Modelling simultaneous anaerobic methane and ammonium removal in a granular sludge reactor

M-K.H Winkler a,b,*, K.F. Ettwig c, T.P.W. Vannecke a, K. Stultiens c, A. Bogdan a, B. Kartal c, E.I.P. Volcke a

a Department of Biosystems Engineering, Ghent University, Coupure Links 653, 9000 Gent, Belgium
b Department of Civil and Environmental Engineering, University of Washington, Seattle, WA 98195-2700, USA
c Microbiology, Radboud University Nijmegen, Heyendaalseweg 135, 6525 AJ, Nijmegen, The Netherlands

Article info
Article history:
Received 27 August 2014
Received in revised form
27 January 2015
Accepted 28 January 2015
Available online 7 February 2015

Keywords:
Anammox
Anaerobic methane oxidation
Granular sludge
Modelling
Simulation

Abstract
Anaerobic nitrogen removal technologies offer advantages in terms of energy and cost savings over conventional nitrification–denitrification systems. A mathematical model was constructed to evaluate the influence of process operation on the coexistence of nitrite dependent anaerobic methane oxidizing bacteria (n-damo) and anaerobic ammonium oxidizing bacteria (anammox) in a single granule. The nitrite and methane affinity constants of n-damo bacteria were measured experimentally. The biomass yield of n-damo bacteria was derived from experimental data and a thermodynamic state analysis. Through simulations, it was found that the possible survival of n-damo besides anammox bacteria was sensitive to the nitrite/ammonium influent ratio. If ammonium was supplied in excess, n-damo bacteria were outcompeted. At low biomass concentration, n-damo bacteria lost the competition against anammox bacteria. When the biomass loading closely matched the biomass concentration needed for full nutrient removal, strong substrate competition occurred resulting in oscillating removal rates. The simulation results further reveal that smaller granules enabled higher simultaneous ammonium and methane removal efficiencies. The implementation of simultaneous anaerobic methane and ammonium removal will decrease greenhouse gas emissions, but an economic analysis showed that adding anaerobic methane removal to a partial nitritation/anammox process may increase the aeration costs with over 20%. Finally, some considerations were given regarding the practical implementation of the process.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Methane is the end product of anaerobic digestion and it can be used for energy generation (van Lier et al., 2008). However, some of the methane remains dissolved in the effluent of anaerobic digesters and potentially escapes to the atmosphere during further downstream processing (Daelman et al., 2012). Given the high global warming potential of methane, about 34 CO₂ equivalents over a 100-year time scale, even small quantities of methane emissions can largely affect the carbon
footprint of a wastewater treatment plant (wwtp) (IPCC et al., 2013). Recently, a bacterial species capable of nitrite-dependent anaerobic methane oxidation (n-damo), ‘Candidatus Methylomirabilis oxyfera’ was discovered (Raghoebarsing et al., 2006; Ettwig et al., 2010). These microorganisms oxidize methane to carbon dioxide coupled to the reduction of nitrite to dinitrogen gas (Ettwig et al., 2010).

Anaerobic nitrogen removal technologies offer clear advantages in terms of energy and cost savings over conventional nitrification–denitrification systems. The n-damo process has so far not been applied in engineered systems, but microorganisms related to Methylomirabilis oxyfera were detected in nitrogen removal processes such as those treating anaerobic digester effluents (Luesken et al., 2011a; Ho et al., 2013), which contain high ammonium concentration and dissolved methane (Bandara et al., 2011). Ammonium can be conveniently removed from such reject waters with a combined partial nitritation – anammox process, as demonstrated at full-scale (van der Star et al., 2007). During partial nitritation, typically half of the ammonium is converted to nitrite, while nitrate formation is prevented. This is followed by anaerobic oxidation of ammonium (anammox) to dinitrogen gas with nitrite as the electron acceptor. As reject water contains high levels of ammonium in proportion to methane, it is potentially interesting to combine anaerobic ammonium and methane oxidizing bacteria to remove nitrogen and methane simultaneously (Luesken et al., 2011a).

