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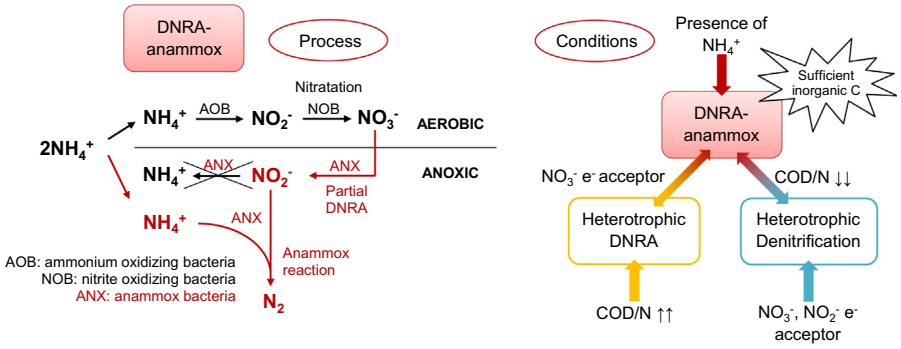
HIGHLIGHTS

- Dissimilatory nitrate reduction to ammonium (DNRA) by anammox bacteria.
- Limitation of COD and presence of ammonium favoured anammox bacteria to pursue DNRA.
- DNRA by anammox bacteria could improve nitrogen removal in the main line.

GRAPHICAL ABSTRACT



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ABSTRACT

Anammox bacteria can perform dissimilatory nitrate reduction to ammonium (DNRA) with nitrite as intermediate coupled to the oxidation of volatile fatty acids (VFA). Batch tests with enriched anammox and a co-culture of anammox and heterotrophic bacteria showed the capacity of *Candidatus* 'Brocadia fulgida' to perform the DNRA coupled to the anammox reaction (DNRA-anammox) at a high rate although the culture was not previously adapted to VFA. From thermodynamic calculations it could be stated that low COD/N influent ratios favour the DNRA-anammox transformation over heterotrophic conversions since more free energy is gained. A process scheme is proposed for an innovative nitrogen removal system in which the nitrate produced by nitrite oxidizing bacteria and/or anammox bacteria is converted during DNRA-anammox pathway, resulting in a sustainable nitrogen removal from municipal wastewater while circumventing the troublesome out-selection of nitrite oxidizing bacteria encountered in mainstream applications.

1. Introduction

The anaerobic ammonium oxidation (anammox) conversion comprises the oxidation of ammonium with nitrite as electron acceptor to nitrogen gas under anoxic conditions. The implementation of the anammox process needs a preceding step in which half of the ammonium in the wastewater is oxidized to nitrite by

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ammonium oxidizing bacteria (AOB) (partial nitritation). For the success of this partial nitritation-anammox system (Fig. 1A), the further oxidation of nitrite to nitrate by nitrite oxidizing bacteria (NOB) must be prevented. Compared to conventional nitrification-denitrification, partial nitritation-anammox requires up to 63% less aeration energy, produces 70–80% less sludge, emits almost no CO_2 and safes up to 100% of costly external organic carbon addition (Siegrist et al., 2008).

Anammox-based processes were successfully implemented at several full-scale plants to treat warm ammonium-rich reject water from anaerobic digesters (sidestream) (Abma et al., 2010; van der Star et al., 2007; Wett, 2007). Due to the advantages of this technology in the sidestream (1% of the volume flow of the wastewater treatment plant, WWTP), current scientific research is focused on the implementation of anammox technology in the mainstream (99% of the volume flow) of the WWTP (Kartal et al., 2010). However, different challenges need to be overcome for successful application of this process in the mainstream. As opposed to the sidestream, the mainstream has lower temperature (down to ca. 10 °C instead of 30 °C), stronger effluent requirements, lower ammonium concentrations (ca. 50 g N m^{-3} instead of 500 g N m^{-3}), and contains higher amount of biodegradable organic matter (higher COD/N ratio). Anammox is known to optimally thrive at 35 °C, but different studies have shown that an implementation of the anammox process at low temperature is also feasible (Hendrickx et al., 2014; Lotti et al., 2014; Vazquez-Padin et al., 2009). In these studies, the nitrite was added directly in the anammox reactor whereas, in practice, the nitrite needs to be formed within the process. In this regard, Lotti et al. (2015) tried to establish partial nitritation-anammox in a pilot-scale reactor to treat a mainstream effluent and found that the formation of nitrite (and suppression of NOB) was the limiting factor of the process. Therefore, one of the main bottlenecks for the application of anammox-based technologies in the mainstream seems to be an effective nitrite supply to the anammox pathway. Although NOB have a lower affinity for oxygen than AOB, the presence of NOB cannot be out ruled in the main line even if operated under oxygen limiting conditions (Pérez et al., 2014). The difficulty to deselect against NOB can be explained by growth rates. At high temperatures, AOB have a faster growth rate than NOB. Thus, NOB can be outcompeted by operating the system at sludge retention times (SRT) below the minimal required growth rate of the nitrite oxidizers (Hellinga et al., 1998). However, at low temperatures (such as in the mainstream) NOB possess a faster growth rate than AOB and therefore NOB cannot be out selected based on SRT control and hardly controlled by oxygen limitation either.

Beside nitritation as a manner to provide nitrite to the anammox reaction during municipal wastewater treatment, other approaches concern the partial denitrification until nitrite only (Fig. 1B). This conversion can be performed by either autotrophic or heterotrophic microorganisms. For the heterotrophic denitrification, the limitation of organic carbon and relatively short anoxic periods positively influence the accumulation of nitrite (Du et al., 2015). In addition, recent studies have shown that low amount of organic compounds (limitation of organic carbon) in partial nitritation-anammox systems can improve the overall nitrogen removal through the partial heterotrophic denitrification, which reduces nitrate to nitrite that can be taken up by anammox organisms (Mozumder et al., 2014).

