A UTILITIES' GUIDE TO STARTING UP ANAMMOX

Octavio Perez-Garcia (Watercare Services Ltd), Filomena Ng (Watercare Services Ltd), Naresh Singhal (The University of Auckland) and Paul Bickers (Watercare Services Ltd)

ABSTRACT

The anammox (anaerobic ammonium oxidation) process has been one of the most innovative developments in biological wastewater treatment in recent years. With its discovery in the 1990s a completely new way of ammonium removal from wastewater has become available. Side-stream deammonification, a process based on anammox activity, can significantly lower the energy consumption and overall operation costs related to the removal of high loads of ammonium nitrogen (~1000 mg-N/L) from biosolids dewatering centrate. The application of Main-Stream Deammonification is likely to be the next biological innovation in the wastewater industry. Watercare has been evaluating the feasibility and benefits of incorporating these processes and have started the development of a viable New Zealand anammox 'inoculum' for wider use. Anammox based technologies have yet to be implemented in New Zealand. One constraint has been the non-availability of anammox cultures to seed full-scale reactors. Biosecurity restrictions by EPA and MPI (Environmental Protection Agency and the Ministry of Primary Industry) have complicated the importation of anammox enrichments from overseas. Watercare have set about the task of developing a culture of our own indigenous anammox biomass, using a combination of molecular biology and bioprocess control techniques. The first step in this process was the confirmation of the presence of small quantities of anammox bacteria in activated sludge samples from the Mangere and Rosedale WWTPs using high-throughput meta-genomics and qPCR techniques. The analysis showed that, although in small quantities, anammox bacteria are present in the activated sludge and mesophillic anaerobic digester reactors at both of these plants.

The second phase of work involved the development of laboratory scale reactors to isolate and enrich the anammox microbes. The bioreactors were seeded with sludge from the activated sludge RAS streams at the Mangere and the Rosedale WWTPs. Ammonium depletion under complete anaerobic conditions started after 110 days of reactor operation. After more than 270 days of continuous operation the bioreactors developed sufficient quantities of anammox culture to take the process 'out of the lab'. So far the ammonium removal rates are in the range of 15-20 mg-N L^{-1} d^{-1}. These rates need to be increased four times (e.g. to achive 100 mg-N L^{-1} d^{-1} or 0.1 kg-N m^{-3} d^{-1}) before the activity would be sufficient for the next stage of 'pilot scale sidestream treatment'. Much of the technical development has focussed on staged scaling up of cultures and optimizing influents characteristics to build-up more anammox biomass and simultaneously increase ammonium removal rates. Pilot scale facilities are currently under design and will be established at the Mangere WWTP 'Wastewater Innovation Centre'.

KEYWORDS

anammox bacteria enrichment, bioreactor design, metagenomics and qPCR, sidestream deammonification, process scale-up

PRESENTER PROFILE

Octavio develops and implements technology to recover resources from waste. He holds Bachelors, Master's and PhD degrees in Science, Biotechnology and Engineering. Author of 32 publications in international journals with more than a 1000 citations, he is currently leading Watercare's biochemistry optimization programme and managing the Wastewater Innovation Centre.

1 INTRODUCTION

Watercare is in the process of evaluating the feasibility and benefits of incorporating deammonification processes at the Mangere and the Rosedale wastewater treatment plants (WWTP). The inclusion of these processes would significantly lower the aeration requirements and overall operational costs. The deammonification process requires that active anammox microbes be present to co-convert ammonium (NH_4^+) and nitrite (NO_2^-) to di-nitrogen (N_2) gas in a single step (Figure 1, Lackner et al, 2014). Enriched cultures of active anammox microbes that could be used as seed to start up the deammonification process are not available in New Zealand. Moreover biosecurity restrictions by EPA and MPI (Environmental Protection Agency and the Ministry of Primary Industry) complicate the importation of anammox enrichments from overseas as pure anammox cultures do not exist. It has therefore become necessary to develop sufficient biomass to seed a full scale deammonification process from first principles.

Watercare have set about the task of developing a culture of our own indigenous anammox microbes, using a combination of molecular biology and bioprocess control techniques. This objective is part of our long term programme to implement New Zealand's first full-scale deammonification reactor for sidestream treatment as summarized in Table 1. This paper covers the methods and results obtained for the first two phases of this programme.

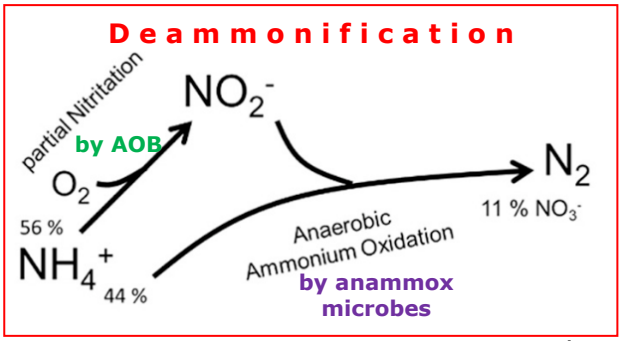


Figure 1: Anammox reaction and microbes as part of the deammonification process

Table 1: Overview of Watercare's deammonification programme, phases and objectives

Phase	Objective	Status up to mid-2018
1	GENETIC DETECTION OF ANAMMOX MICROBES IN WATERCARE's WASTEWATER RESOURCES	**Achieved**
2	ESTABLISHMENT AND ENRICHMENT OF ANAMMOX MICROBES IN LABORATORY BIOREACTORS	**Achieved**
3	SCALE-UP AND ASSESSMENT FOR ACTIVITY/INHIBITION OF DEAMMONIFYING PROCESS	**In progress**
4	ANALYSIS OF PROCESS DESIGN AND TECHNO-ECONOMICS THROUGH PILOT SCALE TRIALS	To start in early 2019
5	PROCUREMENT AND CONSTRUCTION OF A FULL SCALE DEAMMONIFICATION UNIT AT MANGERE WWTP	To start 2020
6	COMMISSIONING AND OPERATION OF FULL SCALE UNIT	Before beginning 2025

2 GENETIC DETECTION OF ANAMMOX MICROBES IN OUR WASTEWATER

2.1 SAMPLING OF SLUDGE AND MIXED LIQUOR

A key first step in the programme of work was to determine whether Watercares wastewater treatment plants contained indigenous anammox microbes sufficient to act as a seed for further intensification. A number of mixed liquor samples were collected from the Mangere and Rosedale treatment plants. Sixty samples were collected in total from both plants (10 from Rosedale and 50 from Mangere). Each sample consisted of 40 mL of sludge collected from mixed liquor or clarified sludge. After collection, samples were immediately placed in a cooler with icepacks and transported to the Environmental Engineering Lab at the University of Auckland. Once there, the samples were stored at -20°C until further processing.

