

Nitrite inhibition and limitation – the effect of nitrite spiking on anammox biofilm, suspended and granular biomass

Markus Raudkivi, Ivar Zekker, Ergo Rikmann, Priit Vabamäe, Kristel Kroon and Taavo Tenno

ABSTRACT

Anaerobic ammonium oxidation (anammox) has been studied extensively while no widely accepted optimum values for nitrite (both a substance and inhibitor) has been determined. In the current paper, nitrite spiking (abruptly increasing nitrite concentration in reactor over $20 \text{ mg NO}_2^- \text{ NL}^{-1}$) effect on anammox process was studied on three systems: a moving bed biofilm reactor (MBBR), a sequencing batch reactor (SBR) and an upflow anaerobic sludge blanket (UASB). The inhibition thresholds and concentrations causing 50% of biomass activity decrease (IC_{50}) were determined in batch tests. The results showed spiked biomass to be less susceptible to nitrite inhibition. Although the values of inhibition threshold and IC_{50} concentrations were similar for non-spiked biomass (81 and $98 \text{ mg NO}_2^- \text{ NL}^{-1}$, respectively, for SBR), nitrite spiking increased IC_{50} considerably (83 and $240 \text{ mg NO}_2^- \text{ NL}^{-1}$, respectively, for UASB). As the highest total nitrogen removal rate was also measured at the aforementioned thresholds, there is basis to suggest stronger limiting effect of nitrite on anammox process than previously reported. The quantitative polymerase chain reaction analysis showed similar number of anammox 16S rRNA copies in all reactors, with the lowest quantity in SBR and the highest in MBBR (3.98×10^8 and 1.04×10^9 copies g^{-1} TSS, respectively).

Key words | anammox, deammonification, nitrite spiking, reject water

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INTRODUCTION

Anaerobic ammonium oxidation (anammox) (Mulder *et al.* 1995) is a wastewater treatment process carried out by chemoautotrophic bacteria from the order *Planctomycetales* (Strous *et al.* 1999a). The process uses NO_2^- as the electron acceptor and oxidizes dissolved NH_4^+ into dinitrogen gas in anoxic conditions (Van Hulle *et al.* 2010). It is a cost-effective method that serves as an alternative to traditional nitrification-denitrification process (Van Hulle *et al.* 2010) due to significant saving on aeration energy, organic carbon consumption and biomass treatment costs (Lotti 2012).

The most critical point for sustaining a stable anammox process with a high total nitrogen removal rate (TNRR) is maintaining the proper substrate NO_2^- concentrations. Nitrite has been recognized as an inhibiting compound for anammox organisms (Strous *et al.* 1999b)

while the specific mechanism of nitrite inhibition is still unknown (Lotti *et al.* 2012). Other inhibiting/limiting parameters for the anammox process are free ammonia, dissolved oxygen (DO) and organic carbon/nitrogen (COD/N) ratio (Ali & Okabe 2015).

Different inhibiting NO_2^- concentrations (IC_{50} ranging from 80 to $430 \text{ mg NO}_2^- \text{ NL}^{-1}$) have been observed for anammox process (Lotti *et al.* 2012) depending on the biomass type (Table 1). The highest IC_{50} values (concentration causing 50% inhibition) have been reported for the long-term tests either with gel biofilm carriers or with granular biomass (Kimura *et al.* 2010; Lotti *et al.* 2012). Lower IC_{50} values have been reported for suspended (flocculated) anammox (Strous *et al.* 1999b; Bettazzi *et al.* 2010). In some cases, the reported high nitrite concentrations may instead be the

Table 1 | IC₅₀ values caused by nitrite concentrations and conditions for batch tests according to various authors

NO ₂ -N IC ₅₀ (mg L ⁻¹)	NO ₂ -N optimum (mg L ⁻¹)	Temperature (°C)	Biomass type	Reference
350	120	30	Suspended	Dapena-Mora <i>et al.</i> (2007)
120	~50	20–43	Suspended	Strous <i>et al.</i> (1999b)
80	37	35	Suspended	Bettazzi <i>et al.</i> (2010)
400	100 ^a	30	Granular	Lotti <i>et al.</i> (2012)
>430	100 ^a	30	Gel biofilm carriers	Kimura <i>et al.</i> (2010)

^aLower inhibiting nitrite concentrations than 100 mg NO₂-NL⁻¹ were not determined.