Regarding the presence of organic carbon compounds in the wastewater, it was recently discovered that anammox bacteria are able to perform dissimilatory nitrate reduction to ammonium (DNRA) with nitrite as intermediate using volatile fatty acids (VFA) as electron donor (organotrophic activity) (Guven et al.,

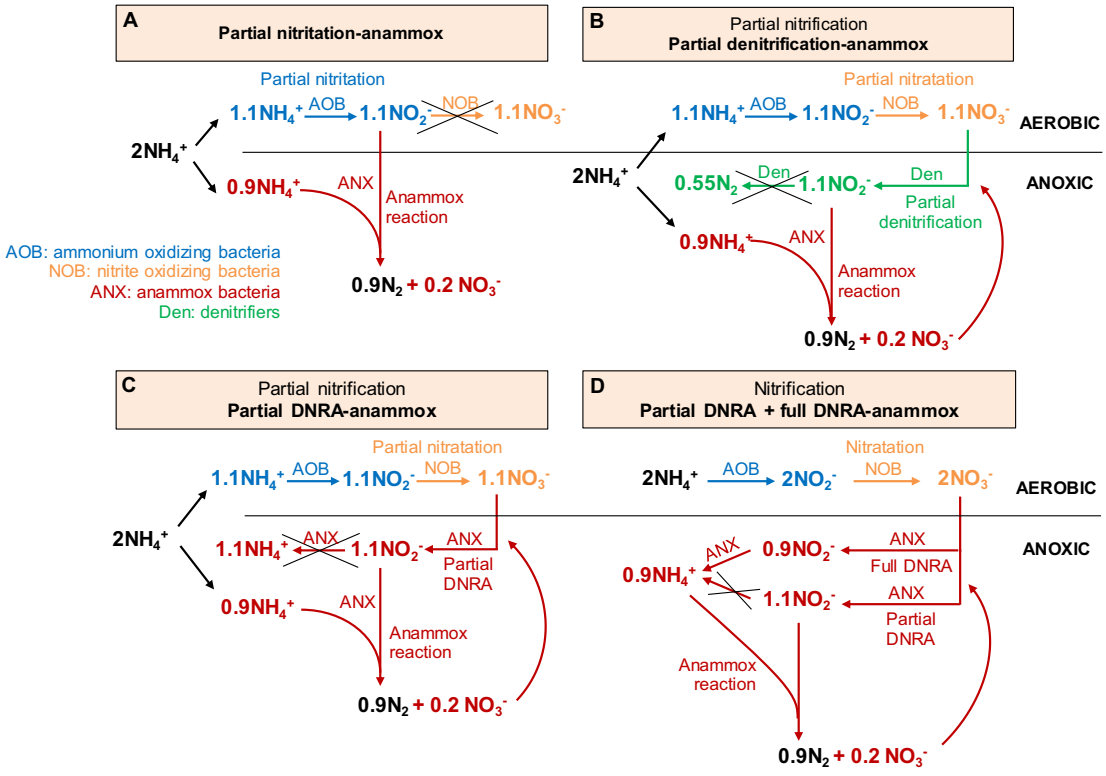

Fig. 1. Comparison of different nitrogen removal configurations involving the anammox process: A) partial nitritation-anammox; B) partial denitrification-anammox; C) partial DNRA (reduction of nitrate to nitrite only by anammox bacteria) and anammox reaction with the nitrite formed and the ammonium available in the environment; D) partial DNRA and anammox reaction with ammonium formed during full DNRA by anammox bacteria.

2005; Kartal et al., 2007a,b; Shu et al., 2015; Winkler et al., 2012a). The VFA are oxidized to CO_2 via acetyl-CoA (Russ et al., 2012) but are not assimilated into biomass (Guven et al., 2005; Kartal et al., 2007b), hence keeping sludge production low. In some experiments, DNRA by anammox bacteria was reported to occur coupled to the anammox transformation (from where the bacteria get energy to grow) in two ways: converting the nitrate to nitrite only (partial DNRA) and combining the nitrite formed with external ammonium present in the environment to yield nitrogen gas in the anammox reaction (Fig. 1C); and also, anammox bacteria were shown to reduce part of the nitrate to the intermediate nitrite and one part to ammonium, the end product of DNRA (full DNRA), to then utilize the formed nitrite and ammonium for anaerobic ammonium oxidation (Fig. 1D) (Kartal et al., 2007a).

The main challenge to establish DNRA by anammox bacteria is their out-competition by heterotrophic bacteria, which compete for nitrate and acetate (heterotrophic denitrification) and which have a much faster growth rate. Heterotrophs are also known to perform DNRA. In general, for heterotrophs, DNRA pathway is promoted over denitrification under nitrate limiting conditions (i.e. high COD/N ratios) and when nitrate (and not nitrite) is the main electron acceptor present (Kraft et al., 2014; van den Berg et al., 2015). However, little is known about the conditions that promote anammox bacteria performing DNRA over the heterotrophic utilization of carbon. Some anammox species, enriched in lab-scale reactors, were found to effectively outcompete heterotrophs for propionate (*Candidatus* 'Anammoxoglobus propionicus', (Kartal et al., 2007b), and acetate (Candidatus 'Brocadia fulgida', (Kartal et al., 2008)) in the presence of ammonium but the conclusions remained descriptive.

The main objective of this study was to gain understanding on the DNRA pathway of anammox bacteria. The bacterial populations were determined in three case-studies and the factors promoting the DNRA by anammox bacteria over heterotrophic transformations evaluated. Thermodynamic calculations were determined to assess the energy gained per mole of substrate and electron transferred during different transformations involving the conversion of nitrate and acetate. The outcome of this research suggests the joint implementation of partial DNRA and anammox processes by anammox bacteria, which can circumvent the accumulation of nitrate in the effluent at the same time providing nitrite to the anammox reaction by partial DNRA, resulting in an innovative and sustainable system to remove nitrogen from municipal wastewater.

2. Materials and methods

Nitrate reduction and organotrophic activity by anammox bacteria were assessed in 3 case-studies involving different conditions. Batch tests were conducted using anammox biomass from a full-scale granular sludge reactor (Case A, described in Section 2.1) and sludge from an anoxic/aerobic sequencing batch reactor (SBR) containing anammox bacteria and heterotrophic phosphate accumulating organisms (PAOs) among other communities (Case B, described in Section 2.2). The third set-up consisted of an anammox SBR operated under a semi-continuous exposure to organic carbon (acetate) (Case C, described in Section 2.3) (see Table 1).

2.1. Batch tests with anammox granular sludge from a full-scale WWTP (Case A)

A series of 5 different batch tests was performed using biomass taken from the anammox granular sludge reactor from the WWTP of Rotterdam (The Netherlands). The biomass was first washed with tap water and then used in each batch test, which were conducted in a 2 L bioreactor. Before each test, nitrogen gas was supplied to the bioreactor for 30 min to ensure anoxic conditions. Different initial substrate concentrations were added in each batch test with a syringe through a septum (Table 1, Case A), together with a mineral medium according to van de Graaf et al. (1996) and Dapena-Mora et al. (2004) (Supplementary Information (SI), Table S1. 1 and Table S1. 2). Inorganic carbon was provided in the mineral medium in excess as $KHCO_3$ (1 g $KHCO_3$ L^{-1}). The temperature was controlled at 30 ± 1 °C by using a heating water jacket connected to a circulating bath and the pH was not controlled. Methanol (MeOH) was added in some batch tests to assess the potential inhibitory effect of the alcohol on the bacterial activities.