2.2 DNA EXTRACTION FROM SAMPLES

Sludge samples were centrifuged at 5000 rpm for 5 minutes, supernatant was discharged, and then 0.25 g of pelletized sludge was used as starting material for DNA extraction. Total genomic DNA was extracted using the standard protocol of the PowerSoil DNA isolation kit (MoBio, Carlsbad, USA). Quantification of extracted DNA purity was done spectrophotometrically using a NanoPhotometer N60 (Thermo Fisher Scientific, USA) at A260/280nm wavelength ratio. Sample's DNA concentration was further confirmed using the Qubit Assay (Qubit 2.0 Fluorometer Invitrogen, USA). After DNA was extracted and quantified the samples were split into three analytical methods. The methods are DNA based molecular biology tests which evaluate the genetic potential of samples. Table 2 summarized these three methods.

Table 2: Molecular methods used to analyse the presence/absence of anammox microbes in sludge and wastewater samples

Method	Purpose
End-point Polymerase Chain Reaction (PCR) of target genes using standard thermocycler	To detect the presence/absence of anammox and AOB (ammonia oxidising bacteria) microbes
Quantitative PCR (qPCR) using spectrophotometric thermocycler	To detect the presence/absence of anammox and AOB microbes
Sequencing of target gene using Illumina MiSeq DNA sequencer	To identify the species of anammox and AOB microbes

2.3 ANAMMOX GENE PCR ANALYSES

End-point Polymerase Chain Reaction (PCR) tests were conducted on DNA samples using molecular probes (primers) specific to anammox and AOB genes. PCR tests were completed using the polymerase reaction mixture KAPA HiFi Hot Start Ready Mix PCR kit together with anammox specific primers. After reaction in thermocycler, PCR products were run in 2% agarose gel electrophoresis to evaluate their molecular size. Gel was visualized and photographed in a Gel Doc XR equipment (BioRad).Quantitative PCR (qPCR) analysis was conducted to evaluate the abundance of anammox and AOB genes in the DNA extracted from sludge samples. qPCR reactions were done using the polymerase reaction mixture PowerUp SYBR Green master mix (Life Technologies, USA) together with the AMX809F/AMX1066R primers (Tsushima et al, 2007). Each sample was analysed in triplicate during each thermocycler run. Ten-fold serial dilutions of PCR products

(*Anammox 16s rRNA amplicons*) were prepared to produce calibration curves for the analysed gene. The DNA concentration of each dilution was quantified in triplicate using Qubit assays. These diluted amplicons were analysed in triplicate during each thermocycler run to confirm the fluorescence signal of target genes at various known DNA concentrations. Finally gene copies numbers were transformed to biomass weight (mg) using the 16S rRNA gene copy number of anammox cells and the average dry weight of bacterial cells.

2.4 ANAMMOX SPECIES IDENTIFICATION

The presence of anammox microbes was confirmed by nucleotide sequence analysis of 16S rRNA genes using the Illumina MiSeq platform. 16S rRNA gene nucleotide sequences are frequently used in phylogenetic classifications such as genus or species in diverse microbial populations. DNA extracts from sludge samples were further prepared for sequencing analysis following the Illumina MiSeq 16S Metagenomic Sequencing Library preparation protocol. Sample preparation involved generating a 16S rRNA amplicons library through end point PCR using the specific Nextera primers and PCR conditions. Confirmation of PCR amplification was done through agarose gel electrophoresis and Gel Doc visualisation. The library was cleaned using the Ampure XP reagent and DNA concentrations quantified and normalized to a concentration of <10 ng/µL using ultrapure PCR water. Once ready, the 16S rRNA library was handed to New Zealand Genomics Ltd (NZGL) for further nucleotide sequencing and raw data analysis.

2.5 ANAMMOX BACTERIA DETECTION

All of the analysed samples provided positive anammox signals (Figure 2). The AMX809F/AMX1066R primers produce DNA fragments of 257 base pair sizes after PCR amplification (Tsushima *et al*, 2007).

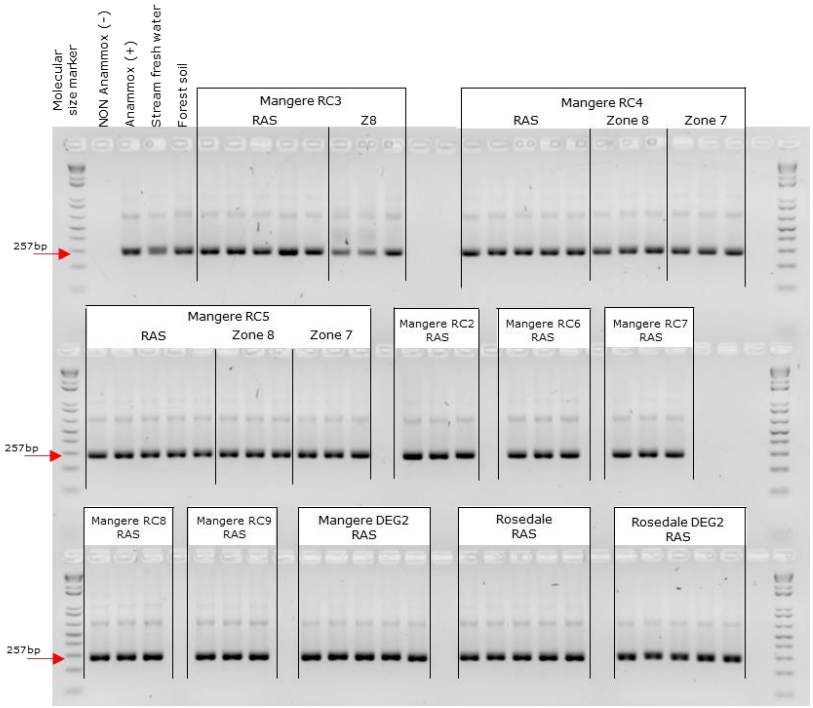


Figure 2: Gel electrophoresis results after PCR sowing positive amplification of anammox 16s rRNA gene copies. Comparison of bands from PCR products against molecular marker demonstrates that products have a size around 250-300 base pairs, which is compatible with the target 257 bp size of anammox gene amplicons.

The electrophoresis run depicted in Figure 2 demonstrates that 257 bp size amplicons were obtained from all samples; this is shown by the position of the dark bands in the electrophoresis gel. This confirms that the probes correctly bound to anammox gene sequences in the DNA extracted from the sludge samples. No false positive results were confirmed as no bands were identified in the DNA samples from non Anammox bacterial (i.e. Escherichia coli).