Lab-scale studies have demonstrated that anammox and n-damo bacteria can coexist in a bioreactor and perform simultaneous removal of methane and ammonium (Luesken et al., 2011b; Zhu et al., 2011). Since both bacteria have a doubling time of more than ten days (Strous et al., 1998; Ettwig et al., 2009), proper biomass retention is needed to handle large volumetric flows and loading capacities such as encountered in a wwtp. Granular sludge consists of biofilm aggregates in the form of dense, fast-settling granules, resulting in compact systems, which allows a high loading rate due to a large biofilm surface area in the reactor (Beun et al., 2006). Therefore, granules offer a good option for simultaneous growth of both n-damo and anammox bacteria. One of the experiments with simultaneous methane and ammonium removal mentioned above (Zhu et al., 2011) was conducted with an inoculum from a granular sludge system, indicating that n-damo grew on granule surfaces already. In these systems, the sludge retention time is uncoupled from the hydraulic retention time. If n-damo would grow in flocs only, they would be washed out due to their slow growth rate. Due to the slow growth rates of both bacteria, experimental work aiming at process optimization can be very time consuming. Mathematical models are useful tools to study new unknown processes as it was demonstrated previously (Picioreanu et al., 1997; Hao et al., 2002; Volcke and van Loosdrecht, 2010). For the construction of a reliable model, realistic parameter values are essential. Therefore, in this study, the nitrite and methane affinity constant of n-damo bacteria were experimentally determined, and the yield coefficient of ndamo was calculated based on thermodynamic state calculations combined with experimental data. A mathematical model was constructed and applied in a simulation study to evaluate the influence of process operation on the coexistence of anaerobic methane and ammonium oxidizing bacteria in a single granule.

2. Material and methods

2.1. Thermodynamic state analysis and experimental determination of the biomass yield of n-damo bacteria

Since no direct measurements are available for the biomass yield of n-damo it was calculated based on experimental batch tests with M. oxyfera (Raghoebarsing et al., 2006; Ettwig et al., 2009) and thermodynamic state analysis. Thermodynamic calculations for the stoichiometric reactions of n-damo bacteria were performed according to the method of (Kleerebezem and Van Loosdrecht, 2010). First the mass and electron balances and reaction stoichiometry are set up, followed by a vector-based calculation method to derive the reaction stoichiometry of the metabolic redox reactions. The calculation combines the anabolic metabolism, which is defined per C-mole of formed biomass with the catabolic reaction, which is calculated by the Gibbs energy needed to produce one C-mole of biomass, to obtain the overall metabolic reaction. All calculations are available as Supplementary material S1.

2.2. Biological conversions – experimental determination of methane and nitrite affinity constants

A mathematical model was set up and the stoichiometric matrix, kinetic expressions are detailed in Supplementary material (S2, S3) and parameter values are listed in Supplementary material S4. It is important to note that, while ammonium (S_{NO3}) is the nitrogen source for biomass growth of anammox bacteria (Strous et al., 1998), n-damo bacteria, are bound to use nitrite (S_{NO2}) – in absence of other nitrogen sources – for incorporation in biomass (Ettwig et al., 2008, 2009; Rasigraf et al., 2012). In principle n-damo bacteria can also grow on ammonium but at high ammonium removal efficiencies and hence low ammonium concentrations they are forced to utilize nitrite for growth. Defining the n-damo yield coefficient as the biomass production on methane substrate, the following n-damo reaction stoichiometry is obtained by closing the COD and N balances:

\[
\begin{align*}
\frac{1}{Y_{n-damo}} & \cdot \mathrm{CH}_4 + \left( \frac{1}{1.71} \cdot \frac{1}{Y_{n-damo}} + i_{N2} \right) \\
\mathrm{NO}_2^- & \rightarrow \left( \frac{1}{1.71} \cdot \frac{1}{Y_{n-damo}} - 1 \right) - 2i_{N2} \mathrm{N}_2 + X_{n-damo}
\end{align*}
\]

Note that this reaction involves a net nitrite consumption, despite of the positive value of i_{N2}, which is resolved from the definition of the yield coefficient on methane. The use of nitrite also for assimilation into biomass leads to a lower overall dinitrogen production than the anabolic n-damo reaction alone. The corresponding reaction rate is given by Eq. (2), which describes substrate dependency of growth of n-damo bacteria by Monod kinetics and also considers nitrite inhibition.
The inhibition constant for n-damo \(K_{N\text{damo}}\) was set to 40 g NO\(_2\)\(-\)/Ngm\(^{-3}\) (He et al., 2013) and for anammox bacteria \(K_{N\text{an}}\) to 400 g NO\(_2\)\(-\)/Nm\(^{-3}\) (Letti et al., 2012) and these inhibition values were considered equal to 50% activity inhibition \((IC_{50})\) for nitrite (Cheng and Prusoff, 1973). The ammonium and nitrite affinity constants for anammox \(N\text{-damo}\) bacteria were based on literature values (Stroos et al., 1999).

