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Enrichment of Anammox biomass from municipal activated sludge: experimental and modelling results

Ana Dapena-Mora,[1] Stijn WH Van Hulle,[2] J Luis Campos,[1] Ramón Méndez,[1]* Peter A Vanrolleghem[2] and Mike Jetten[3]

[1]*Chemical Engineering Department, Instituto de Investigaciones Tenológicas, ETSE, Rua Lope Gómez de marzoa s/n, Campus Sur, University of Santiago de Compostela, E-15782, Spain*

[2]*Department for Applied Mathematics, Biometrics and Process Control (Biomath), Ghent University, Coupure Links 653, B-9000 Gent, Belgium*

[3]*Department of Microbiology, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands*

Abstract: Anaerobic Ammonia Oxidising (Anammox) biomass was enriched from sludge collected at a municipal wastewater treatment plant, employing a Sequential Batch Reactor (SBR). After 60 days Anammox activity started to be detected, by consumption of stoichiometric amounts of NO_2^- and NH_4^+ in the system. Fluorescence *In Situ* Hybridisation analysis confirmed the increase of Anammox bacteria concentration with time. A final concentration of enriched biomass of 3–3.5 gVSS dm^{-3} was obtained, showing a Specific Anammox Activity of 0.18 gNH_4^+-N gVSS^{-1} d^{-1} The reactor was able to treat nitrogen loading rates of up to 1.4 kgN m^{-3} d^{-1}, achieving a removal efficiency of 82 %. On the other hand, the start-up and operation of the Anammox SBR reactor were consequentially modelled with the Activated Sludge Model nr 1, extended for Anammox. The simulations predicted quite well the experimental data in relation to the concentrations of nitrogenous compounds and can be used to estimate the evolution of Anammox and heterotrophic biomass in the reactor. These simulations reveal that heterotrophs still remain in the system after the start-up of the reactor and can protect the Anammox microorganisms from a negative effect of the oxygen.

Keywords: Anammox; modelling; Sequential Batch Reactor (SBR); denitrification; nitrification

NOTATION

Anammox	ANaerobic AMMonium OXidising
ASM1	Activated Sludge Model nr 1
COD	Chemical Oxygen Demand
DO	Dissolved Oxygen (mgO_2 dm^{-3})
FISH	Fluorescence *In Situ* Hybridisation
HRT	Hydraulic Retention Time (h)
NLR	Nitrogen Loading Rate (gN dm^{-3} d^{-1})
PLC	Programmable Logic Controller
SAA	Specific Anammox Activity (gNH_4^+ -N gVSS^{-1} d^{-1})
SBR	Sequential Batch Reactor
SHARON	Single Reactor System for High Ammonia Removal Over Nitrite
VSS	Volatile Suspended Solids (g dm^{-3})
WWTP	Wastewater Treatment Plant

S_{NH}	Ammonium concentration (mgN dm^{-3})
S_{NO_2}	Nitrite concentration (mgN dm^{-3})
S_{NO_3}	Nitrate concentration (mgN dm^{-3})
S_S	Readily degradable substrate (mgCOD dm^{-3})
X_{AN}	Concentration of Anammox organisms (mgCOD dm^{-3})
X_H	Concentration of heterotrophs (mgCOD dm^{-3})
X_I	Concentration of inert biomass (mgCOD dm^{-3})
X_{NH}	Concentration of ammonium oxidisers (mgCOD dm^{-3})
X_{NO}	Concentration of nitrite oxidisers (mgCOD dm^{-3})

* Correspondence to: Ramón Méndez, Chemical Engineering Department, Instituto de Investigaciones Tenológicas, ETSE, Rua Lope Gómez de marzoa s/n, Campus Sur, University of Santiago de Compostela, E-15782, Spain
E-mail: eqrmndzp@usc.es
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X_S Slowly degradable substrate (mgCOD dm^{-3})

INTRODUCTION

Biological nitrogen removal from wastewater having a high nitrogen content can be costly, particularly when the wastewater contains only small amounts of biologically-degradable carbon compounds (eg effluents from anaerobic digestion of sludges from WWTP or food and agriculture industry wastewaters, fertilisers and leachate effluents).[1]

A novel way to remove nitrogenous compounds of this type of wastewaters is the combination of:

1. A partial nitrification system of the type SHARON (Single Reactor System for High Ammonia Removal Over Nitrite), that oxidises 50% of the ammonium to nitrite by controlling the HRT, pH and temperature.[2]
2. An Anammox system, where ammonium is oxidised anaerobically, using the nitrite produced in the previous SHARON system as electron acceptor (eqn. (1)).

$$\begin{aligned} NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \\ \rightarrow 1.02\ N_2 + 0.256\ NO_3^- \\ + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O \end{aligned} \quad (1)$$

Application of the combined SHARON–Anammox process would reduce the required oxygen input by 60% (compared with a conventional nitrification–denitrification process) and would alleviate the need for addition of methanol (and concomitant increased sludge production).[2]

The Anammox process was discovered by Mulder *et al* in 1995[3] and the first identified Anammox organism was named *Candidatus* 'Brocadia Anammoxidans'.[4] Nowadays different Anammox organisms have been detected by PCR, phylogenetic analysis or FISH in both wastewater treatment and natural systems where nitrogen losses occurred: *Candidatus* 'Kuenenia stuttgartiensis', *Candidatus* 'Scalindua brodae', *Candidatus* 'Scalindua wagneri' and *Candidatus* 'Scalindua sorokinii'.[5,6] The presence of Anammox organisms has been detected in several types of wastewater treatment processes.[7] For example, Anammox activity has been observed in a rotating biological contactor treating ammonium-rich leachate,[8] a trickling filter treating wastewater,[9] fixed- and fluidised-bed reactors and SBRs treating a synthetic medium.[10,11]

Application of the Anammox process is limited by the availability of Anammox biomass. The isolation and enrichment of Anammox biomass from a mixture of bacterial populations requires the optimisation of conditions favouring the Anammox process, while limiting the growth of any other kind of microbial population. In particular, since the Anammox process is anaerobic, the exclusion of oxygen is essential especially during the start-up of reactors. As the maximum specific growth rate of the Anammox bacteria is very low ($0.003\,h^{-1}$),[11] it is important to use a system that minimises biomass washout, in order to maximise the biomass concentration in the system. Strous *et al* performed an enrichment of Anammox biomass in a fluidised-bed reactor, treating a sludge digester effluent, reaching a NLR of $1.5\,kgN\,m^{-3}\,d^{-1}$.[11] However, the retention capacity of the system was not good and the biofilm structure in the reactor was not homogeneous, making it difficult for the biomass to access the substrate.[10] Strous *et al* showed that the SBR is a suitable system to grow Anammox organisms.[11] The strongly selective conditions achieved in this system permitted a 74% enrichment of Anammox microorganisms in the SBR. Using this type of reactor, Strous *et al* could determine, for the first time, the stoichiometric and kinetic parameters of the Anammox process, which can be used for modelling and subsequent optimisation of the Anammox process.[11]

Hitherto, few studies have been conducted on modelling start-up and dynamic behaviour of the Anammox process. Hao *et al* described a simulation study on the behaviour of a partial nitrification–Anammox system under different process conditions, such as varying temperatures and dissolved oxygen concentrations.[12,13] However, no verification with real experimental data was performed and no start-up dynamics were included in the study. Koch *et al*. performed simulations with a similar system, but also did not include any start-up or long-term dynamic effects.[14] Furthermore, it should be noted that, in both studies, total nitrogen concentration amounted to about $150\,mgN\,dm^{-3}$, while in practice this concentration would be ten-fold greater in an Anammox reactor.

The purpose of the present study was two-fold. Firstly, to isolate and produce Anammox biomass from sludge of a municipal WWTP by using an SBR system.[15] If successful, this would make the application of the Anammox process feasible in situations where this type of biomass may not be directly accessible. Secondly, to interpret the results of this enrichment using the IWA Activated Sludge Model nr 1, extended with the submodels for a two-step nitrification–denitrification model and the Anammox process.[12,13,16–18] Simulation results from both start-up and dynamic operation of the reactor were compared with the measured values. Total nitrogen concentrations in the reactor amounted to $900\,mgN\,dm^{-3}$ as would be the case in an industrial reactor. This simulation study also quantified the kinetic properties of the enriched Anammox biomass.

MATERIALS AND METHODS

Description of the SBR

Inoculum

The reactor was inoculated with a mixture of sludges from the municipal WWTPs of Bertamiráns, Padrón

and Negreira (A Coruña, Spain). All three WWTPs are operated in oxic/anoxic conditions, with a HRT of 11 h, a carbon loading rate of 0.86 kgCOD m^{-3} d^{-1} and a nitrogen loading rate of 0.065 kgN m^{-3} d^{-1}. About 90% of the incoming COD and about 60% of the incoming nitrogen are removed.