Table 1
Initial substrate concentrations for the batch tests with biomass from a full-scale anammox granular sludge reactor (Case A), from a bubble column SBR containing anammox bacteria and PAOs among other communities (Case B), and feeds used during the operation of the SBR of Case C.

		NH_4^+ (g N m^{-3})	NO_3^- (g N m^{-3})	NO_2^- (g N m^{-3})	Acetate (g COD m^{-3})	MeOH (mM)
Case A						
Batch test						
A.1	$NH_4^+ + NO_2^-$	200		200		
A.2	$NH_4^+ + NO_2^-$ + MeOH	200		200		12
A.3	$NH_4^+ + NO_3^-$ + acetate	200	100		460	
A.4	$NH_4^+ + NO_3^-$ + acetate + MeOH	100	50		230	12
A.5	NO_3^- + acetate		50		230	
Case B						
Batch test						
B.1	$NH_4^+ + NO_2^-$	147		50		
B.2	$NH_4^+ + NO_2^-$ + MeOH	147		50		5
B.3	$NH_4^+ + NO_3^-$ + acetate	147	50		76	
B.4	NO_3^- + acetate		50		76	
B.5	NO_3^- + acetate + MeOH		50		76	5
B.6	$NH_4^+ + NO_2^-$ + acetate	147		50	76	
B.7	NO_2^- + acetate			50	76	
B.8	NO_2^- + acetate + MeOH			50	76	5
Case C						
'Anammox regime'						
Feed C.1	$NH_4^+ + NO_2^-$	125–200		100–200		
'Regime under semi-continuous exposure to acetate'						
Feed C.2	$NH_4^+ + NO_3^-$ + acetate (no HCO_3^-)	50–100	50–100		50–100	
Feed C.3	$NH_4^+ + NO_2^-$ (no HCO_3^-)	50–200		50–200		

Liquid samples were collected at different times along the batch tests to determine the consumption rates.

2.2. Batch tests with anammox and heterotrophic bacteria (Case B)

A series of 8 batch tests was performed with granular biomass collected from a bubble column SBR operated under intermittent anoxic/aerobic conditions, carrying out nitritation-anammox and organic carbon removal via DNRA process as described in Winkler et al. (2012a), but with the difference that the reactor operation was changed to a twofold higher carbon/nitrogen ratio in the influent (from 0.5 to 0.9) (SI, PHASE III in Fig. S2. 1). The biomass was collected from the SBR, washed with tap water and aerated to remove excess of ammonium. All batch tests were completed in glass flasks containing 250 mL, in which nitrogen gas was supplied for 25 min prior to the start of the tests to ensure anoxic conditions. Each test was induced with a different set of substrates as listed in Table 1 (Case B) and same mineral medium and trace elements as used during the operation of the reactor (Winkler et al., 2012a). Temperature and pH were not controlled during the batch tests. As in the batch tests of Case A (Section 2.1), methanol was added in some tests. Samples were taken over time during each batch test and the volatile suspended solids were determined.

2.3. Sequencing batch reactor under semi-continuous acetate supply (Case C)

A SBR of 2.3 L was inoculated with anammox granular sludge from the CANON reactor from the wastewater treatment plant of Amersfoort (The Netherlands). The reactor was operated in 6 h cycles with two different regimes (SI, Fig. S3. 1).

'Anammox regime' lasted 1 month and compromised 150 min of continuous feeding + mixing (Feed C.1 (Table 1, Case C), NH_4^+ + NO_2^-), 120 min of mixing, 60 min settling, and 30 min decanting.

'Regime under semi-continuous exposure to acetate' lasted 1 month, during which the bacteria were intermittently fed with acetate as the only carbon source. The reactor was operated in different phases. The phase, which was operated with acetate consisted of 90 min of continuous feeding + mixing (Feed C.2 (Table 1, Case C), NH_4^+ + NO_3^- + acetate) and 30 min of mixing. The phase, which was operated without acetate consisted of 120 min of continuous feeding + mixing (Feed C.3 (Table 1, Case C), NH_4^+ + NO_2^-) and 30 min of mixing. Then a settling phase of 60 min and decanting of 30 min was applied.

For both regimes, 1 L of medium was fed each cycle. The feeding pump for the 'Anammox regime' was set at 6.67 mL min^{-1}, while the feeding flow rate of the 'regime under semi-continuous exposure to acetate' was 4.76 mL min^{-1}. The modifications on the composition of the different feeding conditions used are summarized in Table 1, Case C. In addition to the compounds of these feeding strategies, a mineral medium was supplied (SI, Table S1. 1 and S1. 2). The effect of methanol was not assessed during the operation of the SBR in Case C to avoid the irreversible inhibition to the bacterial population during reactor operation. Although the reactor was inoculated with granular sludge, the granules disintegrated after 2 days of operation due to the mixing applied by a magnetic stirrer to keep homogenous conditions. The temperature was controlled at 30 ± 1 °C by using a heating water jacket connected to a circulating bath and the pH was kept at 7.4 ± 0.4.

2.4. Analytical methods and FISH analysis

The total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to standard methods (APHA, 1998). Liquid samples were taken and immediately filtered through disposable Millipore filters (0.45 μm pore size). Ammonium, nitrate, nitrite and COD (acetate) were determined with standard test kits Hach-Lange and Macherey-Nagel for Cases A and C. The nitrogen compounds in Case B were analysed with QuikChem 8500 Flow Injection Analysis (FIA) from Lachat Instruments and the acetate was analysed with a High Performance Liquid Chromatography (HPLC) system.

Fluorescence in situ hybridization (FISH) and slicing was performed on the granular biomass from the bubble column SBR for Case B according to the method proposed by Winkler et al. (2011) in order to observe the population spatial distribution inside the granules. Likewise, FISH was accomplished on the floccular biomass from the anammox SBR operated with a transient exposure to acetate (Case C).

3. Results and discussion

Nitrate reduction by heterotrophic and anammox bacteria was evaluated in three separate case studies: (i) batch tests with enriched anammox biomass from a full-scale reactor (Case A), (ii) batch tests with anammox and heterotrophic biomass from an anoxic/aerobic SBR (Case B), and (iii) sequencing batch operation of a bioreactor with anammox biomass (Case C) (see Table 1 for more information on the conditions of each Case study). The results from bacterial population determination are presented in Section 3.1, and the anammox and nitrate reduction activities assessed in the absence and presence of organic carbon (acetate) are described in Section 3.2. The factors influencing the acetate and nitrogen transformations of both heterotrophs and anammox bacteria are discussed in Section 3.3 and 3.4 (i.e. competition heterotrophs vs. anammox bacteria). Finally, a scheme to apply the combined partial DNRA-anammox by anammox bacteria is presented in Section 3.5.