N-damo bacteria were only recently discovered and not all parameters relevant for this microorganism have been experimentally determined so far. For this reason, several parameters were estimated based on available experimental data. Bacterial growth can be described with a first order system with a time constant \(1/\mu\), which is related to the observed doubling time by \(\mu = \ln 2/t_{\text{doubling time}}\). For n-damo bacteria, a doubling time as long as 14 days has been reported (Ettwig et al., 2009). As this value was observed during near-optimal growth conditions (no substrate limitation, no nitrite inhibition), the corresponding growth rate \(\mu\) can be considered as the maximum growth rate and is thus calculated as \(\mu_{\text{max}} = \ln 2/14 = 0.0495\) d\(^{-1}\). The methane affinity constant \(K_{\text{CH4}}\) of n-damo bacteria was estimated as 0.19 g COD m\(^{-3}\) based on reanalysis of published data (Ettwig et al., 2008; Rasigraf et al., 2012) and unpublished results. The nitrite affinity constant \(K_{\text{NO2}}\) was estimated from nitrite depletion in an enrichment culture under methane saturation (duplicate experiments) similar to the setup of previous research (Ettwig et al., 2008) and was found to be 0.6 g NO\(_2\)\(-\)/Nm\(^{-3}\) (Supplementary material S5).

### 2.3. Model implementation: reactor configuration and influent characteristics

A one-dimensional biofilm model was set up to describe the growth and decay of anaerobic methane oxidizers (n-damo) and anaerobic ammonium oxidizers (anammox) in a granular sludge reactor. The Aquasim model can be downloaded as Supplementary material S6. Spherical biomass particles (granules) were modelled to grow from an initial radius of 0.01 mm to a granule radius of 0.75 mm such that the reactor eventually contained 100 m\(^3\) of biomass, comprising both active biomass as well as inerts. The reactor volume was set to 400 m\(^3\). Nitrite was assumed to be supplied by partial nitrification in a separate compartment, which was not further addressed in this study. The influent was considered to be fed at a flow rate of 2500 m\(^3\) d\(^{-1}\) at a total nitrogen concentration of 1000 g N m\(^{-3}\) and ~100 g COD m\(^{-3}\) of methane (1.5 mM, close to saturation), respectively. The values are based on measurements from reject water (Hartley and Lant, 2006; Bandara et al., 2011) (Supplementary material S7). A schematic view of the biofilm reaction model describing the combined methane and ammonium removal process in a granule is depicted in Fig. 1. The model was implemented in the Aquasim software (Reichert, 1994). The model was applied in a simulation study to determine the influence of the influent nitrite: total nitrogen ratio, the substrate loading rate, and the granule size on substrate removal and granule composition. This was accomplished by varying one parameter at a time, while keeping all others constant.

### 2.4. Stoichiometric calculations and economic feasibility

An optimal supply of nitrite, ammonium and methane for n-damo and anammox was calculated based on the stoichiometric coefficients (Supplementary material S4) and previously set values for the yield coefficients. The stoichiometric nitrite concentration needed for the n-damo bacteria for the given methane concentration (100 g COD m\(^{-3}\)) was then calculated as \(S_{\text{NO2}} / S_{\text{CH4}} = 1 / 1.71(1 / Y_{\text{N-damo}} - 1) + i_{\text{NxB}} / 1 / Y_{\text{N-damo}}\). The nitrogen concentration (1000 g N m\(^{-3}\)) was assumed to be a mixture of nitrite and ammonium in a stoichiometric optimal ratio for anammox bacteria being \(S_{\text{NO2}} / S_{\text{NH4}} = 1 / Y_{\text{an}} + 1 / 1.14 / Y_{\text{an}} + i_{\text{NxB}}\). This results in an overall stoichiometric-optimal influent composition in terms of nitrite (555 g NO\(_2\)\(-\)/Nm\(^{-3}\)), ammonium (445 g NH\(_4\)\(+\)/Nm\(^{-3}\)), and methane (100 g COD m\(^{-3}\)), allowing for complete methane and ammonium removal. To evaluate the economic feasibility of simultaneous methane and ammonium removal in respect to the partial nitrification/anammox process a value of 0.5 € per kg nitrogen oxidized (to nitrite) was used to estimate the aeration costs for both processes (van Dongen et al., 2001).