Reactor mineral medium

The composition of the mineral medium fed to the SBR reactor is shown in Table 1. Concentrations of ammonium and nitrite were added as specified in the results section.

Operation of the SBR

The process was carried out in an SBR with a working volume of 1 dm^3, a diameter of 10 cm and a height/diameter ratio of 1 (Fig 1). The system was maintained at 35 °C and a pH between 7.8 and 8, maintained without any deliberate control. The HRT was fixed at 0.62 days. The medium was mixed at 50 rpm with a paddle stirrer with a diameter of one-third of the internal diameter of the reactor.

The SBR worked in cycles of 6 h, controlled by a PLC (CPU224, Siemens). Each cycle had three phases: in the first phase (5.5 h), the reactor was fed continuously. In the second phase (0.33 h), the stirrer and the influent supply were stopped and the biomass was allowed to settle. Finally, in a third phase (0.17 h), the supernatant was pumped out of the reactor. The reactor was flushed continuously with a mixture of 95% Ar/5% CO_2 to maintain anaerobic conditions.

The operational strategy consisted of increasing the inlet concentrations of ammonium and nitrite as the limiting substrate (nitrite) was totally consumed.

Table 1. Composition of the mineral medium fed to the reactor

Compound	(g dm^{-3})
$(NH_4)_2SO_4$	0.132–1.88
$NaNO_2$	0.069–2.46
$NaNO_3$	0–0.85
$KHCO_3$	1.25
NaH_2PO_4	0.05
$CaCl_2.2H_2O$	0.30
$MgSO_4.7H_2O$	0.20
$FeSO_4$	0.00625
EDTA	0.00625
H_2SO_4	0.5–1.25 cm^3 dm^{-3}
Trace elements solution[a]	1.25 cm^3 dm^{-3}

[a] van de Graaf *et al* (1996).[19]

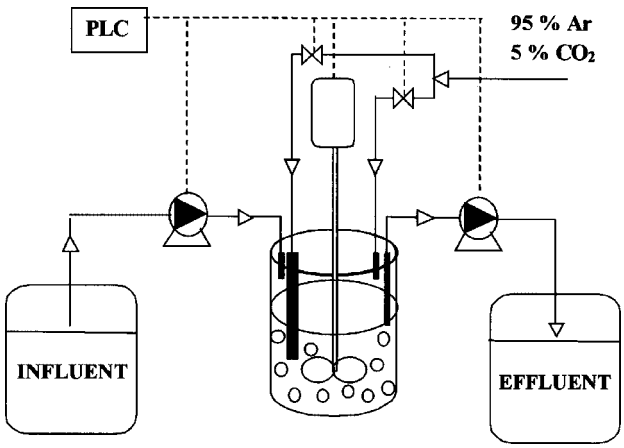

Figure 1. Experimental set-up of the sequential batch reactor for Anammox enrichment.

Analytical methods

Ammonium was analysed by the phenol–hypochlorite method.[20] Nitrite and nitrate were analysed by spectrophotometry.[21] Biomass concentration was determined as gVSS dm^{-3}.[21] The bacterial population was monitored by FISH analysis, according to Schmid *et al*.[5] The applied oligonucleotide probes were: PLA46 [S-P-Planc-0046-a-A-18] (planctomycetes), EUB338 [S-D-Bact-0338-a-A-18] (eubacteria), AMX820 [S-*-Amx-0820-a-A-22] (Anammox of the type 'Brocadia Anammoxidans' and 'Kuenenia stuttgartiensis') and KST1273 ('Kuenenia stuttgartiensis').

Modelling the Anammox SBR

The SBR

All modelling was performed in the modelling and simulation environment WEST®.[22] A standard SBR model in the WEST® model base was used to describe the SBR's behaviour. In this model the three SBR-cycle phases were described. In view of the size of the experimental reactor (1 dm^3) and the presence of a stirrer system, the SBR reactor was assumed to be ideally mixed. Although settling was ideal, the fraction of biomass withdrawn with the effluent was allowed at 0.5%, to take account of sampling. It was considered that 0.5% of the biomass contained in the reactor was washed-out in every withdrawal. The implementation of the SBR in the WEST® software is depicted in Fig 2.