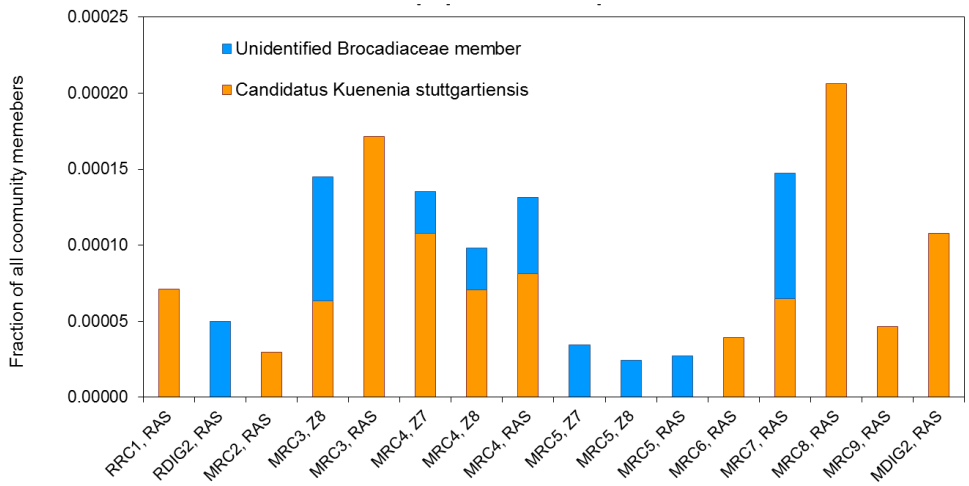
2.6 THE MICROBES *CANDIDATUS* KUENENIA STUTTGARTIENSIS AND AN UNIDENTIFIED ANAMMOX FAMILY (BROCADIACEAE) MEMBER FORM THE ANAMMOX POPULATIONS IN SLUDGE SAMPLES

Results from Illumina MiSeq sequencing analysis confirm a rich population of Planctomycetia microbes in all the analysed samples. Within this class, two species of the anammox family Brocadiaceae, *Candidatus* Kuenenia stuttgartiensis and another unidentified species, were detected in the sludge samples. The unidentified species is yet to be confirmed as a previously reported or as new species. Only seven anammox species are currently known worldwide. The proportion of these anammox microbes within the whole bacterial community ranges from 0.00003 to 0.0002 fraction (or from 0.003 to 0.02 %) (Figure 3a). This indicates that although ubiquitous, anammox species form a fraction of the species present in the sludge and mixed liquor samples.

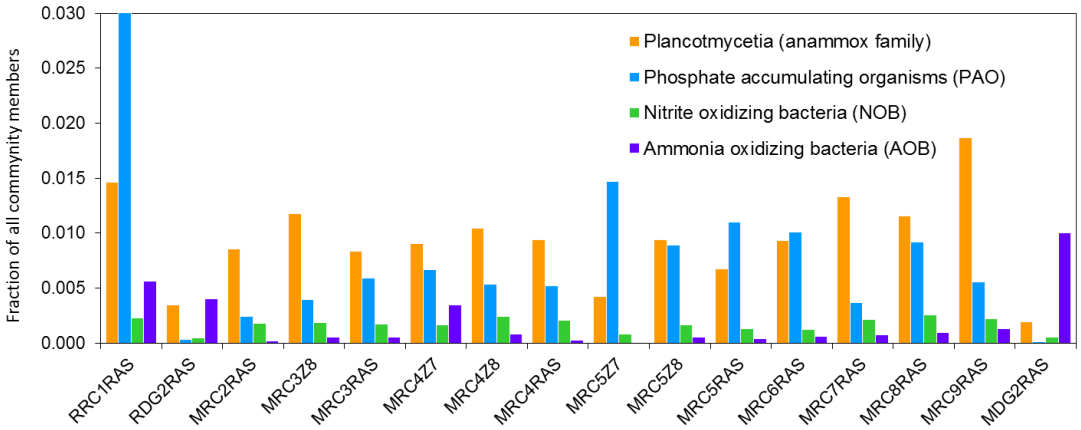
Sequencing results indicated that members of the Planctomycetia family, which anammox species belong to, form 0.5 to 2% of the all identified species in samples (Figure 3b). PAO organisms were present in all samples except in those from digesters at a percentage ranging from 0.5 to 3.5% of the identified species. The PAO species, Candidatus *Accumulibacter phosphatis,* was detected as a prominent member of PAOs population. NOB organisms were slightly more abundant than AOB organisms, although the number of identified NOB species were only four while that for AOB was eight. As depicted in

Figure 4c, members of the phylum Bacteroidetes form the core of the microbial community of all analysed samples. Gama and Beta proteobacteria populations were prominent in samples from wastewater treatment bioreactors but not in digester samples. Also, results indicate that the microbial species in communities of Rosedale RAS differ from those of Mangere RAS, although phylum distribution is similar.

a) Relative abundance of anammox species detected in sludge



b) Relative abundance of key functional microbial groups in sludge samples



c) Composition of microbial communities in sampled sites

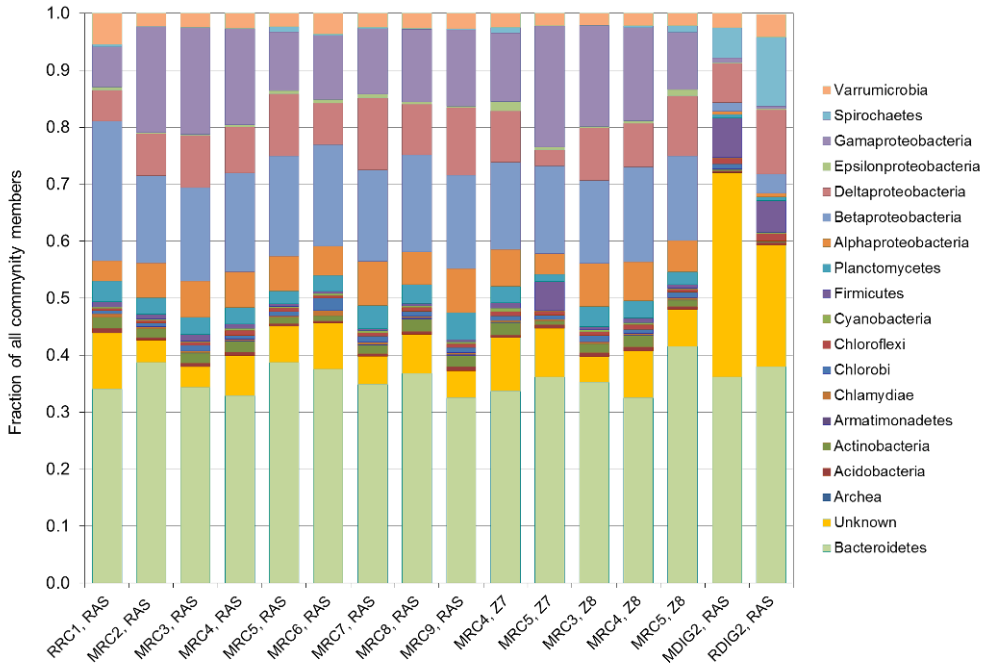


Figure 3: Microbial species determination based on 16s rRNA gene sequence analysis: a) Species composition of anammox populations in sampled sites; b) Fractions of microbial functional groups in communities of sampling sites; c) Composition of microbial communities in sampled sites. Community fractions are organized in different phylum.