### 3. Results and discussion

#### 3.1. Yield determination of n-damo bacteria by measurements and thermodynamic calculations

N-damo bacteria grow autotrophically, and thus fix carbon dioxide by the Calvin-Benson-Bassham cycle (Rasigraf et al., 2014). In principle, they are able to assimilate ammonium as nitrogen source, but were grown without ammonium in the medium, and are hence bound to assimilate nitrite (Ettwig et al., 2008, 2009). In the presence of ammonium, as in the simulated case, anammox bacteria are co-enriched, and consume all ammonium supplied, so that most likely no ammonium is left for assimilation by M. oxyfera (Luesken et al., 2011b). Therefore, unlike to the study of Chen et al. (2014), nitrite and not ammonium was assumed to be assimilated into biomass. Assuming an optimal energy usage of methane and nitrite yielded a stoichiometric equation of:

\[
1\text{NO}_2^- + 0.434\text{CH}_4 + 1\text{H}^+ \rightarrow 0.492\text{N}_2 + 1.28\text{H}_2\text{O} + 0.335\text{CO}_2 + 0.099\text{C}_2\text{H}_4\text{O}_3\text{N}_2
\]

(A)

However, the stoichiometric nitrite: methane ratio of 2.3 (1/0.43) did not fit the measured ratio from experimental data exactly. Kinetic measurements from previous research reported a nitrite over methane ratio of 1/0.415 = 2.4 (Raghoebarsing et al., 2006; Ettwig et al., 2009). The stoichiometric Equation (A) was hence fitted by changing the biomass yield such that the reported nitrite over methane ratio of 2.4 was met, resulting in the following equation:
NO_2^- + 0.415CH_4 + 1H^+ → 0.493N_2 + 1.27H_2O + 0.348CO_2 + 0.066C_14H_20O_5N_2  (B)

A sensitivity analysis was conducted in which the optimal stoichiometric media composition was used. The yield of Anammox bacteria was kept constant (0.17 g COD g^{-1} N) whereas the yield of n-damo bacteria was varied beyond its default value of 0.16 g COD g^{-1} N (corresponding with 0.066 mol biomass per mol of nitrite) to determine its effect on the competition. N-damo bacteria could compete for substrate as long as their yield coefficient was higher than 0.13 g COD g^{-1} N; at smaller values n-damo bacteria were washed out (Supplementary material S9).

3.2. Granule structure and influence of nitrite: total nitrogen ratios

The model in this study showed that anammox bacteria mostly grew on outer layers whereas n-damo bacteria mainly grew underneath the layer of anammox bacteria, which can be explained by slower growth rates of n-damo bacteria with respect to anammox bacteria. The most inner part of the granules consisted of inert compounds (Fig. 1).

In the model, different NO_2^- – N: total N ratios (total N was kept at 1000 g N m^{-3}) were tested at a fixed methane concentration (100 g COD m^{-3}) to study at which influent composition both bacteria could coexist. At lower ratios (ammonium in excess), n-damo bacteria were outcompeted by anammox bacteria. However, at ratios close to the stoichiometric equilibrium, needed to completely remove methane and ammonium, n-damo bacteria remained in the system. At high ratios (nitrite in excess), anammox bacteria got limited by ammonium, leading to an accumulation of nitrite while methane was completely removed, until an inhibitory concentration of nitrite was reached (Fig. 2). N-damo bacteria can accordingly only grow in a narrow range close to the stoichiometric optimum influent composition.

