000	0.000	0.3145	0.000	0.17	1.827	-0.105	0.959	-0.021	0.002	-0.002	1
07-Jan	09:00	7898	0.5	72	0.000	0.000	0.000	0.342	0.000	0.1895	1.997	-0.105	1.080	-0.082	0.002	-0.051	1
14-Jan	09:00	8066	0.5	168	0.000	0.000	0.000	0.2915	0.000	0.18855	1.686	-0.105	1.074	-0.004	0.002	-0.022	1
20-Jan	09:00	8210	0.5	144	0.000	0.000	0.000	0.1185	0.000	0.1855	0.620	-0.105	1.055	0.113	0.002	-0.020	1
28-Jan	09:00	8402	0.5	192	0.000	0.000	0.000	0.19	0.000	0.134	1.061	-0.105	0.737	-0.061	0.002	0.013	1
02-Feb	09:00	8522	0.5	120	0.000	0.000	0.000	0.1375	0.000	0.112	0.737	-0.105	0.602	0.030	0.002	0.007	1
03-Mar	09:00	9266	0.5	744	1.739	0.000	2.270	0.5565	0.000	0.304	0.553	-0.105	1.785	0.029	0.002	-0.017	1
04-Apr	09:00	9986	0.5	720	1.739	0.000	2.270	0.0155	0.0965	0.1405	-0.002	0.459	0.777	0.049	-0.023	0.055	1
13-Apr	10:30	10204	0.5	218	1.739	0.000	2.270	0.0195	0.000	0.1335	0.002	-0.105	0.734	0.036	0.046	0.035	1
15-Apr	10:30	10252	0.5	48	1.739	0.000	2.270	0.048	0.000	0.3725	0.031	-0.105	2.208	0.025	0.002	-0.521	1
22-Apr	09:00	10418	0.5	167	1.739	0.000	2.270	0	0.000	0.136	-0.018	-0.105	0.750	0.042	0.002	0.181	1
26-Apr	10:30	10516	0.5	98	1.739	0.000	2.270	0	0.045	0.0965	-0.018	0.158	0.506	0.037	-0.049	0.079	1
30-Apr	15:00	10616	0.5	101	1.739	0.000	2.270	0.000	0.0145	0.0915	-0.018	-0.020	0.475	0.037	0.031	0.043	1
05-May	09:00	10730	0.5	114	1.739	0.000	2.270	0.0595	0.0115	0.1075	0.043	-0.038	0.574	0.026	0.003	0.021	1
10-May	12:00	10853	0.5	123	1.739	0.000	2.270	0.0265	0.0355	0.037	0.009	0.102	0.139	0.041	-0.022	0.104	1
14-May	12:00	10949	0.5	96	1.739	0.000	2.270	0.007	0.007	0.1185	-0.011	-0.064	0.642	0.040	0.031	-0.055	1
20-May	12:00	11093	0.5	144	2.320	5.000	2.270	0.0005	0	0.1045	-0.018	-0.105	0.555	0.049	0.111	0.046	1
24-May	15:00	11192	0.5	99	2.320	5.000	2.270	0.0095	0.0305	0.124	-0.009	0.073	0.676	0.047	0.072	0.013	1
28-May	14:00	11287	0.5	95	2.320	5.000	2.270	0.327	0.3305	0.167	0.317	1.826	0.941	-0.017	-0.248	-0.020	1
08-Jun	11:00	11548	0.5	261	2.320	5.000	2.270	0.5505	0.0465	0.0775	0.547	0.167	0.389	0.022	0.209	0.075	1
16-Jun	11:00	11740	0.5	192	2.320	5.000	2.270	1.144	0	0.0565	1.156	-0.105	0.260	-0.030	0.130	0.053	1
23-Jun	09:00	11906	0.5	166	0.000	0.000	2.270	0.6665	0	0.115	0.666	-0.105	0.620	0.036	0.002	-0.003	1
08-Jul	09:00	12266	0.5	360	0.000	0.000	2.270	0.3115	0	0.077	0.301	-0.105	0.386	0.011	0.002	0.050	1
19-Jul	09:00	12530	0.5	264	0.000	0.000	2.270	0.5175	0	0.038	0.513	-0.105	0.145	-0.024	0.002	0.060	1
27-Jul	09:00	12722	0.5	192	0.000	0.000	2.270	0.4775	0	0.1325	0.472	-0.105	0.728	-0.006	0.002	-0.019	1