3 ESTABLISHMENT AND ENRICHMENT OF ANAMMOX MICROBES

3.1 OVERVIEW OF CULTIVATION APPARATUS

A laboratory scale reactor apparatus was designed and built to optimize the development of anammox microbes enrichments. The apparatus consists of three 3L bioreactors (Photo 1). Each bioreactor is equipped with peristaltic pumps; magnetic stirrers; and pH, temperature and dissolved oxygen probes. These components were integrated into the apparatus to control bioreactor temperature, hydraulic retention time, ammonia and nitrite concentrations and anaerobic conditions. The three bioreactors were designed to operate as sequential batch reactors (SBR). This operation mode was selected to maximise the biomass retention in the bioreactor. The target Hydraulic Retention Time (HRT) is 23 h.

Each of the bioreactors was seeded with activated sludge collected from i) RAS system of Rosedale WWTP; ii) RAS system of RCs 2-9 of Mangere WWTP (composite sample); and iii) RAS system of digesters 1-4 of Mangere WWTP (composite sample). These sources were selected as they were previously identified as promising sources of anammox microbes, during genetic detection analysis. The three cutlers are respectively identified as "R", "M" and "D" for simplification.

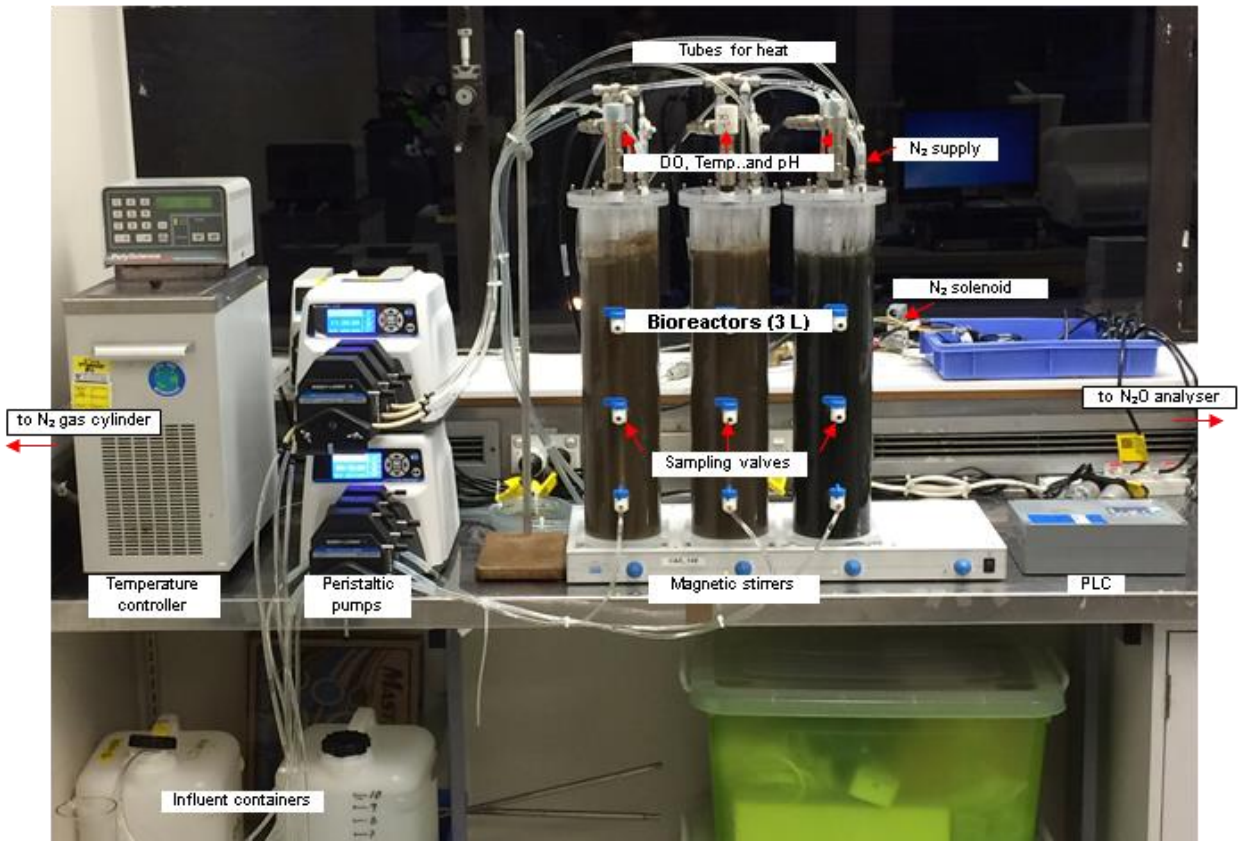


Photo 1: The cultivation apparatus to enrich anammox microbes from environmental samples. The apparatus was firstly installed at the Environmental Engineering Laboratory of the University of Auckland. On March 2018, the cultures were transferred to the new Wastewater Innovation Centre at Watercare's Mangere wastewater treatment plant.

3.2 CULTIVATION PERIOD

The cultures have been running since November 2017. The bioreactors average pH is 7.1±0.2 and have complete anaerobic conditions (i.e. dissolved oxygen concentration = 0.0 mg-O_2 L^{-1}). Two different major operation changes have been done on the cultures in order to improve ammonium removal performance. Firstly, pumping of a nitrite solution started on day 10; and secondly, strict pH control started at day 55. These two changes have helped to increase the ammonium removal rate, as described below, although the biomass has not yet developed the characteristic red colour of anammox biomass (Laureni et al, 2015).

3.3 AMMONIUM REMOVAL ACTIVITY

The cultures started to present anaerobic ammonium removal 20 days after establishing true anaerobic conditions and the start of nitrite dosing (Figure 4). The culture seed with Rosedale RAS, removes 90 mg-N of ammonium in 12 days. The culture achieved a cyclic behaviour of ammonium removal and so ammonium has been iteratively added to achieve increasing concentrations. Cultures "M" and "D" have a slower ammonium removal rate, so that they remove 90 mg-N in 30 days, although the ammonium removal rate has been increasing in the last 170 days.