Extension of IWA ASM1

For modelling purposes the Activated Sludge Model nr 1 extended with a two-step nitrification–denitrification model[23] was further extended with the Anammox process.[12–14] In this model the death–regeneration concept[16] is preferred over the endogenous respiration concept as used in other simulation studies to describe decay.[12–14] This preference follows from the fact that the behaviour of the Anammox biomass under substrate-limiting conditions is not completely clear yet and from the observation that heterotrophs were shown to be active in the reactor.

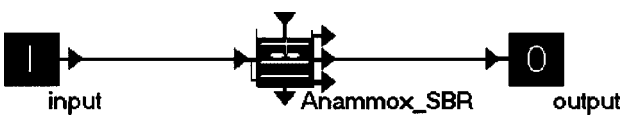

Figure 2. Implementation of the Anammox reactor in WEST. Influent is supplied through an influent file (I) and effluent is withdrawn through the output file. The reactor is a 1 dm^3 SBR reactor.

Only the application of this death–regeneration concept can explain this activity, since no biodegradable substrate (BOD) was added with the influent.

In the extended ASM1 model, ammonium (S_{NH}) is oxidised to nitrite (S_{NO_2}) by the ammonium oxidisers (X_{NH}). This nitrite can be further oxidised to nitrate (S_{NO_3}) by nitrite oxidisers (X_{NO}). Both nitrite and nitrate as well as oxygen (S_O) can be used as electron acceptors by heterotrophs (X_H) for growth and energy generation, while readily biodegradable substrates (S_S) are used as electron donors. No biodegradable substrate was supplied directly in the influent here (Table 1), but a value of 2 mg COD dm^{-3} biodegradable substrate in the influent was assumed to account for any biodegradable substrate present in the water used for medium preparation, and biodegradable substrate originating from EDTA in the trace element solution. Readily degradable substrates are also formed through the hydrolysis of slowly degradable substrate (X_S). This slowly degradable substrate is produced during decay of biomass, along with inert biomass (X_I). This extended ASM1 model was calibrated and validated in a previous study.[23]

In the Anammox process, which is added to the model in this study, ammonium and nitrite are combined to form nitrogen gas, while a small amount of nitrate is also produced, as shown in eqn (1). The stoichiometry of the Anammox process is represented in Peterson matrix format[16] in Table 2 for the growth and decay processes of Anammox in the first and second rows, respectively.

Monod kinetics were used to describe the dependency of the growth rate of Anammox on ammonium and nitrite concentrations. An additional Monod term was used to describe an eventual inhibition of the Anammox organisms by oxygen (eqn (2)):

$$\rho_{growth} = \mu_{AN} \frac{K_{O,AN}}{K_{O,AN} + S_O} \frac{S_{NO_2}}{K_{NO_2,AN} + S_{NO_2}} \times \frac{S_{NH}}{K_{NH,AN} + S_{NH}} X_{AN} \quad (2)$$

where S_O is the dissolved oxygen concentration (mg O_2 dm^{-3}) and $K_{O,AN}$ is the affinity constant for oxygen of Anammox microorganisms (mg O_2 dm^{-3}).

It should be noted, however, that nitrite is not only a substrate, but also can inhibit the Anammox process.[24] Therefore Haldane kinetics are perhaps more appropriate than the Monod kinetics applied, but the measured nitrite concentrations in the reactor were never high enough to suggest an inhibitory effect, which prevented any calibration of the inhibition constant.

For the decay rate of Anammox the following expression was used (eqn (3)):

$$\rho_{decay} = b_{AN} X_{AN} \quad (3)$$

The maximum specific growth rate of the Anammox biomass (μ_{AN}) was derived from Strous *et al*[11] and set to a value of 0.08 d^{-1} at 35 °C. The decay coefficient (b_{AN}) and the affinity constants were manually fitted to the available data. The decay coefficient was set to 0.0011 d^{-1} at 35 °C, which is an order of magnitude below the maximum growth rate, while affinity constants for ammonium ($K_{NH,AN}$) and nitrite ($K_{NO_2,AN}$) were both set to 0.3 gN m^{-3}. This value is higher than the one proposed by Strous *et al*,[24] indicating possible but minor substrate diffusion limitation in the SBR reactor.