3.1. Bacterial populations

FISH analysis was performed to determine the dominant bacterial populations (see SI, Fig. S4. 1). The anammox species observed in each of the three cases of this study was *Candidatus* 'Brocadia fulgida', which is known to have the ability to carry out DNRA and outcompete heterotrophs for volatile fatty acids (VFA) (Kartal et al., 2008; Winkler et al., 2012a,b). Concerning their relative abundance, anammox bacteria were the dominant organisms present in the experiments of Case A and Case C, with negligible amount of other microorganisms. For the Case A, the biomass was taken from a full-scale anammox reactor in which the COD/N ratio was relatively low, hindering the development of heterotrophic bacteria. The SBR of Case C was not exposed continuously to acetate (see Fig. S3. 1 in the SI), which disfavoured the presence of heterotrophs.

The granular sludge for the batch tests of Case B was harvested from a lab reactor exposed to acetate (Winkler et al., 2012a). The FISH determination of this biomass revealed anammox bacteria and polyphosphate-accumulating organisms (PAOs) in the inner core of the granules, in addition to AOB in the outer zone. In the reactor the produced nitrate from the aerated phase was recycled together with the influent containing acetate. However, over time partial nitritation-anammox worked well and only little nitrate accumulated. Thus, anaerobic conditions (no electron acceptor present) were established and the growth of PAOs promoted, which accumulated acetate internally as poly-β-hydroxybutyrate (PHB) under anaerobic conditions and under aerobic or anoxic conditions the internally stored PHB was oxidized and used for cell growth.

3.2. Anammox conversion and organotrophic nitrate reduction

The anammox activity was assessed in the absence (Section 3.2.1) and presence of acetate (Section 3.2.2). Also, the nitrate and acetate consumption rates were measured in the experiments with acetate addition.

3.2.1. Anammox activity in the absence of acetate

The anammox activity without acetate present was assessed during Case A (batch tests A.1 and A.2 in Table 1), Case B (batch tests B.1 and B.2 in Table 1), and the 'Anammox regime' of Case C (Feed C.1 in Table 1) (Fig. 2). The determination of the substrate consumption rates is described in Section S5 of the SI. As expected the ammonium (r_{NH4}) and nitrite (r_{NO2}) specific consumption rates (i.e. expressed per g VSS, total biomass) were higher for the reactors containing highly enriched anammox biomass (Cases A and C) and accordingly lower for the reactor containing also PAOs (Case B) (Fig. 2).

The addition of methanol inhibited the anammox activity (r_{NH4}) by 94.0% and 89.3% in Cases A and B, respectively (Fig. 2A and B). The inhibition observed was not complete as previously reported (Guven et al., 2005), likely due to diffusion limitations or a protective environment inside the granules.

3.2.2. Anammox and organotrophic nitrate reduction activities in the presence of acetate

The anammox activity (r_{NH4}) decreased by 42.1% in Case A and 41.9% in Case B (Fig. 3.1) in the presence of acetate, nitrate and ammonium, when compared to the case with ammonium and nitrite, but not acetate (Fig. 2). When nitrite was not added, it had to be formed from the reduction of nitrate. Nitrite production would then become the limiting reaction, because the nitrate reduction rates (r_{NO3}) for both Cases A and B in Figs. 3.1 and 3.2 were slower than the nitrite reduction rates (r_{NO2}) performed during regular anammox reaction in Fig. 2A and B.

In order to clarify which communities (anammox or heterotrophic bacteria) reduced the nitrate and oxidized acetate (Fig. 3), an inhibitor of anammox bacteria (methanol) was supplied in the medium together with ammonium, nitrate and acetate for Case A (batch test A.4, Fig. 3.1). The addition of methanol inhibited the nitrate reduction and acetate oxidation by 99.0% and 98.4%, respectively, compared to the test without methanol addition (see batch tests A.3 and A.4 in Fig. 3.1). Since earlier studies have shown that heterotrophs are not inhibited at concentrations used in this study (12 mM for Case A, Table 1) (Jensen et al., 2007) it could be concluded that anammox bacteria were the major responsible microorganisms for the consumption of nitrate and acetate in Case A, which is in agreement with the lower abundance of other bacteria than anammox on the FISH images (Fig. S4. 1). It is important to note that, the anammox biomass used for the experiments in Case A was not adapted to organic matter. This biomass was taken from a full-scale anammox reactor preceded by a SHARON reactor in which organic carbon would have been oxidized. Despite this fact, in the present study it was shown that the anammox species *Candidatus* 'Brocadia fulgida' performed DNRA along with anaerobic ammonium oxidation at a high rate. In the present study the nitrate reduction rate by anammox bacteria was 1.9 slower than the conventional anammox activity (ammonium oxidation rate in the test with ammonium and nitrite only), which is significantly higher compared to previous studies (nitrate reduction was 21 times slower than regular anammox activity in the tests from Kartal et al. (2007b) and 10 times slower in Kartal et al. (2007a)).

In Case B, where experiments were conducted with biomass from a laboratory reactor with high presence of heterotrophic populations and PAOs, it could not be concluded to which extent heterotrophic and/or anammox bacteria contributed to the reduction of nitrate and oxidation of acetate when supplying ammonium, nitrate and acetate (B.3 in Fig. 3.1). A higher acetate consumption rate (r_{COD}) was found during the batch tests with biomass from the lab-scale reactor containing PAOs (Case B, 21.1 mg COD $(g\ VSS)^{-1}\ h^{-1}$) than in the batch tests with anammox biomass from the full-scale system (Case A, 6.99 mg COD $(g\ VSS)^{-1}\ h^{-1}$). This higher activity can be attributed to PAOs, which are known to have a high acetate uptake rate due to their capacity storing acetate as PHB. Note that during the tests with nitrate and acetate (B3, B4 and B5 in Fig. 3) uptake of phosphorus was observed (SI, Table S5. 2), confirming PAO activity.