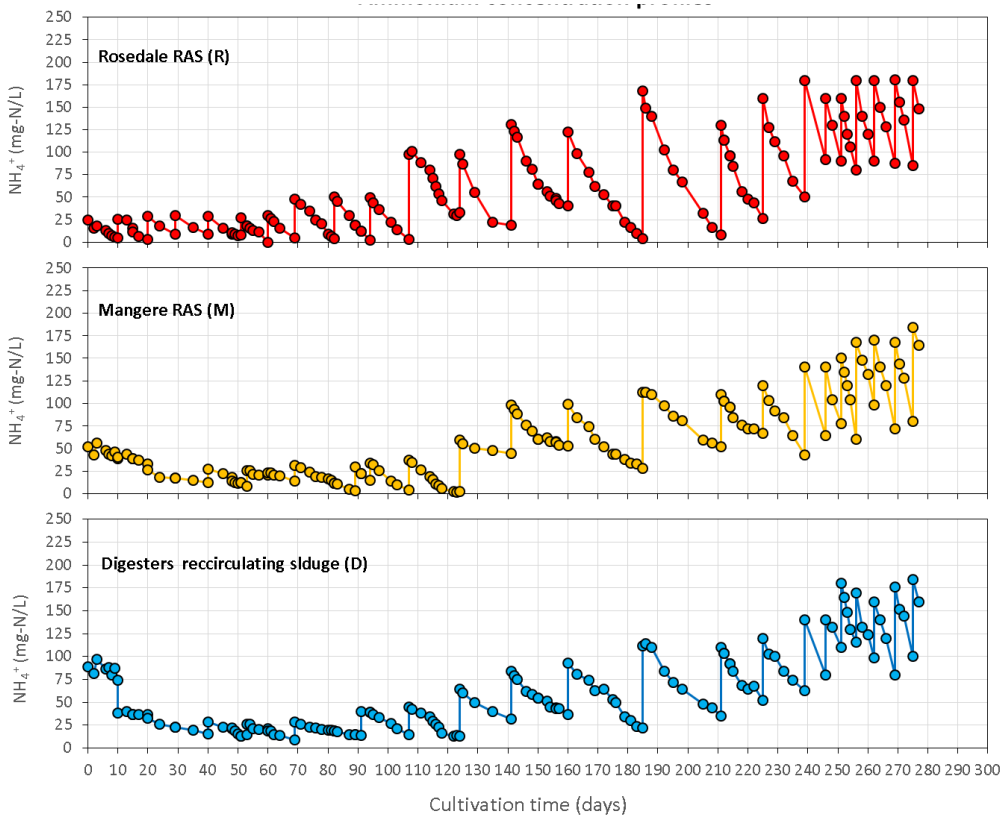


Figure 4: Ammonium concentration profiles of the three lab-scale reactors.

Ammonium consumption rates are depicted in Figure 5a, the rates are presented in negative values as they represent loss of ammonium from the bioreactors. Average values obtained for the three cultures have increased across time, from -0.4±0.7 to -20.6±3.2. Cumulative ammonium removal shows that "R" reactor has consumed around 5000 mg of NH_4-N during a period of 270 days through the anammox reaction (Figure 5b), which is equivalent to an ammonium removal volumetric rate of 18 mg-N L^{-1} d^{-1}. Cultures "D" and "M" have removed 3000 mg of NH_4-N during the same 270 days.

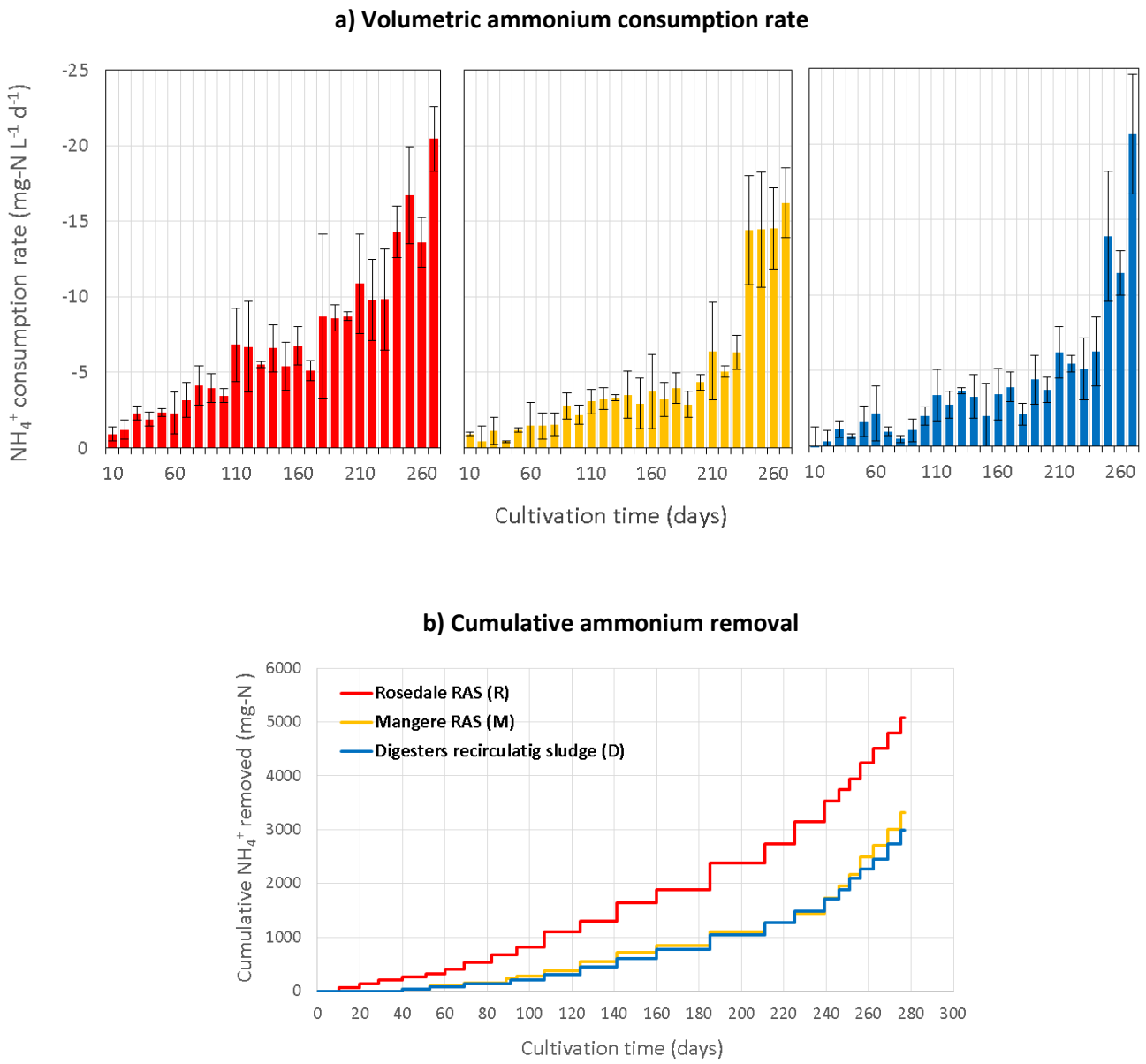


Figure 5: Ammonium removal performance. a) volumetric ammonium consumption rates of the three lab-scale reactors. The Graphic below depicts Mean±St.Dev values over periods of 10 days. The rates are presented in negative values as represent loss of ammonium from the system; b) cumulative ammonium removal

Figure 6 presents the correlation between ammonium and nitrite consumption rates during the last 170 days of cultivation. The graphs show that the "R" culture is consuming ammonium faster than nitrite and that the ratio is below the theoretical 1.3:1 NO_2:NH_4 ratio, which indicates nitrite consumption rates lower than the expected value. This can be explained if the "R" culture is consuming NO_2 as well producing NO_x compounds (indeed a normal behaviour in anammox cultures), which would increase NO_2 concentration and conversely decrease the expected NO_2 consumption rate. Nitrite consumption rate in "M" and "D" cultures is higher than the expected value to achieve the theoretical 1.3:1 ratio, indicating the presence of denitrification activity, which might be occurring given the presence of organic carbon on the sludge used to seed the reactors.