3.3. Influence of substrate loading rate

Previous simulation studies have evaluated the effect of inflow variations on a partial nitrification–anammox biofilm process even though at that time it was not known whether AOB and anammox bacteria could grow in a biofilm (Hao et al., 2002). A similar approach was chosen here to assess the effect of the total biomass volume on the bulk liquid concentrations (Fig. 3A) as well as on the biomass fractions of n-damo and anammox bacteria (Fig. 3B). The competition of both bacteria was evaluated for influent containing the stoichiometric ratios needed for complete ammonium and methane removal at a fixed biomass volume retained in the system. In a wastewater treatment reactor (such as in an aerobic granular sludge reactor), the sludge retention time is controlled by selectively removing sludge from the system. Considering a fixed biomass volume is therefore a reasonable assumption. Since
the substrate loading was kept constant in the model, the biomass specific substrate loading was higher at lower sludge volumes.

For a low amount of biomass, the biomass specific substrate loading of all substrates was too high for a complete substrate removal. This was leading to high ammonium and (inhibitory) nitrite concentrations and consequently to low removal efficiencies (Fig. 3A and B operating zone I). When the biomass volume was increased stepwise from 10 to 50 m³, anammox bacteria consumed nitrite to levels at which nitrite

![Fig. 2](image1)

![Fig. 3](image2)

**Fig. 2** – (A) Influence of influent nitrite fraction at fixed methane concentration (100 g COD m⁻³) on reactor performance in terms of (●) ammonium, (■) methane, (▲) nitrate, (♦) nitrite, and (●) nitrogen gas as well as (B) biomass fraction of (◊) inerts, (-keys) anammox, and (keys) ndamo bacteria at different influent nitrite: total nitrogen ratios [%] and at a fixed influent methane concentration of 100 g COD m⁻³. The influent total nitrogen concentration was kept at 1000 g N m⁻³. At the intersection (denoted by grey line) the media contained the ideal stoichiometric ratio for simultaneous removal of ammonium and methane (A) enabling coexistence of anammox and ndamo (B).

**Fig. 3** – Influence of biomass volume (A) on reactor performance in terms of (○) biomass specific loading rate, (+) biomass specific removal capacity, (□) nitrogen removal efficiency, and (▲) methane removal efficiency as well as (B) biomass profiles of (▲) anammox bacteria, (●) ndamo bacteria, and (■) inerts at different biomass volume fractions in the reactor. The total volume of the reactor was considered to be 400 m³. In operating zone I (A) no methane was removed and (B) no ndamo were present. In operating zone II methane was removed (A) and ndamo grew in the system (B). For biomass volumes between 50 and 100 m³ oscillations occurred (grey area; (A, B, C)). Graph C was obtained for a biomass volume of 80 m³. The denotation is NH₄⁺ (black -), NO₂⁻ (black –), CH₄ (grey--) effluent concentrations (g N m⁻³ and g COD m⁻³).
inhibition was lower, leading to a stepwise increase in their biomass activity (and growth rate). In this operating zone (10–50 m³) ammonium was in excess and despite stoichiometric ratios of substrates in the influent (555 g NO₂⁻ m⁻³; 445 g NH₄⁺ m⁻³; 100 g COD m⁻³ (CH₄)) n-damo bacteria lost the competition for nitrite to anammox bacteria, which dominated the system (Fig. 3B). At these conditions did the low methane affinity constant of n-damo bacteria not give any competitive advantage and was overruled by the high nitrite affinity of anammox bacteria compared to n-damo bacteria (Figs. 2A and 3 operating zone I).

Only when the biomass volume was set high enough (>50 m³), corresponding to a sufficiently low biomass loading rate (<1gN/gVSS/d) such that anammox could consume almost all ammonium, n-damo bacteria were able to grow (Luesken et al., 2011b). Under these conditions the biomass specific removal capacity got close to what is needed to completely remove all nitrogen and methane. However, only from a biomass volume of 100 m³ and higher, the biomass specific removal rate coincided with the nitrogen loading rate, corresponding with complete and stable methane and ammonium removal. The results showed that even if the influent contained the ideal stoichiometric ratios for complete ammonium and methane removal, the biomass concentration needed to be sufficiently high (or the biomass specific substrate loading sufficiently low) to allow simultaneous methane and ammonium removal. At lower biomass concentrations (too high biomass loading rate), n-damo bacteria were outcompeted by anammox bacteria. Note that the same results in terms of the biomass loading rate were obtained when varying the influent flow rate instead of the biomass concentration.