3.3. What triggers DNRA by anammox bacteria over heterotrophic DNRA and heterotrophic denitrification?

The present study aims at assessing when denitrification and DNRA take place and if they are carried out by either heterotrophs (including PAOs) or anammox bacteria. Factors influencing the

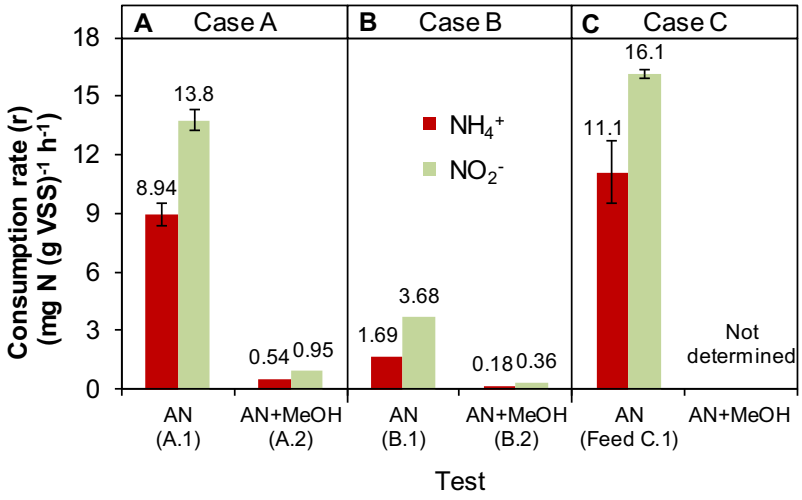

Fig. 2. Anammox activities during the A) batch tests of Case A, with biomass from a full-scale anammox granular sludge reactor (A.1, A.2 and A.3); B) batch tests of Case B, with biomass from a SBR with high presence of heterotrophic bacteria (B.1 and B.2), and C) operation of the SBR of Case C under the 'Anammox regime' (Feed C.1); when having a media composition with (AN + MeOH) and without (AN) methanol. The corresponding concentrations of each test are listed in Table 1.

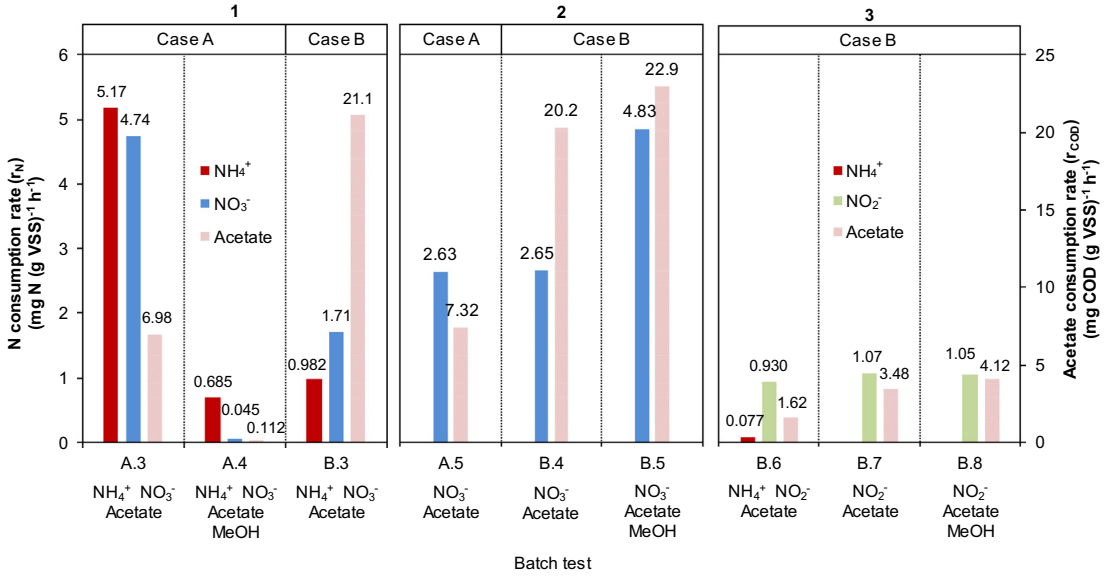

Fig. 3. Influence of acetate on the ammonium, nitrate, nitrite and acetate consumption rates (r_{NH4}, r_{NO3}, r_{NO2} and r_{COD}). Results during the batch tests with enriched anammox biomass from a full-scale granular sludge reactor of Case A (A.3 to A.5, Table 1) and from a SBR containing anammox and heterotrophic bacteria of Case B (B.3 to B.8, Table 1) under different feeding conditions: nitrate as electron acceptor with (1) and without (2) ammonium in the medium; and nitrite as electron acceptor (3).

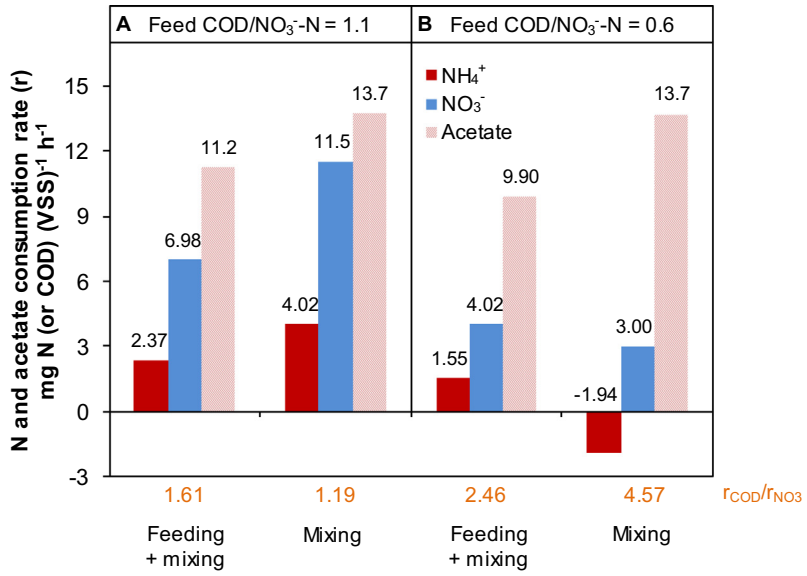

Fig. 4. Effect of inorganic carbon absence on the ammonium, nitrate and acetate consumption rates (r_{NH4}, r_{NO3} and r_{COD}) for the 'Regime under semi-continuous exposure to acetate' in Case C under two COD/NO_3^--N feeding ratios.

DNRA pathway of anammox bacteria were studied experimentally (Figs. 3 and 4) and by thermodynamic calculations (Table 2) to distinguish the carbon and nitrogen transformations among heterotrophs (including PAOs) and anammox bacteria.