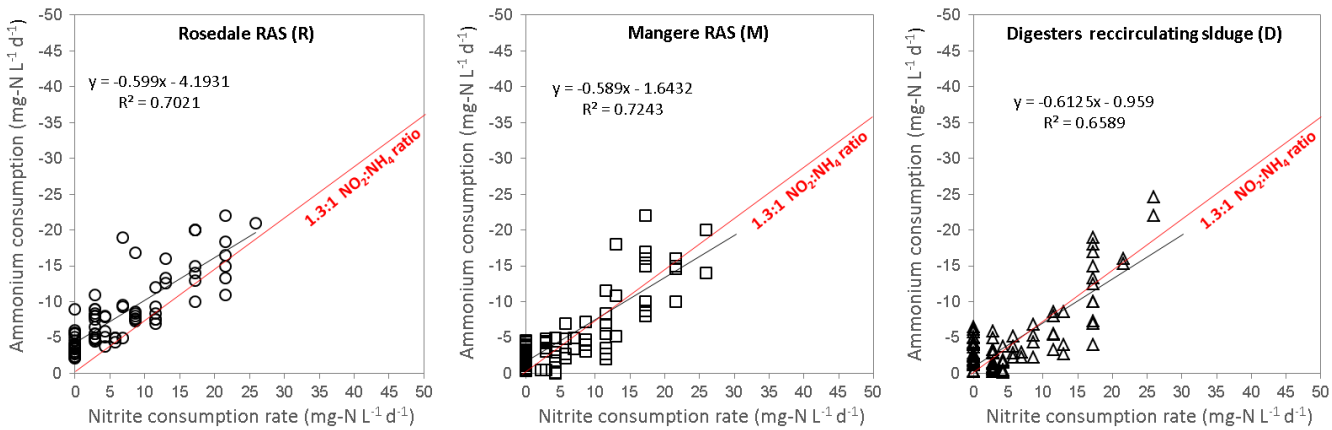


Figure 6: Correlation between nitrite and ammonium consumption rates. Theoretical anammox activity has a rate ratio of 1.3:1 NO_2:NH_4.

3.4 AMMONIUM TRANSFORMATION TO NITRATE AND NITROGEN GAS

As expected from anammox cultures, ammonium and nitrite consumed are converted to nitrogen gas and residual nitrate (Lackner et al, 2014). Figure 8a shows ammonium and nitrite consumption from in reactor R. In every SBR cycle residual nitrate is removed from the reactor then formed during the reaction phase of the cycle. Nitrite concentration is flat as is immediately consumed after addition.

Figure 7a shows complete depletion of NH_4-N during a SBR cycle, although NO_3-N plus NO_2-N mass is exceeds the balance with the ammonium consumed; the ammonium is likely to be converted to di-nitrogen gas. Indeed continuous gas formation and bubbling have been observed in the reactor under complete anaerobic conditions (Figure 7b), which is evidence that the anammox reaction is indeed occurring.

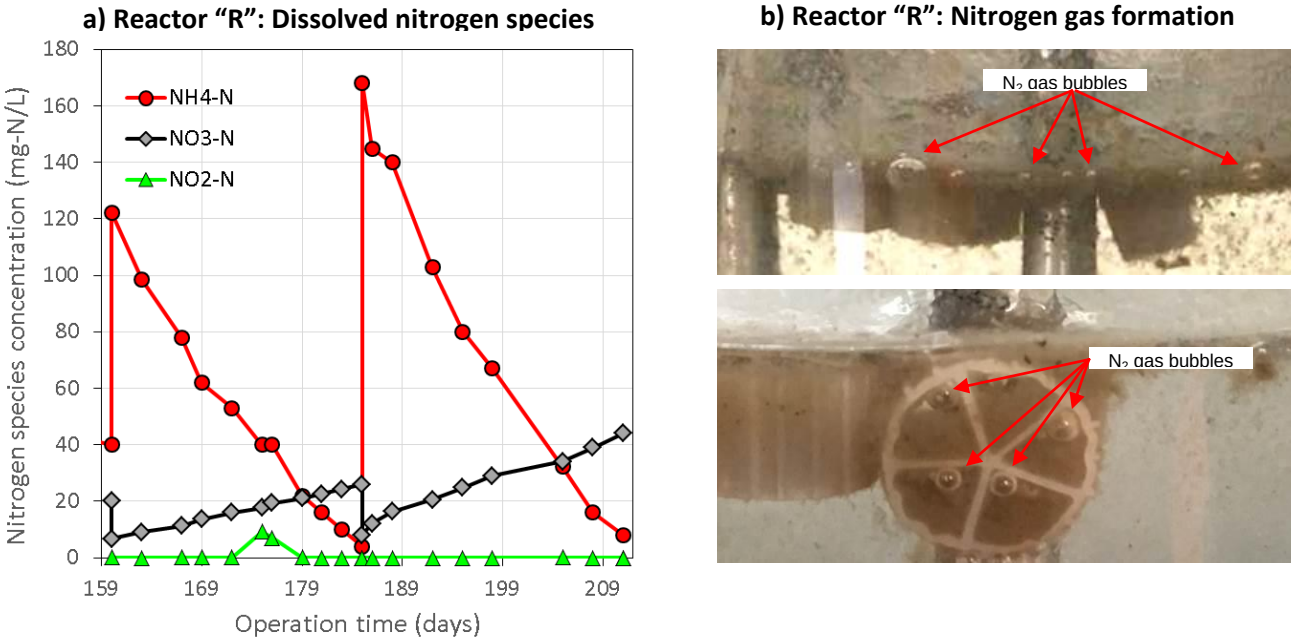


Figure 7: Nitrogen conversion in reactor "R": a) Dissolved nitrogen species. Ammonium and nitrite consumption with residual nitrate production; b) Nitrogen gas bubbles in reactor

3.5 BUILD UP OF ANAMMOX BIOMASS

Figure 8 shows the qPCR based anammox quantification results. The graphs shows the results corresponding to the first 270 days of cultivation, samples collected between day 50 and 150 of cultivation got spoiled due to storage fridge failure and were not analysed. From day 160 a significant increase of gene copies associated to anammox cells was observed in the three reactors. Reactor "R" presented the most substantial increase of anammox bugs genes, jumping from $2.4x10^2$ to $3.0x10^6$ gene copies per uL of sample DNA (x/µL) within 150 days, indicating an exponential growth of anammox concentration. Anammox gene copies of reactor M are also increasing in an exponential trend with the concentration increasing from $1.9x10^2$ to $5.5x10^3$ x/µL in 100 days, and then to $1.1x10^6$ x/µL in 60 days. Anammox growth in reactor "D" is slower, rising to $3.4x10^4$ x/µL in 60 days.