A sensitivity analysis was conducted under conditions with excess ammonium to evaluate the effect of a range of realistic nitrite affinity constants of n-damo bacteria on their competition with anammox bacteria. This test was conducted to evaluate to which extent the model outcome relied on a very accurate experimental determination of the nitrite affinity constant (Supplementary material S4, S8). The results confirmed that under the entire range of currently known realistic nitrite affinity constants, n-damo bacteria would suffer washout at high ammonium concentrations, as previously observed (Luesken et al., 2011b). Hence, n-damo bacteria can only survive if nitrite is in excess of ammonium, so that additional nitrite cannot be utilized by anammox bacteria. Under high ammonium concentrations, n-damo bacteria cannot be favoured through elevated methane concentrations because the competition depends on nitrite and not on methane.

### 3.4. Oscillatory reactor behaviour

During the transition period from near-complete (50 m³) to full substrate removal (80 m³), the removal efficiency was not stable and resulted in oscillating substrate removal efficiencies, similar to a competition behaviour known from general ecology (Berryman, 1992) (Fig. 3A, B and C grey area). The oscillating pattern (Fig. 3C, Supplementary material S10) can be explained by the affinity constants of both bacteria. In microbiology the competition of two species for one substrate is driven by their substrate affinity (Andrews and Harris, 1986) and their doubling time. A good example is the competition between two common genera of nitrite oxidizing bacteria: *Nitrospira* (K-strategist winning at low substrate concentrations) and *Nitrobacter* (R-strategist winning at high substrate concentration). *Nitrospira* possess a low maximum specific growth rate (in this case like n-damo), but is well adapted to low nitrite concentrations (unlike n-damo), whereas *Nitrobacter* is known to be a relatively fast-growing NOB (like anammox) with low affinities to nitrite (unlike anammox) (Schramm et al., 1999; Kim and Kim, 2006). According to the r-K selection theory, n-damo bacteria cannot compete with anammox for nitrite in the presence of excess ammonium due to their lower affinity constant and growth rate. This means that, n-damo and anammox bacteria can only grow together if enough nitrite remains for methane oxidation. At a biomass volume of 50 m³ the amount of anammox bacteria was sufficient to oxidize ammonium almost completely (Fig. 3 operating zone I). At biomass volumes from 50 to 80 m³ (Fig. 3 grey area), the biomass specific loading (loading rate per VSS) closely (but not fully) matched the biomass specific removal capacity (removal rate per VSS). Since the reactor was fed in all scenarios with a medium fulfilling the stoichiometric influent conditions needed for complete methane and ammonium removal, low effluent ammonium concentration and lower maximum specific growth rates of anammox bacteria corresponded to elevated methane and incomplete nitrite effluent concentrations. Consequently, n-damo bacteria had a high maximum specific growth rate and a high nitrite reducing activity. This in turn resulted in the collapse of the growth of anammox bacteria and a peak in ammonium and nitrite effluent concentrations. At higher ammonium and nitrite concentrations anammox bacteria started to grow again, leading to decreasing nitrite concentrations, an increase in growth rate of anammox bacteria, a decrease in methane removal and a lower n-damo growth rate thereby starting a new oscillation cycle (Fig. 3C). It should be stressed that n-damo bacteria did not actively compete for nitrite. If in the presented scenario (Fig. 3C) the influent nitrite concentrations would be lowered such that anammox bacteria could consume all nitrite with ammonium, n-damo bacteria would be washed out.