3.3.1. Effect of ammonium in the medium on the organotrophic nitrate reduction by anammox bacteria

The effect of ammonium in the medium on the nitrate (r_{NO3}) and COD (r_{COD}) conversion rates was assessed through batch tests with the enriched anammox biomass in Case A (test A3 vs. A5, Fig. 3), in which it was concluded earlier (Section 3.2.2) that anammox bacteria were the dominating culture and responsible for the nitrate and acetate consumption. With ammonium in the medium, the reduction of nitrate was faster (tests A.3 vs. A5, Fig. 3) and no accumulation of nitrite was observed. Therefore, nitrite for anaerobic ammonium oxidation (r_{NH4} = 5.173 mg N $(g\,VSS)^{-1}\,h^{-1}$, A.3 in Fig. 3) must have been supplied through the anammox mediated DNRA pathway in which nitrite is formed as intermediate (Fig. 1C). The absence of ammonium (and nitrite) in the medium (A.5 in Fig. 3) led to the accumulation of nitrite in the beginning of the test (SI, Fig. S7. 1A), which can be explained by the fact that the reduction of nitrate to nitrite by anammox bacteria is faster than the conversion of nitrite to ammonium (Kartal et al., 2007a)). Within this test no ammonium was accumulated, suggesting that anammox bacteria immediately metabolized the ammonium obtained through the full DNRA pathway for anaerobic ammonium oxidation (Fig. 1D).

The capacity of anammox bacteria of using DNRA expands their growth niche from a pure autotrophic environment to ecological niches in which organic carbon occurs along with nitrate. The

Table 2
Catabolic reaction stoichiometry, Gibbs free energy per substrate, N_2 produced and electron transferred, and stoichiometric ratio COD/NO_3^- for different pathways.

	Biological process	Reaction	kJ $(e^-eq)^{-1}$	kJ (mol NO_3^- or NO_2^-)$^{-1}$	kJ (mol acetate)$^{-1}$	kJ (mol N_2)$^{-1}$	Stoichiometric COD/NO_3^- (g COD (g N)$^{-1}$)
1	Denitrification	$8NO_3^- + 5CH_3COO^- + 8\,H^+ \rightarrow 5CO_2 + 4N_2 + 5HCO_3^- + 9H_2O$	−99.60	−498.0	−796.8	−996.0	2.86
2	DNRA	$NO_3^- + CH_3COO^- + 2\,H^+ \rightarrow CO_2 + NH_4^+ + HCO_3^-$	−62.51	−500.1	−500.1		4.57
3	Partial DNRA/ Denitrification*	$4NO_3^- + CH_3COO^- \rightarrow CO_2 + 4NO_2^- + HCO_3^- + H_2O$	−69.05	−138.1	−552.4		1.14
4	Nitrite reduced via Anammox	$NO_2^- + NH_4^+ \rightarrow N_2 + 2H_2O$	−119.3	−357.8		−357.8	
5	Nitrite reduced via DNRA	$4NO_2^- + 3CH_3COO^- + 8\,H^+ + H_2O \rightarrow 3CO_2 + 3HCO_3^- + 4NH_4^+$	−60.33	−362.0	−482.6		3.43
6	Nitrite reduced via Denitrification	$8NO_2^- + 3CH_3COO^- + 8\,H^+ \rightarrow 3CO_2 + 3HCO_3^- + 4N_2 + 7H_2O$	−119.96	−359.9	−959.7	−719.8	1.71
7	Partial DNRA + Anammox**	$4NO_3^- + CH_3COO^- + 4NH_4^+ \rightarrow CO_2 + HCO_3^- + 4N_2 + 9H_2O$	−99.18	−495.9	−1984	−495.9	1.14
8	Full DNRA + Anammox***	$8NO_3^- + 5CH_3COO^- + 8\,H^+ \rightarrow 5CO_2 + 5HCO_3^- + 4N_2 + 9H_2O$	−99.60	−498.0	−796.8	−996.0	2.86

* Partial DNRA = partial denitrification, i.e. reduction of nitrate to nitrite only.
** All the NH_4^+ comes from the environment, i.e. only the first step of the DNRA process occurs ($NO_3^- \rightarrow NO_2^-$) (Fig. 1C).
*** All NH_4^+ comes from the DNRA process, i.e. all NO_3^- is converted to NO_2^- and half of the NO_2^- is further converted to NH_4^+ (Fig. 1D).

DNRA pathway has not been shown to deliver net energy to anammox bacteria but supplies them with their catabolic substrates. Depending on the conditions, only nitrite is produced (via partial DNRA) which is then reduced with ammonium present in the environment (Fig. 1C) or DNRA produces both nitrite an ammonium to fuel the anammox reaction (Fig. 1D).

The presence of ammonium enhances the organotrophic reduction rate of nitrate by anammox bacteria since the slow formation of ammonium through DNRA is avoided and the produced nitrite can be immediately utilized for the energy yielding anaerobic ammonium oxidation with nitrite. This is in line with other studies which have shown that the presence of ammonium gives anammox bacteria advantage to perform DNRA over heterotrophic transformations (Guven et al., 2005; Kartal et al., 2007a,b; Winkler et al., 2012a,b).

3.3.2. Effect of substrate limitation – energy perspective

The energy obtained per substrate consumed, N_2 formed, and the electrons transferred were calculated for different microbial pathways (see SI, Section S6 for description of the calculation) since it is one of the main driving forces explaining microbial competition (Gonzalez-Cabaleiro et al., 2015) (Table 2).

According to the energy per electron transferred for the heterotrophic transformations (Table 2), a higher theoretical yield is obtained during the denitrification of nitrite (reaction 6, −120 kJ e^--eq^{-1}) than is the case for the denitrification of nitrate (reaction 1, −100 kJ e^--eq^{-1}) whereas the DNRA is the least favoured process of the heterotrophic transformations (reaction 2, −62.5 kJ e^--eq^{-1}). Therefore, under no substrate limiting conditions, heterotrophic denitrification over nitrite would be more favoured than other heterotrophic transformations of nitrogen if nitrite is available in the medium, as shown by Kraft et al. (2014). With respect to substrate competition, van den Berg et al. (2015) selected heterotrophic communities performing DNRA over denitrification when limiting the nitrate in the system (high COD/N ratio). However, from a thermodynamic point of view, the limitation of nitrate cannot be used to explain the dominance of the heterotrophic DNRA pathway because a similar energy is harvested per mole of nitrate during denitrification (reaction 1) and DNRA (reaction 2, Table 2). Instead, the limitation of acetate (low COD/N ratio) would promote the heterotrophic denitrification (−797 kJ mol-acetate^{-1}) over the heterotrophic DNRA (−500 kJ mol-acetate^{-1}).