The yield on ammonium for Anammox organisms was set to 0.159 gCOD gCOD^{-1}.[11] The amount of ammonium incorporated in the biomass (i_{nbm}) was set to 0.0583 gN gCOD^{-1},[16] while the fraction of inert biomass produced during cell decay was set to 0.08 gCOD gCOD^{-1}.[16]

RESULTS AND DISCUSSION

Start-up of the reactor

An SBR reactor was selected for the process because of its high biomass retention capacity.[11] The reactor was inoculated with a high concentration of VSS since during the first days a decrease in the biomass concentration into the system occurs, due to decay of the original biomass.

Another point to consider is possible diffusion of oxygen into the system. This is a critical factor especially during the enrichment since Anammox microorganisms are inhibited by low DO concentrations.[25]

Operation of the reactor

An appreciable consumption of NH_4^+ and NO_2^- was observed in the system after 2 months of operation (Fig 3). As these compounds were consumed, their concentrations in the feed were increased stepwise.

Initially, denitrifying activity was the favoured process (anaerobic atmosphere and presence of NO_3^-), eliminating the organic matter present in the medium from the lysis of any aerobic bacteria.

Table 2. Stoichiometric matrix for the Anammox process in Peterson matrix format[16]

Name:	Ammonium	Nitrite	Nitrate	Nitrogen gas	Anammox	Slowly degradable substrate	Inert	Process rate
Symbol:	S_{NH}	S_{NO_2}	S_{NO_3}	S_{N_2}	X_{AN}	X_S	X_i	ρ
Unit:	gN m^{-3}	gN m^{-3}	gN m^{-3}	gN m^{-3}	gCOD m^{-3}	gCOD m^{-3}	gCOD m^{-3}	gCOD m^{-3} d^{-1}
Growth of X_{AN}	$-1/Y_{AN}-i_{nbm}$	$-(1.52+1/Y_{AN})$	1.52	$2/Y_{AN}$				ρ_{growth}
Decay of X_{AN}	$i_{nbm}-f_i i_{Xp}$				-1	$(1-f_i)$	f_i	ρ_{decay}

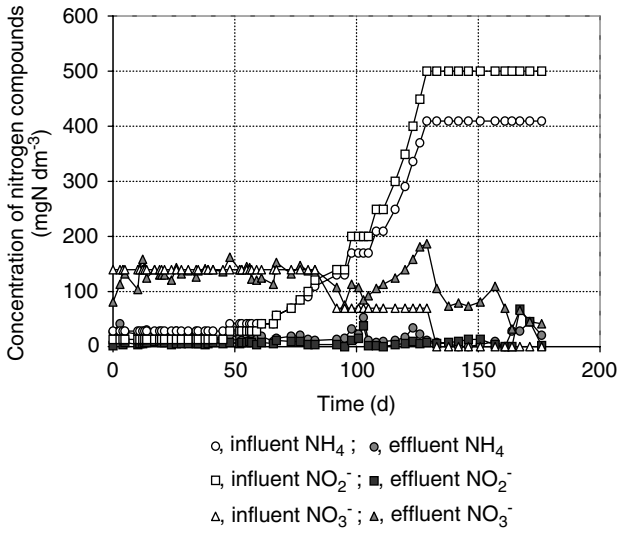

Figure 3. Concentration of nitrogen compounds in the influent to the reactor and in the effluent.

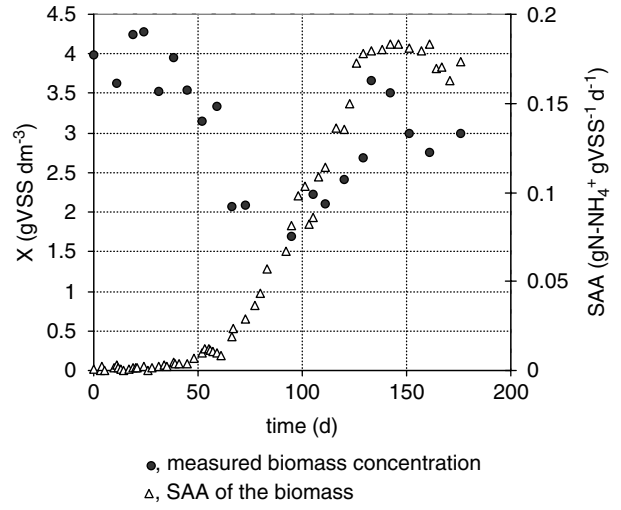

Figure 4. Measured biomass concentration in the reactor and SAA of the biomass.