result of biomass activity loss, not the main cause of it (Lotti *et al.* 2012). Balancing between optimal substrate concentrations and nitrite toxicity range is a challenge for the development of anammox technology and the wide range of observed nitrite inhibition levels can make it even more difficult to design or operate anammox-based systems. Developing biomass with high tolerance to nitrite could make wider use and application of a more robust and cheaper N-removal technology possible. Our previous studies have suggested that tolerance to higher nitrite concentrations could be established by applying high nitrite concentration spikes in the reactor (Zekker *et al.* 2014a). The tolerance to nitrite could also be linked to the anammox species present in the biomass, as *Candidatus Brocadia anammoxidans* is reportedly less tolerant to nitrite (IC₅₀ 7 mmol nitrite) than both *Candidatus Brocadia sinica* (<16 mmol nitrite) and *Candidatus Kuenia stuttgartiensis* (13 or 25 mmol nitrite) (Oshiki *et al.* 2011). Though, as not all species of anammox bacteria have been thoroughly characterised and not all anammox studies have conducted microbial sequencing, this current article focuses more on the links between biomass type and nitrite spiking than microbiological differences.

This study aimed to elucidate the reasons behind the wide variety of published results about the effect of nitrite inhibition on different types of anammox biomass. Moreover, the link between nitrite spiking (abruptly increasing the nitrite concentrations inside the reactor over 20 mg NO₂-NL⁻¹) and biomass reaction to short-term exposure to extremely high nitrite concentrations was studied in batch tests for three different types of biomass (biofilm, suspended, granular). Both the limiting and inhibitory effect of nitrite was researched in order to determine whether the biomass can adapt to high nitrite concentrations. Microbial research via the quantitative polymerase chain reaction (qPCR) method (with 16S rRNA primers Amx694F and Amx960R) was carried out in order to describe the most

common species of anammox bacteria present in used biomasses.

MATERIALS AND METHODS

Continuous reactors setups, operation and inoculums

Biomasses from three different laboratory-scale reactors – a moving bed biofilm reactor (MBBR (volume 20 L)), a sequencing batch reactor (SBR (10 L)) and an upflow anaerobic sludge blanket (UASB (2 L)) were used in this study. The specific details for all three biomasses during their continuous reactor operation period are presented in Table 2. All reactors were fed with reject water (composition in Zekker *et al.* (2012)) coming from the anaerobic tank of Tallinn wastewater treatment plant (Tallinn WWTP). The influent was fed using a peristaltic pump (Seko, Italy). The movement of biomass in all the reactors was ensured by mechanical stirring at approximately 100–200 rounds per minute (rpm) and additionally by coarse-bubble aeration.

MBBR was an 20 L anammox reactor, operated under anoxic conditions (DO concentration <0.2 mg L⁻¹) at 25.8 (±1.3)°C. Around 10,000 ring-shaped carrier elements made of polyethylene (Bioflow 9) were used for microorganisms' attachment material. The carriers occupied about 50% of the liquid volume of the reactor with total specific surface of 800 m² m⁻³ (carriers' interior protected specific surface of 500 m² m⁻³). Hydraulic retention time (HRT) of 18–48 h was applied. Influent TN (total nitrogen) concentrations of up to

Table 2 | Specifications of the biomass used in batch tests

Reactor type	Biomass type	Inoculum's origin	TNLR (g N m ⁻³ d ⁻¹)
MBBR	Biofilm	Tallinn WWP	240–440
SBR	Suspended	Hannover WWP	20–50
UASB	Granular	Rotterdam WWP	400–600

900 mg N L⁻¹ (NH₄⁺-N/NO₂⁻-N ratio about 1/1.3) were maintained by feeding the reactor with a mixture of reject water and NaNO₂ solution. The biomass wet weight at the time of the batch tests was about 6 mg per carrier (Zekker et al. 2012).

SBR was a 10 L deammonification reactor with intermittent aeration, inoculated with the biomass from Hannover anammox pilot plant (Germany) anammox reactor (Li et al. 2004). The plexiglass reactor was equipped with a water jacket and connected with a thermostat (Assistant 3180, Germany) operated at 26.0 (±0.5)°C, DO was measured and controlled by DO controller (Elke Sensor, Estonia). Relatively short HRT of 15 h was applied. SBR was fed with a flow rate of ~0.5 L h⁻¹. The SBR was fed spikily fewer than 5% of the cycle time maintaining a high (50%) volumetric exchange ratio. SBR ran in cycles of 30 min aerobic (with DO up to 3 mg L⁻¹) and 30 min anoxic phases.