03-Aug	09:00	12890	0.5	168	0.000	0.000	2.270	0.468	0	0.0985	0.462	-0.105	0.518	-0.009	0.002	0.058	1
12-Aug	09:00	13106	0.5	216	0.000	0.000	2.270	0.4685	0	0	0.463	-0.105	-0.089	-0.010	0.002	0.097	1
29-Aug	09:00	13514	0.5	408	0.000	0.000	2.270	0.3865	0	0	0.378	-0.105	-0.089	-0.004	0.002	0.049	1
14-Sep	09:00	13898	0.5	384	0.000	0.000	2.270	0.061	0	0	0.044	-0.105	-0.089	0.014	0.002	0.049	1

REACTOR 4 17.0

Date	Time	T	Q	Δt	Influent Conc. (mM)			Abs Ave (A)			Conc. Ave (mM)			Removal (mmol/hr)			toxic N
	hh:mm	h	l/d	hr	Nitrite	Nitrate	Amm	Nitrite	Nitrate	Amm	Nitrite	Nitrate	Amm	Nitrite	Nitrate	Amm	mmol NO2
17-Aug	09:00	0	0.75	0	1.739	1.764	2.270	0.188	0.0655	0.2265	1.048234	0.277615	1.307645	undefined	undefined	undefined	1
21-Aug	09:00	96	0.75	96	1.739	0.000	2.270	0.218	0.0935	0.2635	1.232999	0.441262	1.535758	-0.016906	-0.042769	-0.01745	1
24-Aug	09:00	168	0.75	72	1.739	0.000	2.270	0.203	0.0985	0.223	1.140616	0.470485	1.286067	0.040512	-0.021602	0.089703	1
28-Aug	09:00	264	0.75	96	1.739	0.000	2.270	0.156	0.049	0.166	0.851151	0.181181	0.934649	0.079005	0.045569	0.10396	1
31-Aug	09:00	336	0.75	72	1.739	0.000	2.270	0.2135	0.0945	0.244	1.205284	0.447107	1.415536	-0.066936	-0.07676	-0.086841	1
04-Sep	09:00	432	0.75	96	1.739	0.000	2.270	0.2035	0.078	0.248	1.143696	0.350672	1.440197	0.02951	0.006118	0.021564	1
14-Sep	09:00	672	0.75	240	1.739	0.000	2.270	0.2035	0.0225	0.2785	1.143696	0.0263	1.628237	0.018603	0.022154	0.006736	1
18-Sep	09:00	768	0.5	96	2.32	0.000	2.42	0.192	0.0595	0.2485	1.072869	0.242548	1.44328	0.038524	-0.043347	0.053101	1
22-Sep	09:00	864	0.5	96	2.32	0.000	2.42	0.1665	0.0515	0.2485	0.915819	0.195792	1.44328	0.057065	0.004201	0.020348	1
25-Sep	09:00	936	0.5	72	3.396	0.000	2.727	0.211	0.0515	0.2725	1.189887	0.195792	1.591245	-0.01875	-0.004079	-0.011275	1
30-Sep	09:00	1056	0.5	120	3.396	0.000	2.727	0.2135	0.0425	0.2435	1.205284	0.143191	1.412454	0.043459	0.004469	0.052715	1
02-Oct	09:00	1104	0.5	48	3.774	0.000	3.030	0.238	0	0.303	1.356176	-0.105202	1.779285	-0.003069	0.090164	-0.103863	1
06-Oct	09:00	1200	0.5	96	3.774	0.000	3.030	0.183	0	0.297	1.01744	-0.105202	1.742293	0.117413	0.002192	0.033378	1
09-Oct	09:00	1272	0.5	72	3.774	0.000	3.030	0.271	0	0.422	1.559417	-0.105202	2.512947	-0.08183	0.002192	-0.171188	1
13-Oct	09:00	1368	0.5	96	3.774	0.000	3.030	0.258	0.042	0.288	1.479353	0.140269	1.686806	0.061983	-0.046391	0.174279	1
16-Oct	09:00	1440	0.5	72	3.774	0.000	3.030	0.2855	0.0125	0.2855	1.648721	-0.032145	1.671393	0.004287	0.041378	0.031944	1