For biomass volumes of 80 m³, oscillations in bulk liquid concentrations stopped and the biomass specific removal capacity fully matched the biomass loading rate leading to a complete, stable and simultaneous methane and ammonium removal and an equal distribution between anammox and n-damo bacteria (Fig. 3B). At high biomass volumes, the system operated below the maximal substrate oxidizing capacity as indicated by the lower biomass specific removal capacity and hence activity, respectively (Fig. 3 operating zone II). However, in this operation zone the removal capacity turned out to be robust. A system designed for simultaneous methane and ammonium removal should hence not be operated close to its maximum biomass specific substrate removal capacity (Fig. 3 intersection operating zone I and II). These insights into the possible oscillatory process operation are of vital importance for a successful implementation of stable and reliable simultaneous ammonium and methane removal in wastewater treatment systems.
3.5. **Influence of granule size on the reactor performance**

An influence of the granule size on bacterial fractions occupying the granule can be expected from the increasing substrate surface loading for increasing granule diameter (lower surface: volume ratio) and diffusion limitations of different substrates (Winkler et al., 2011; Volcke and van Loosdrecht, 2012). For relatively small granules (<0.3 mm), the granule consisted entirely out of active biomass and therefore there was no accumulation of inerts. Under these conditions, the nitrogen and methane removal efficiencies remained quite constant with increasing granule size, similar to other simulation studies (Ni et al., 2009), while the fraction of n-damo bacteria increased at the expense of anammox bacteria. The reason for this is that n-damo bacteria were located more in the inner part of the granule and thus were faced with lower substrate concentrations for increasing granule size, leading to a decreased activity and thus implying the need for more biomass to perform the same conversion.

As the granule size increases, substrate diffusion into the centre of the granules becomes more difficult, which resulted in the accumulation of inert material (Fig. 4B) thus implying less space for active biomass growth at the given constant total biomass volume. As a result, the anammox conversion efficiency decreased with increasing granule size (Fig. 4A). The methane removal efficiency also decreased for larger granules and was more pronounced than the decrease in the anammox conversion (Fig. 4A). Anammox bacteria grow faster and have a higher affinity for nitrite than n-damo bacteria, which constitutes an advantage for the former in the competition for space on the surface of the granule. This effect is more severe on larger granules which have a smaller specific surface. Overall, it is recommended to keep the granule size small enough to maintain high ammonium and methane removal efficiencies, but not too small to still ensure good settling properties and a high biomass retention capacity. A proper biomass wasting should hence be applied to select for a certain granule size.

3.6. **Cost estimation and implications for practice**

Aeration costs account for approximately half of the costs created during wastewater treatment. Aeration is therefore a sensitive parameter for the economic feasibility of new treatment processes. Anammox-based treatment systems are economically interesting because ammonium only needs to be partly oxidized to nitrite (not to nitrate) compared with conventional nitrification—denitrification systems, hence reducing aeration costs by approximately two-fold (van Dongen et al., 2001). The theoretical cost estimation for the simultaneous removal of ammonium (1000 g N m⁻³) and methane (100 g COD m⁻³) from reject water (scenario B) showed that the costs are increased by 23% compared to a partial nitritation anammox two-stage treatment system (scenario A) (Table 1). The reason is that more ammonium needs to be oxidized to nitrite to remove both ammonium and methane present in the reject water than for anammox-based ammonium removal only. Currently there are no penalties for high greenhouse gas emissions from wastewater treatment, but it is highly likely that future regulations for threshold values of emitted greenhouse gases from wastewater may justify the application of *M. oxyfera* for the treatment of reject water or other streams containing high ammonium and methane concentrations.

A challenge in the application of n-damo bacteria (scenario B) is that methane needs to be kept in the liquid phase. A preceding partial nitritation step to yield the desired nitrite: ammonium mixture would strip all methane from the liquid phase. An alternative option could be to split the reject water in two streams: one for the oxidation of all ammonium to nitrite and another stream containing methane and ammonium. However, in this option, half of the methane would still be stripped in the aerated tank. In addition, the required complete oxidation of ammonium to nitrite will entail additional costs, for caustic addition, in the typical case that the wastewater does not contain enough alkalinity to buffer the acidifying nitrification.