This view was supported by an initial consumption of nitrate together with a significant decrease in biomass concentration during the first days (Figs 3 and 4). After lysis of the aerobic bacteria, lysis of the denitrifying bacteria began from lack of organic substrates. At that time denitrifying activity ceased and consumption of nitrate was no longer observed.

The biomass concentration in the system decreased during the first days, reaching a minimum of 1.7 gVSS dm^{-3} at day 100 (Fig 4). After that day, Specific Anammox Activity (SAA, gNH_4^+-N $gVSS^{-1}$ d^{-1}) in the reactor increased exponentially and a concomitant increase in the biomass concentration was observed (Fig 4), as well as a gradual colour change, from brownish to reddish, the typical colour of Anammox biomass.[19]

An average of 0.24 g NO_3^--N were produced and 1.25 g NO_2^--N were consumed for every g NH_4^+-N consumed. These values approach the ones obtained by Strous *et al* for the stoichiometry of the Anammox process.[11]

FISH analyses were realised to follow the evolution of the bacterial populations in the reactor. The probe EUB338 was used to see the total quantity of active cells in the sample. Since Anammox organisms have many features in common with planctomycetes, the probe PLA46 also hybridises with Anammox bacteria and was used to detect their presence in the reactor. A more specific probe (AMX820), which hybridises with *Candidatus* 'Kuenenia stuttgartiensis' and *Candidatus* 'Brocadia Anammoxidans', was also employed. Hybridisation with PLA46 and AMX820 was not detectable at the beginning of the experiment but after 2 months an increasing positive signal with these two probes was observed. All the biomass that hybridised with PLA46 hybridised also with AMX820, and the proportion of this biomass over eubacteria was increasing, together with the Anammox activity, in the reactor. Final FISH analysis of the enriched microorganisms showed that they were of the type 'Kuenenia stuttgartiensis' (hybridised with KST1273).

The biomass showed an activity of 0.18 g NH_4^+-N $gVSS^{-1}$ d^{-1} and the reactor was loaded at a NLR of 1.4 kgN m^{-3} d^{-1} achieving a removal efficiency of 82%. The maximum concentrations of ammonium and nitrite in the influent were 0.4 g NH_4^+-N dm^{-3} and 0.5 g NO_2^--N dm^{-3}.

Model simulations

The SBR was inoculated with 4 g sludge. This corresponds to about 6 g COD sludge. No distinction was made between Anammox and other organisms. Therefore it was assumed that this 6 g COD biomass consisted of approximately 1.8 g COD heterotrophs, 50 mg COD ammonia oxidisers, 50 mg COD nitrite oxidisers, 4 g COD inert biomass and 10 mg COD Anammox biomass based on a steady state analysis of the municipal WWTP biomass as proposed by Petersen *et al*.[26] These values were used as initial conditions for the simulations with the mathematical model.

The calculated values of the ammonium (S_{NH}), nitrite (S_{NO_2}) and nitrate (S_{NO_3}) concentrations are compared with the measured ones in Fig 5. From these data the calculated values agree well with the measured ones. Also the effluent concentrations are low, indicating the possibility of the Anammox reactor to treat nitrogen-rich streams.

In Fig 6, the time evolution of the Anammox organisms' concentration (X_{AN}), as well as the concentration of inert biomass (X_I) is depicted. The quantity of Anammox biomass is initially very low and starts to increase in an exponential way after 2 months. This is quantitatively in agreement with the results of the FISH analysis, ie after 2 months an increasing signal was observed. The simulations further show that Anammox become dominant after day 100. This