19-Oct	09:00	1512	0.5	72	3.774	0.000	3.030	0.272	0.038	0.2875	1.565576	0.116891	1.683724	0.06564	-0.037624	0.025136	1
22-Oct	09:00	1584	0.5	72	3.774	0.000	3.030	0.312	0.073	0.286	1.81193	0.321449	1.674476	-0.01729	-0.054995	0.030424	1
26-Oct	09:00	1680	0.5	96	3.774	0.000	3.030	0.3295	0.016	0.2805	1.919709	-0.011689	1.640567	0.019545	0.059237	0.034951	1
03-Nov	09:00	1872	0.5	192	3.774	0.000	3.030	0.1847	0.000	0.1995	1.02791	-0.105202	1.141184	0.136172	0.010471	0.083567	1
05-Nov	09:00	1920	0.5	48	3.774	0.000	3.030	0.3495	0	0.303	2.042886	-0.105202	1.779285	-0.323406	0.002192	-0.199938	1
10-Nov	09:00	2160	0.5	240	3.774	0.000	3.030	0.328	0.000	0.326	1.910471	-0.105202	1.921085	0.048203	0.002192	0.013058	1
01-Dec	09:00	2664	0.5	504	3.774	0.000	3.030	0.3295	0.000	0.278	1.919709	-0.105202	1.625154	0.038319	0.002192	0.039249	1
04-Dec	09:00	2736	0.5	72	3.774	0.000	3.030	0.3545	0.000	0.3165	2.07368	-0.105202	1.862515	-0.000931	0.002192	-0.031721	1
09-Dec	09:00	2856	0.5	120	3.774	0.000	3.030	0.2775	0.000	0.4475	1.59945	-0.105202	2.67016	0.112486	0.002192	-0.10692	1
14-Dec	09:00	2976	0.5	120	3.774	0.000	3.030	0.3235	0.000	0.281	1.882756	-0.105202	1.64365	-0.000734	0.002192	0.174305	1
19-Dec	09:00	3096	0.5	120	3.774	0.000	3.030	0.3295	0.037	0.3385	1.919709	0.111046	1.99815	0.033396	-0.032949	-0.028724	1
21-Dec	09:00	3144	0.5	48	3.774	0.000	3.030	0.362	0.000	0.3425	2.119871	-0.105202	2.022811	-0.03643	0.078779	0.012249	1
24-Dec	09:00	3216	0.5	72	0.000	0.000	0.000	0.3905	0.000	0.347	2.295398	-0.105202	2.050555	-0.089265	0.002192	-0.04927	1
28-Dec	09:00	3312	0.5	96	0.000	0.000	0.000	0.37	0.000	0.2935	2.169142	-0.105202	1.720715	-0.022833	0.002192	0.022561	1
04-Jan	09:00	3480	0.5	168	0.000	0.000	0.000	0.3625	0.0215	0.3115	2.122951	0.020456	1.831689	-0.039554	-0.013142	-0.04939	1
07-Jan	09:00	3552	0.5	72	0.000	0.000	0.000	0.392	0.000	0.296	2.304637	-0.105202	1.736128	-0.090911	0.031861	-0.013606	1
14-Jan	09:00	3720	0.5	168	0.000	0.000	0.000	0.3515	0.000	0.293	2.055204	-0.105202	1.717633	-0.017577	0.002192	-0.033912	1
20-Jan	09:00	3864	0.5	144	0.000	0.000	0.000	0.28	0.000	0.2855	1.614847	-0.105202	1.671393	0.018344	0.002192	-0.029362	1
28-Jan	09:00	4056	0.5	192	0.000	0.000	0.000	0.2365	0.000	0.232	1.346938	-0.105202	1.341554	-0.00434	0.002192	0.001256	1
02-Feb	09:00	4176	0.5	120	0.000	0.000	0.000	0.2615	0.000	0.1595	1.500908	-0.105202	0.894575	-0.053081	0.002192	0.044685	1
03-Mar	09:00	4920	0.5	744	1.739	0.000	2.270	0.3795	0.000	0.1695	0.371275	-0.105202	0.956227	0.054306	0.002192	0.025962	1