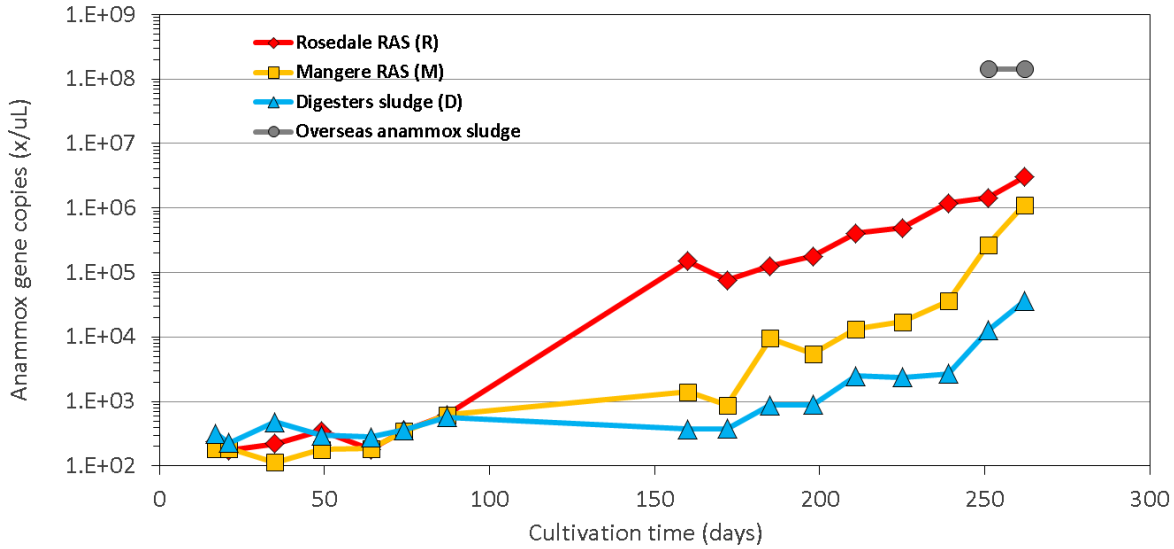


Figure 8: Net quantification of populations of Anammox (AMX) in cultures' biomass obtained with the qPCR method. Vertical axis is in logarithmic scale

Figure 9 shows the volatile suspended solids (VSS) concentration of the reactors. After losing significant amounts of solids during the first 100 days, the reactors have now stabilised their dry weight concentration. Early loss of solids happened most likely due to the break down and lysis of organic matter and cells of non anammox organisms. VSS values are currently stable, indicating that the reactors have equilibrium between biomass formation and endogenous decay. Although during the last 30 days VSS concentration has increased in the three reactors indicating of biomass formation.

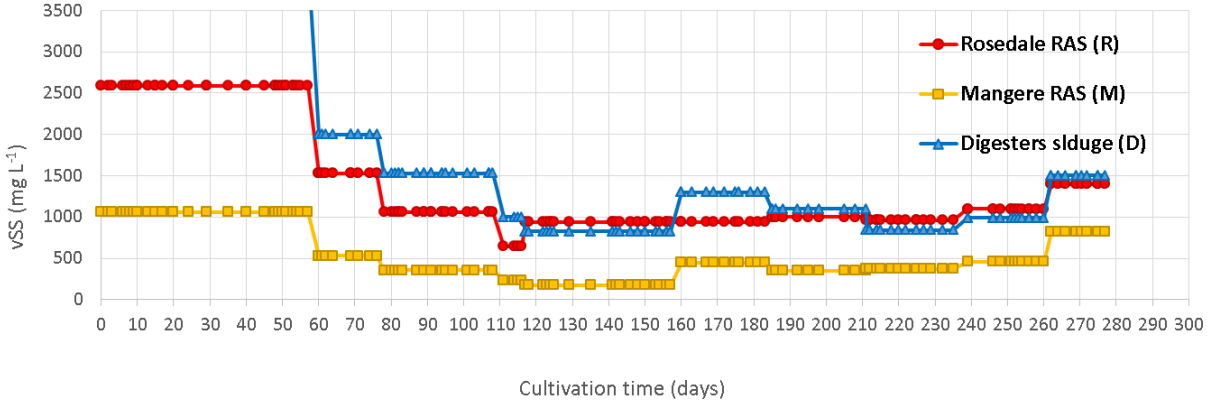


Figure 9: Total Suspended Solids (TSS) of bioreactors.

Photo 2a and 2b shows the floccular biomass in the cultures during settling stage. The biomass forms dense large flocks of rapid settleabilty that start resembling granular biomass. After 200 days of cultivation none of the biomass has yet developed the characteristic reddish colour of anammox biomass. Photos 2c to 2f show a flock with granule shape observed with microscope. The images have been amplified with objectives. The flock presents a compact structure similar to those of granules, although formal settlability and density tests have yet to be conducted.