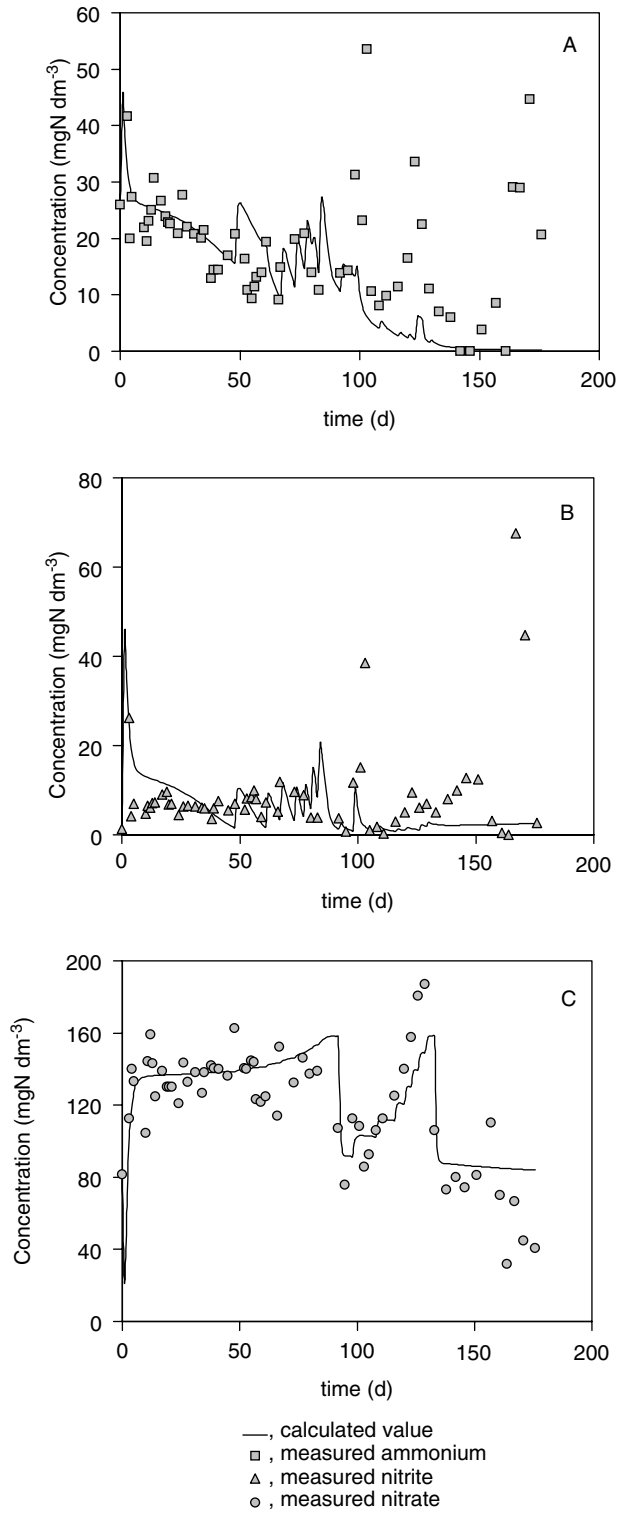

Figure 5. Comparison between the calculated and measured values of ammonium, nitrite and nitrate in the effluent of the reactor. After start-up these concentrations are low, showing the capability of the Anammox process to treat highly-loaded streams.

explains the colour change from brownish to reddish, as mentioned above.

At first, most of the biomass is heterotrophic (X_H) and autotrophic (X_{NH} and X_{NO}), as the reactor was inoculated with sludge from a municipal WWTP, but these populations decrease because they do not have substrates. No or very little autotrophic (X_{NH} and X_{NO}) biomass could remain in the reactor after start-up. This can be seen from the strong decrease in autotrophic biomass concentration in Fig 7.

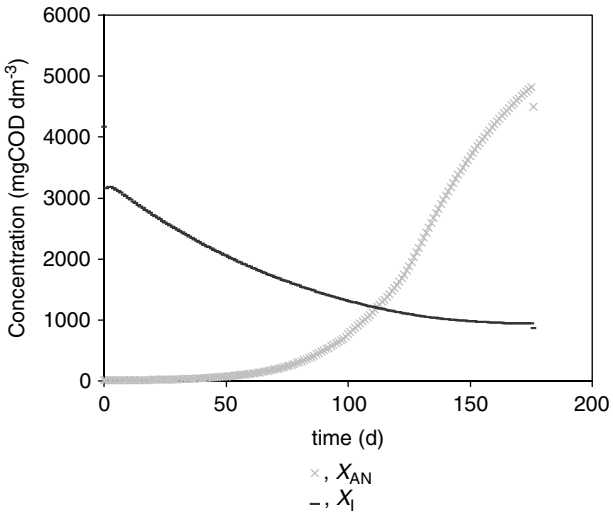

Figure 6. The calculated amounts of Anammox biomass (X_{AN}) and inert biomass (X_I) in the reactor. After day 60 the Anammox organisms start to grow exponentially and after day 100 the Anammox organisms become dominant.