04-Apr	09:00	5640	0.5	720	1.739	0.000	2.270	0.1475	0.0875	0.145	0.133134	0.406195	0.805179	0.039078	-0.020537	0.034084	1
13-Apr	10:30	5858	0.5	218	1.739	0.000	2.270	0.089	0.000	0.292	0.073085	-0.105202	1.711467	0.0394	0.042163	-0.0592	1
15-Apr	10:30	5906	0.5	48	1.739	0.000	2.270	0.0695	0.0825	0.2825	0.053069	0.376973	1.652898	0.042213	-0.178624	0.0336	1
22-Apr	09:00	6072	0.5	167	1.739	0.000	2.270	0	0	0.231	-0.018271	-0.105202	1.335388	0.043894	0.051423	0.051889	1
26-Apr	10:30	6170	0.5	98	1.739	0.000	2.270	0.123	0.000	0.2365	0.107985	-0.105202	1.369297	0.011966	0.002192	0.012852	1
30-Apr	15:00	6270	0.5	101	1.739	0.000	2.270	0.091	0.0535	0.281	0.075138	0.207481	1.64365	0.04022	-0.057214	-0.033359	1
05-May	09:00	6384	0.5	114	1.739	0.000	2.270	0.0655	0.0095	0.318	0.048963	-0.049679	1.871763	0.039112	0.039383	-0.02572	1
10-May	12:00	6507	0.5	123	1.739	0.000	2.270	0.081	0.0435	0.2225	0.064873	0.149036	1.282984	0.032679	-0.030569	0.101939	1
14-May	12:00	6603	0.5	96	1.739	0.000	2.270	0.191	0.0025	0.1995	0.177785	-0.09059	1.141184	0.01253	0.044321	0.048627	1
20-May	12:00	6747	0.5	144	2.320	5.000	2.270	0.204	0.0055	0.2575	0.191129	-0.073057	1.498767	0.042776	0.103619	-0.026147	1
24-May	15:00	6846	0.5	99	2.320	5.000	2.270	0.2375	0.308	0.5875	0.225516	1.694915	3.533292	0.03773	-0.234735	-0.375682	1
28-May	14:00	6941	0.5	95	2.320	5.000	2.270	0.126	1.086	0.257	0.111064	6.241964	1.495684	0.0665	-0.839557	0.380756	1
08-Jun	11:00	7202	0.5	261	2.320	5.000	2.270	0.383	0.038	0.1375	0.374868	0.116891	0.75894	0.023341	0.500683	0.079468	1
16-Jun	11:00	7394	0.5	192	2.320	5.000	2.270	0.6305	0	0.2875	0.62892	-0.105202	1.683724	0.012737	0.126023	-0.069668	1
23-Jun	09:00	7560	0.5	166	2.320	5.000	2.270	0.8115	0	0.246	0.814711	-0.105202	1.427867	0.012333	0.106358	0.043747	1
08-Jul	09:00	7920	0.5	360	0.000	0.000	2.270	0.3875	0	0.2775	0.379487	-0.105202	1.622072	0.012646	0.002192	0.004328	1
19-Jul	09:00	8184	0.5	264	0.000	0.000	2.270	0.258	0	0.289	0.246559	-0.105202	1.692972	0.003423	0.002192	0.007456	1
27-Jul	09:00	8376	0.5	192	0.000	0.000	2.270	0.3935	0	0.275	0.385646	-0.105202	1.606658	-0.020349	0.002192	0.021462	1
03-Aug	09:00	8544	0.5	168	0.000	0.000	2.270	0.421	0	0.482	0.413874	-0.105202	2.882861	-0.011479	0.002192	-0.141907	1
12-Aug	09:00	8760	0.5	216	0.000	0.000	2.270	0.4735	0	0.289	0.467764	-0.105202	1.692972	-0.013986	0.002192	0.10567	1
29-Aug	09:00	9168	0.5	408	0.000	0.000	2.270	0.2295	0	0	0.217304	-0.105202	-0.088779	0.005909	0.002192	0.123381	1
14-Sep	09:00	9552	0.5	384	0.000	0.000	2.270	0.133	0	0	0.11825	-0.105202	-0.088779	0.001922	0.002192	0.049141	1