Regarding the conversions involving anammox bacteria, the theoretical Gibbs free energy obtained per N_2-mol produced is higher for the partial DNRA-anammox (reaction 7, Fig. 1C) and full DNRA-anammox reaction (reaction 8, Fig. 1D) than the energy obtained if only performing conventional anaerobic ammonium oxidation (reaction 4). Nevertheless, whether or not anammox bacteria can use this extra energy when performing the DNRA pathway is not yet understood. Current literature suggests that anammox bacteria do not use the organic carbon for cell assimilation and therefore energy usage from this pathway remains unsolved (Kartal et al., 2013). Following this thermodynamic approach, the limitation of acetate could be the key to promote the partial DNRA-anammox (reaction 7, −1984 kJ mol-acetate^{-1}) over both heterotrophic DNRA (reaction 2) and heterotrophic denitrification (reaction 1), as well as the full DNRA-anammox pathway (reaction 8). This can be explained because more energy is generated when supplying externally the ammonium (partial DNRA-anammox, Fig. 1C) than when forming the ammonium through DNRA and then converting it during the regular anammox reaction (full DNRA-anammox, Fig. 1D). Also, the partial DNRA-anammox is the only pathway in which anammox bacteria could outcompete heterotrophic denitrifiers from a thermodynamic perspective when limiting the carbon source (more energy is produced, 1984 kJ acetate-mol^{-1} during partial DNRA-anammox vs. 797 during heterotrophic denitrification). Although the energy usage of the anammox driven DNRA pathway is not unravelled yet, experimental results have shown that heterotrophic transformations can be outcompeted by anammox bacteria performing DNRA at low COD/N ratios (Winkler et al., 2012b). Overall it must be noted that anammox can generate ammonium and/or nitrite from the DNRA pathway and hence support their autotrophic pathway under conditions in which they are lacking their electron acceptor or donor (or both). This therefore yields in an energy gain irrespective from the DNRA pathway.

3.3.3. Effect of inorganic carbon

The DNRA reaction yields inorganic carbon (reaction 2 in Table 2), while the anammox reaction uses inorganic carbon for biomass growth. The effect of inorganic carbon on the combined partial DNRA-anammox transformation by anammox bacteria

was investigated with an anammox SBR, which was operated with alternating feeding regimes of heterotrophic conditions (ammonium, nitrate and acetate), and autotrophic conditions (ammonium and nitrite). The reactor was run without the addition of inorganic carbon and therefore CO_2 must have been provided biotically via the oxidation of acetate through the DNRA pathway (Case C, Fig. 4).

The r_{NH4}, r_{NO3} and r_{COD} were studied for two different COD/NO_3^--N ratios in the influent (1.1 and 0.6 g COD (g N)$^{-1}$, Fig. 4A and B, respectively). For the feeding ratio COD/NO_3^--N = 1.1, the consumption rate ratios obtained (r_{COD}/r_{NO3} = 1.61 and 1.19 in Fig. 4A) were very similar to the stoichiometric ratios COD/NO_3^--N corresponding with partial DNRA-anammox (1.14 in Table 2 reaction 7). This, together with the fact that no significant biomass growth was observed (average biomass concentration during the operation of the SBR = 1.14 ± 0.02 g VSS L^{-1}), suggests that partial DNRA-anammox by anammox bacteria dominated over heterotrophic conversions during the feeding ratio COD/NO_3^--N of 1.1. The inorganic carbon produced during the partial DNRA by anammox bacteria presumably supported the regular autotrophic anammox reaction.

When a COD/NO_3^--N ratio of 0.6 was fed, the r_{NH4} became negative during the mixing step, indicating formation of ammonium through DNRA, and the r_{NO3} decreased by 62.1%. With a lower feeding ratio, the inorganic carbon formation through partial DNRA was lower since less COD was added and lower oxidation of acetate by DNRA occurred, thus limiting the anammox activity. Overall, the inorganic carbon supplied by the DNRA pathway needs to be sufficient to maintain the energy yielding anaerobic ammonium oxidation.

3.4. Anammox conversion in the presence of heterotrophic bacteria

Batch tests were performed with nitrite, nitrate, and acetate for Case B (high presence of heterotrophic bacteria, PAOs) (Fig. 3) to assess the competitiveness of the anammox pathway in presence of heterotrophic bacteria and organic carbon compounds. Since the addition of methanol did not inhibit the nitrate/nitrite and acetate consumption rates in the absence of ammonium (B.4 vs. B.5 and B.7 vs. B.8 in Fig. 3), it is clear that heterotrophic denitrification was the dominant pathway (Jensen et al., 2007) consuming nitrate/nitrite and acetate for these batch tests. When ammonium was added (together with nitrate/nitrite, and acetate) (B.7 and B.8 in Fig. 3.3), it was found that anammox bacteria oxidized ammonium in the presence of nitrate (r_{NH4} = 0.982 mg N (gVSS)$^{-1}$ h^{-1}), but only a negligible consumption was found in the experiments with nitrite (r_{NH4} = 0.077 mg N (g VSS)$^{-1}$ h^{-1}). Thus, anammox bacteria were more competitive for the nitrite formed from the reduction of nitrate (most likely by heterotrophs) than for the nitrite supplied directly in the medium, when having acetate present. This could be explained by a limitation on the heterotrophic denitrification since the acetate was completely consumed after 20 min (see SI, Fig. S7. 1C, D and E). When nitrite was supplied, it was taken up by the heterotrophs, but when nitrate was added, only partial denitrification ($NO_3^- \rightarrow NO_2^-$) took place, which was indicated by nitrite accumulation in the system (Fig. S7. 1C, D and E). Therefore, the organic carbon limitation (low COD/N ratio) together with the presence of nitrate can benefit the anammox reaction, since the heterotrophic denitrification yields nitrite that can be used along with ammonium by anammox bacteria (partial denitrification-anammox, Fig. 1B). This finding also confirms the results obtained in the modelling study of Mozumder et al. (2014), in which the authors pointed out that low concentrations of COD could benefit the anammox reaction by the heterotrophic reduction of nitrate to nitrite.

3.5. Partial DNRA-anammox by anammox bacteria for more sustainable municipal wastewater treatment

The main bottleneck to implement anammox technology in the mainstream of the WWTP is an effective supply of nitrite to the anammox reaction (Ma et al., 2016), which is troublesome due to the suppression of NOB at low temperatures (Lotti et al., 2015). If the DNRA pathway of anammox bacteria could be promoted in the mainstream, the suppression of NOB would not be so crucial anymore since nitrate could be reduced by anammox bacteria with some organic carbon, while keeping sludge production low given that anammox bacteria do not grow on organic substrates (Kartal et al., 2007b; Winkler et al., 2012a). Also, stable operational conditions are a critical point during mainstream wastewater treatment, which are more difficult to obtain when aiming at nitritation ($NH_4^+ \rightarrow NO_2^-$) instead of nitrification ($NH_4^+ \rightarrow NO_3^-$). The feasibility of the partial DNRA-anammox conversion (reaction 7 in Table 2 and Fig. 1C) by anammox bacteria was demonstrated in this study (Figs. 3 and 4) and in previous research (Guven et al., 2005; Kartal et al., 2007a; Winkler et al., 2012a,b).