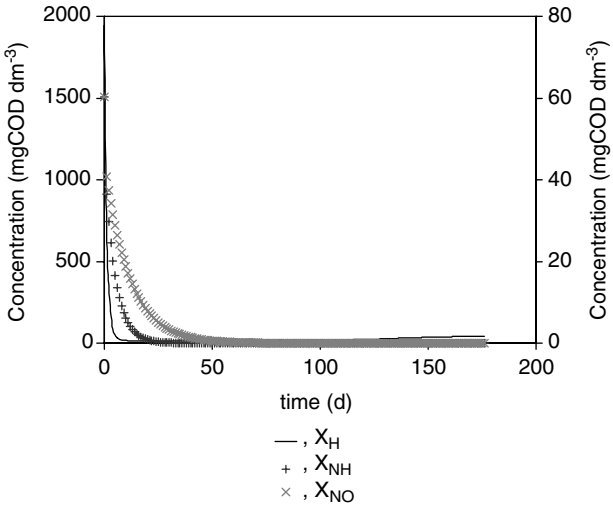

Figure 7. The calculated amounts of X_H, X_{NH} and X_{NO} over the total experimental period. No or very little autotrophic (X_{NH} and X_{NO}) biomass could remain in the reactor after start-up.

From Fig 7 it can be seen that the concentration of heterotrophic biomass also decreases markedly after start-up of the reactor. However, a detailed plot of the concentration of heterotrophic organisms from day 100 to the final day of the experiment (Fig 8) reveals that heterotrophs still remain in the system after this time and perform a 'background' process. This is probably because heterotrophic biomass is able to live on cell lysis products (cryptic growth) and from the biodegradable substrate in the influent even in the absence of oxygen, since these organisms can use nitrate and nitrite as electron acceptor. Autotrophic biomass is not able to do this and thus these organisms cannot survive in a strictly anoxic environment.

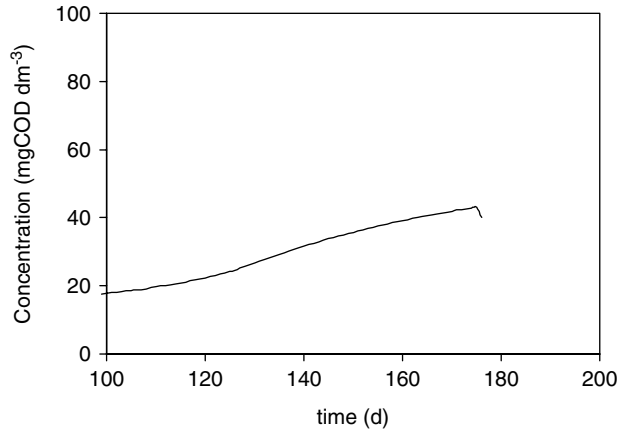

Figure 8. The calculated amounts of X_H from day 100 onwards indicating the presence of heterotrophs in the Anammox reactor during operation.

If, however, in a full-scale application oxygen was present, for instance from leaks, the aerobic bacteria would consume this oxygen for growth and thus allow the Anammox bacteria to grow in an anoxic environment.

CONCLUSIONS

The isolation of Anammox biomass from WWTP sludge requires an anaerobic atmosphere. The presence of oxygen, even in low quantities, will inhibit the process during the enrichment procedure because of the low initial concentration and low growth rate of the Anammox biomass.[11]

The SBR is a suitable system for the isolation of a microbial community with an extremely slow growth rate.[11] An efficient retention of the biomass was achieved, and biomass concentrations in the effluent were always very low. The SBR permits a homogeneous distribution of substrates, products and biomass, preventing the formation of local accumulations of nitrite that could inhibit the Anammox process.[24] The operation of the SBR here was stable and high nitrogen removal efficiency was reached (82%).

The Anammox SBR reactor was modelled in the modelling and simulation environment WEST®.[22] The Activated Sludge Model nr 1 was extended with a two-step nitrification–denitrification model and with the Anammox process to interpret the experimental results. Simulations were in good agreement with the measurement data and showed that both the Anammox biomass as heterotrophs were active in the reactor. The presence of these heterotrophs is important, because they can consume any oxygen that might leak into the system, as could the nitrifying bacteria if they were present. This way the reactor can still stay anoxic, which favours the activity of the Anammox biomass.

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