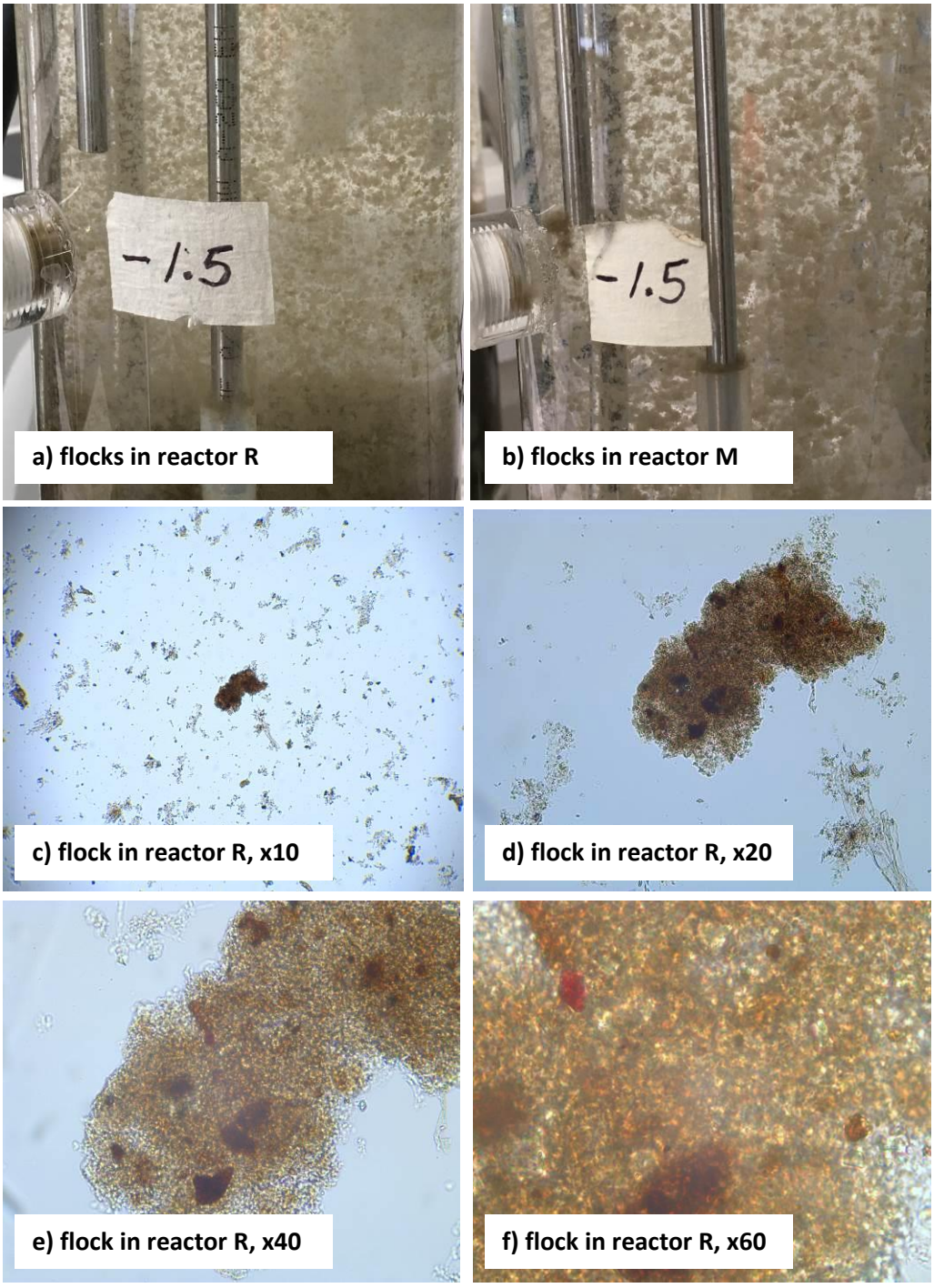


Photo 2: Biomass in anammox reactors. Photos a) and b) show biomass flocks settling in reactors "R" and "M"; Images c), d), e) and f) are light microscope photos of biomass sampled from reactor R after 160 days of cultivation.

4 SCALE-UP AND ASSESSMENT OF THE DEAMMONIFYING PROCESS

4.1 NEXT STAGE

Achievement of three further process evaluations is required before scaling-up the deammonification process to pilot trails: i) to increase influent concentrations from 150 mg-N L^{-1} to 900 mg-N L^{-1} and at the same time increase anammox biomass concentration (with ammonium influent concentration adjusted depending on removal rate); ii) adaptation of cultures from an anammox process to a full deammonification process (by complementing anammox activity with aerobic AOB activity); and finally iii) to test biomass deammonification activity under real feed conditions (in this case dewatering centrate high in ammonia). This last stage will involve evaluating the inhibitory effects of centrate on biomass activity in batch cultures and obtaining kinetic values for the further design of pilot and full scale processes. Achieving these millstones will take between 6 to 12 months of further work.

From the current 3 L cultures we plan to scale up to 15 L cultures (Photo 3a), then to 50 L, then to 250 L and finally ~2000 L cultures, at which point we will conduct centrate fed pilot trials (Figure 10). We will do the above work during the coming months at the Watercare Wastewater Innovation Centre (Photo 3b)

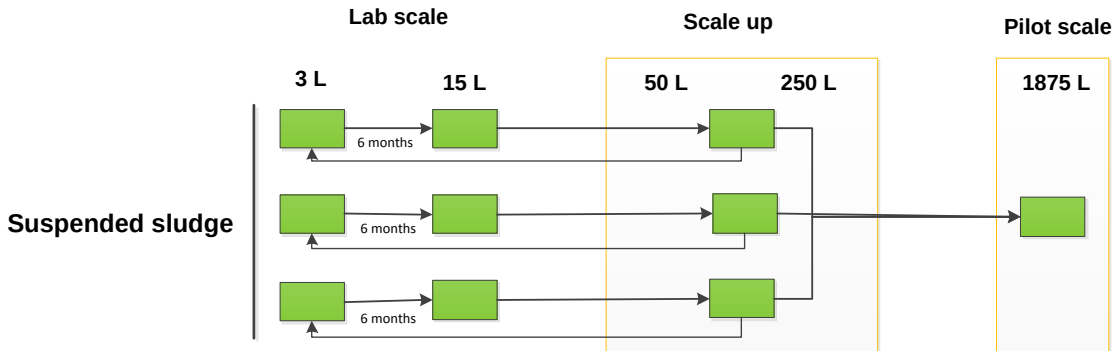


Figure 10: Process scale up strategy for the coming months.

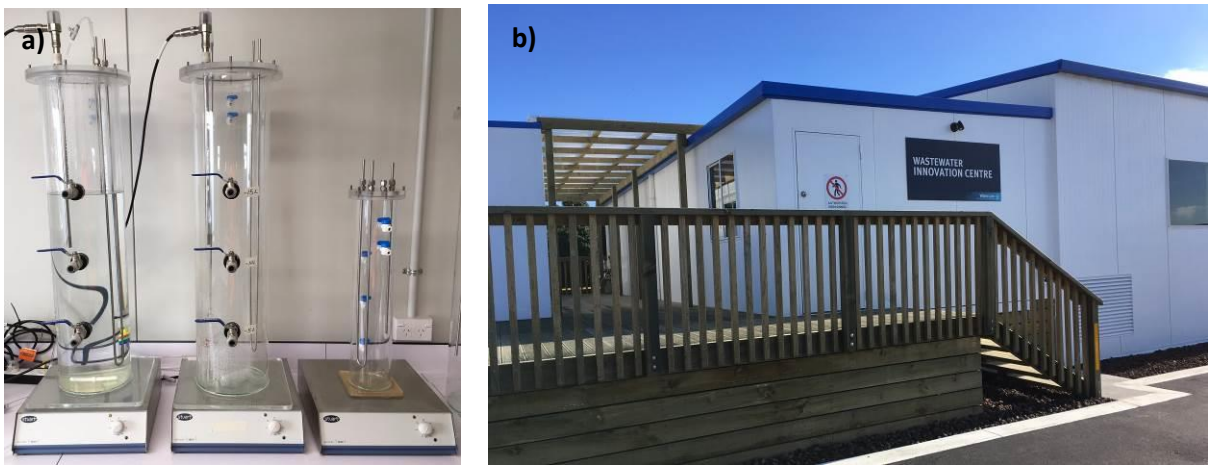


Photo 3: Scale up gear: a) 15 L bioreactors alongside a 3 L bioreactor; b) The new Watercare's Wastewater Innovation Centre, home of the anammox bioreactors.

5 CONCLUSIONS

Anammox microbes are the key component needed to develop the deammonification process. The presence of anammox microbes in Activated Sludge samples from Mangere and Rosedale WWTP was confirmed during this project. We have developed an active and vigorous culture enriched with anammox microbes, which is a New Zealand first. It is also relevant to highlight that anammox enrichment from environmental samples has been done only infrequently internationally, so that obtaining an indigenous New Zealand enrichment is a significant milestone.

So far we have found that the key operational variable to maintain optimal conditions for our anammox cultures are: i) complete anaerobiosis (DO = 0 mg L-1); ii) ammonium concentration below 30 mg-N L^{-1} to avoid ammonia toxicity during the first days of cultivation; and iii) a strict pH between 7.0 and 7.4.

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