In order to decrease the nitrate accumulation produced by NOB (or by anammox itself) in the mainstream, the partial DNRA-anammox process could be used and implemented for enhanced nitrogen removal according to the scheme shown in Fig. 5. In the suggested approach the organic matter from the wastewater is removed in a high rate activated sludge (HRAS) system and captured in biomass, which is used to maximize the energy recovery in the digester (Jetten et al., 1997). Since the HRAS system is operated at low hydraulic retention time, nitrification is prevented and the effluent of the HRAS is high in ammonium. This effluent could be treated in two steps: conversion of half of the ammonium into nitrate by means of partial nitrification in an aerobic reactor; and partial DNRA-anammox by anammox bacteria in an anoxic unit (see Fig. 5). The advantage would be to not only remove the nitrate generated by NOB but also the nitrate produced during the anammox conversion itself, thus increasing the effluent quality. In order to promote the partial DNRA pathway of anammox bacteria, volatile fatty acids (VFA) need to be supplemented to the system since anammox bacteria have not been reported to be able to utilize e.g. sugars or particulate COD. Therefore, it is suggested to generate VFAs onsite in a small fermentation unit receiving sludge from the HRAS reactor. This external supply of VFA would be associated with some costs but if compared to conventional nitrification-denitrification systems, up to 50% energy savings in aeration would be obtained by applying partial DNRA-anammox, since only half of the ammonium would be oxidized to nitrate. A fraction of 21% of the COD contained in the sludge from the HRAS system would need to be converted into VFA in the fermenter to promote the partial DNRA-anammox (see Section S8 in the SI for details on the calculation of the COD needed to carry out the partial DNRA-anammox).

Regarding the practical implementation of the nitrogen removal system in Fig. 5, a two reactor configuration is suggested, since for mainstream applications the use of two reactors would not increase significantly the overall investment costs, while the control in two-unit configuration would be easier than in one reactor. The first aerated reactor could still aim for partial nitritation and the accumulation of nitrite, which has however been shown to be the major bottleneck in the application of anammox in the main line (Lotti et al., 2015). It is therefore a fair assumption that nitrate and not nitrite will be dominant.

The use of granular sludge technology for the partial DNRA-anammox reactor circumvents the necessity of settlers for the biomass separation, leading to compact and high rate process units. Besides, literature suggests that in granular sludge reactors

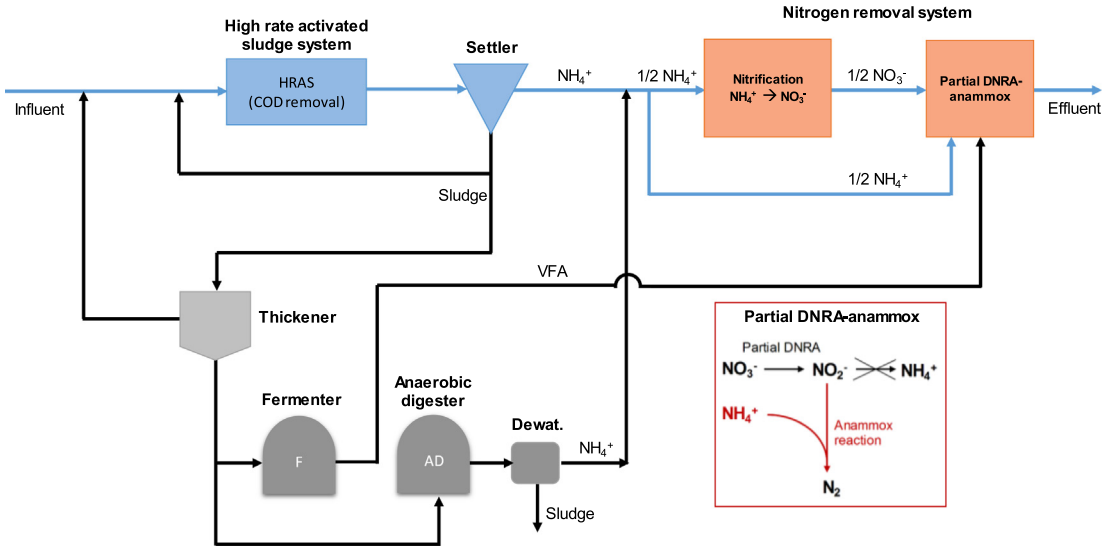

Fig. 5. Suggested scheme for more sustainable municipal wastewater treatment with nitrification of half of the ammonium + partial DNRA-anammox by anammox bacteria as nitrogen removal system.

heterotrophs could be more easily washed out from the system since these microorganisms grow mainly in flocs (Lotti et al. (2015)).

Overall, all aspects discussed in Section 3.3 and 3.4 would facilitate the success of the partial DNRA-anammox process. The presence of ammonium in the medium would favour the outcompetition of heterotrophs, as shown in this study (Fig. 3) and by others (Kartal et al., 2007b, 2008). Also, when ammonium is available in the medium, the conversion of nitrite to ammonium by anammox bacteria is prevented (full-DNRA) since this conversion is slower than the nitrate reduction to nitrite (Fig. 3 in this study and Kartal et al. (2007a)), thus promoting partial DNRA-anammox (conversion in Fig. 1C instead of D). The presence of nitrate instead of nitrite could make anammox bacteria competitive for the nitrite formed during partial DNRA or partial denitrification (Fig. 3 and Mozumder et al. (2014)). However, as shown in Fig. 4, the insufficient availability of inorganic carbon for anammox bacteria to grow could impede the combined partial DNRA-anammox process. Furthermore, *Candidatus* 'Brocadia fulgida', a common species in full-scale WWTPs and found in this study (Fig. S4. 1), has proven to be a suitable organism for performing partial DNRA-anammox, without previous adaptation to VFA (Fig. 3.1).

This study is the first one suggesting the exploitation of the organotrophic nitrate reduction capacity of anammox bacteria to facilitate the implementation of the regular anammox conversion in the main line of the wastewater treatment plant. However, further research is needed to proof the stability of the process long-term.

4. Conclusions

The competition among heterotrophs and anammox bacteria for organic carbon and nitrate was studied to gain understanding on the DNRA capacity of anammox bacteria. The species *Candidatus* 'brocadia fulgida' showed high DNRA performance even when the culture was not previously adapted to organic carbon. Gibbs free energy calculations showed that limiting organic carbon (low COD/N influent ratio) favoured the partial DNRA ($NO_3^- \rightarrow NO_2^-$)-anammox conversion over the heterotrophic DNRA and heterotrophic denitrification. Also, the presence of ammonium and sufficient inorganic carbon enhanced the process. Overall, the combined partial DNRA-anammox could be applied for sustainable nitrogen removal from municipal wastewater.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.02.063.

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