A more plausible solution could be to implement methane and ammonium removal in a single reactor system, in which the oxygen level is controlled to ensure simultaneous oxidation of part of the ammonium on the one hand and anaerobic methane and ammonium oxidation on the other hand. Methane stripping might be remedied to a certain extent by recirculation of the aeration gas, which might allow redissolution of stripped methane into the liquid phase. The feasibility of such an operation needs further investigation, e.g. with respect to the competition of n-damo with aerobic methane oxidizing bacteria. Another challenge in practice will be the control of the produced nitrite: ammonium ratios to

![Fig. 4 – Influence of granule size on steady state (A) reactor performance in terms of (●) methane removal and (●) ammonium removal efficiency (▲) and nitrate effluent concentrations as well as (B) biomass concentrations of (●) anammox, (●) ndamo, and (●) inerts. Simulations were conducted at stoichiometric influent concentrations of methane (100 g COD m⁻³) and nitrogen components (1000 g total N m⁻³, namely 555 g NO₂⁻ – Nm⁻³ and 445 g NH₄⁺ – Nm⁻³).](image-url)
needed to evaluate the profit of such a solution. A complete and more elaborated economic feasibility study is required for understanding the inner workings of anaerobic methane oxidizing bacteria only. However, a complete study would include the expensive addition of organic electron donor (presently mostly methanol) would be required (Peng et al., 2007). In such a case, methane originating from anaerobic digestion could be applied without ammox bacteria, such as for the treatment of wastewater which is lacking organic carbon and for which the expensive addition of organic electron donor (presently mostly methanol) would be required (Peng et al., 2007). In such a case, methane originating from anaerobic digestion could be used for denitrification purposes in a dedicated reactor for anaerobic methane oxidizing bacteria only. However, a complete and more elaborated economic feasibility study is needed to evaluate the profit of such a solution.

4. Conclusions

- The nitrite and methane affinity constant of anaerobic methane oxidizing (dnamo) bacteria was determined experimentally and the yield coefficient of n-damo bacteria was calculated from experimental data combined with thermodynamic state analysis.
- Simultaneous anaerobic methane and ammonium removal in a granular sludge reactor is feasible through the coexistence of anammox and n-damo bacteria, provided that nitrite, ammonium and methane are supplied in optimal stoichiometric amounts, and that the biomass loading rate is sufficiently low (i.e., high biomass concentration in reactor or low influent flow rate).
- A system designed for simultaneous methane and ammonium removal should not be operated close to its maximum biomass specific substrate removal capacity due to the risks of oscillating removal efficiency.
- Smaller granules resulted in higher ammonium and methane removal efficiencies due to lower diffusion limitations.
- The additional removal of methane besides ammonium may increase the aeration requirements by over 20% compared to partial nitritation – anammox treatment systems.

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Scenario A Partial nitritation – anammox</th>
<th>Scenario B Partial nitritation – anammox + ndamo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium oxidized</td>
<td>g NH₄⁺ – Nm⁻³</td>
<td>430</td>
<td>555</td>
</tr>
<tr>
<td>Aeration costs</td>
<td>€ m⁻³</td>
<td>0.21</td>
<td>0.28</td>
</tr>
<tr>
<td>Additional costs of scenario B</td>
<td>%</td>
<td>23%</td>
<td></td>
</tr>
</tbody>
</table>

Loosdrecht for discussions as well as Katinka van de Pas-Schoonen for technical support. Mari Winkler was funded by a Marie Curie Intra-European Fellowship (PIEF-GA-2012-329328). Katharina Ettwig and Boran Kartal were funded by veni grants (Projects 863.13.007 and 863.11.003, respectively) from the Dutch Science Foundation (NWO). Karin Stultiens was supported by an ERC Advanced grant on anaerobic ammonium oxidation (No. 2322937). Thomas Vannecke is supported by the Research Foundation-Flanders (FWO) through a Ph.D. fellowship.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2015.01.039.

REFERENCES

Cheng, Y., Prusoff, W., 1973. Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108.

Acknowledgements

The authors thank R. Kleerebezem for help with the thermodynamic calculations and Huub op den Camp and MCM van Loosdrecht for discussions as well as Katinka van de Pas-Schoonen for technical support. Mari Winkler was funded by a Marie Curie Intra-European Fellowship (PIEF-GA-2012-329328). Katharina Ettwig and Boran Kartal were funded by veni grants (Projects 863.13.007 and 863.11.003, respectively) from the Dutch Science Foundation (NWO). Karin Stultiens was supported by an ERC Advanced grant on anaerobic ammonium oxidation (No. 2322937). Thomas Vannecke is supported by the Research Foundation-Flanders (FWO) through a Ph.D. fellowship.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2015.01.039.