UASB was a 2 L anoxic anammox reactor inoculated with granular biomass from full-scale anammox UASB (Rotterdam, The Netherlands (van der Star et al. 2007)). The plexiglass reactor was equipped with a water jacket and thermostated at 34 (±1)°C. This reactor system was operated at higher temperatures than the other two in order to keep the biomass in similar conditions as in the Rotterdam WWTP (original temperature between 30 and 40°C (van der Star et al. 2007)). The average HRT of the system was 22 h and the reactor was fed with the effluent of a 1.5 L nitritation reactor with TN concentrations up to 800 mg N L⁻¹ (NH₄⁺-N/NO₂⁻-N ratio ~ 1/1.3). In order to maintain a stable upflow rate for the granular sludge and help granules grow in size (maximum measured granule size >1 mm), upflow velocity of 4 L h⁻¹ was used (Zekker et al. 2014a).

Firstly, continuous reactor operation was carried out to cultivate anammox biomass and to achieve sufficient TNRR. In order to cultivate higher tolerance to nitrite, the MBBR and UASB reactors were spiked with nitrite by abruptly raising the nitrite concentration inside the reactor to over 20 mg

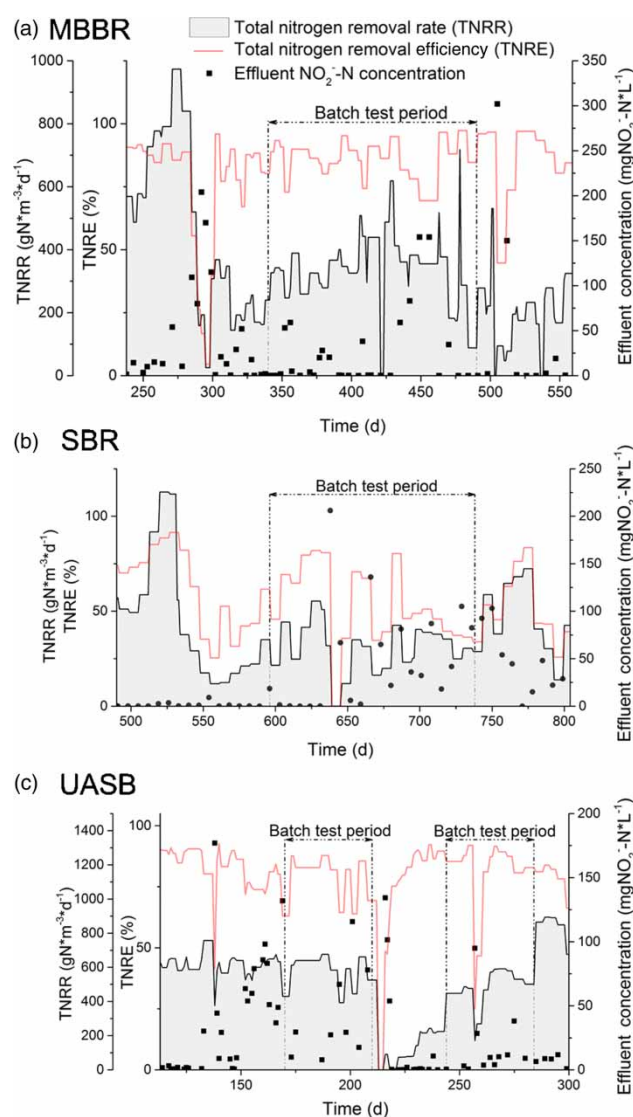


Figure 1 | Measured nitrite concentrations and TNRRs during both pre-batch and batch periods in (a) MBBR, (b) SBR and (c) UASB reactor.

NO₂-N L⁻¹ (Figure 1; Table 3). In this study, the nitrite values over 20 mg N L⁻¹ were considered as spikes, as the value is used commonly as a threshold of nitrite inhibition

Table 3 | Summary of reactor nitrite spiking and batch test results.

Reactor			Batch		
Type	Abundance (Anammox 16S rRNA copies g ⁻¹ TSS)	Fraction of spikes (pre-batch) ^a	Fraction of spikes (batch) ^a	NO ₂ -N IC ₅₀ (mg L ⁻¹)	NO ₂ -N optimum (mg N L ⁻¹)
MBBR	1.04 × 10 ⁹	9/24 (38%)	11/30 (37%)	~85	40
SBR	3.98 × 10 ⁸	No spiking	12/21 (57%)	98	81
UASB	4.72 × 10 ⁸	15/30 (50%)	9/21 (43%)	240	83

^aNumber of reactor samples with measured nitrite values over 20 mg N L⁻¹ out of all measured samples.

(Wett 2007). No nitrite spiking was applied in SBR as no nitrite was added externally due to the deammonifying biomass performing aerobic and anaerobic ammonium oxidation process was present in the reactor.

As all reactors were operated with reject water from the Tallinn WWTP with inconsistent concentrations, fluctuations in reactor total nitrogen removal efficiency (TNRE) can be seen for all of the reactors (Figure 1). Some bigger decreases in reactor efficiencies could be the result of nitrite inhibition, which in some cases for the MBBR and UASB might have been unintentionally caused by the spiking. During the batch test period, drops in TNRR (up to 40%), can be the result of biomass being taken out and then returned to the reactor after batch testing. Calculated TNRR, TNRE values and maintained NO_2^- -N concentrations for both pre-batch (before batch tests) and batch periods are presented on Figure 1 for each of the systems. All figures, efficiency calculations and regression statistics were visualised and calculated with Origin.

Batch tests

The inhibiting/limiting effect of different nitrite concentrations on three different biomasses (MBBR biofilm, SBR sludge and UASB granular sludge) was studied in series of batch tests. The tests were performed in air-tight 800 mL, while the test bottles were mixed by magnetic stirrers (mixing rate ~ 100 rpm). All batch tests were performed at 25°C in order to achieve comparability between different biomasses. The influent NO_2^- -N/ NH_4^+ -N ratio was prepared based on the anammox stoichiometric ratio 1.32/1 described by Strous *et al.* (1998). Acidic and alkaline solutions of micro- and macronutrients were added according to Zekker *et al.* (2012) to maintain sufficient nutrient balance for the biomass.

After the addition of substrates, the batch cell together with the biomass was deaerated for 15 min with argon, to ensure anoxic conditions for the test. Samples were taken after every 2 h during the 6-h period using the overpressure of argon.

For the MBBR (biofilm) 200 carriers were used in each batch test, the average total suspended solids (TSS) concentration in the test cell was 2.2 g L^{-1} . In the tests with suspended biomass the average TSS concentrations for SBR and UASB biomass were 2.4 g L^{-1} and 5.5 g L^{-1} , respectively. The average TSS used for UASB was higher due to significant mineral part in the granules (40–60% of TSS weight), while both the extracted biofilm and the suspended biomass were almost fully composed of organic

matter. The batch test TNRRs were calculated based on the linear regression of substrate concentrations in time for a gram of biomass TSS. The IC_{50} values have been calculated based on the slopes of the acquired regression equations. As all batch tests were performed at similar pH values (8 ± 0.6), free nitrous acid (FNA) concentrations were not considered (Puyol *et al.* 2014). Furthermore, whether nitrite inhibition is caused by NO_2^- or FNA was not determined in the current study.

Chemical analysis

Prior to analysis, the water samples were centrifuged at 4,000 rpm for 10 min to remove the solids. NH_4^+ , NO_2^- , NO_3^- concentrations were measured spectrophotometrically according to Greenberg *et al.* (1992). The samples pH value was measured with a pH-meter connected with Jenway pH electrode (Germany) and DO was measured by Marvet Junior (Estonia) electrodes, respectively. The measurement of TSS was performed according to Greenberg *et al.* (1992).

PCR methodology

In case of biofilm, five biomass carriers were taken from the reactor and biomass was mechanically removed using a vortex mixer, followed by DNA extraction by MoBio Power-soil DNA isolation kit (USA) according to the manufacturer's instructions. For sludge samples, the same DNA isolation procedures were applied as for biofilm samples. The PCR products were purified with the JETquick Spin Column Kit (GENOMED GmbH) and then sequenced. 25–50 mg of biomass was applied for DNA extraction (Zekker *et al.* 2012).

Pla46f /Amx368r primers were used for targeting anammox bacteria. Nested PCR was carried out according to the thermocycling parameters described by Sánchez-Melsió *et al.* (2009). PCR-denaturing gradient gel electrophoresis (PCR-DGGE) for detecting diversity of the most abundant microorganisms was conducted using the eubacterial primer set GC-BacV3f/907r (Koskinen *et al.* 2007). The sequencing was carried out according to Zekker *et al.* (2012).

Quantitative polymerase chain reaction

qPCR was conducted with primer sets Amx694F(GGGGA-GAGTGGAAGCTTCTG) and Amx960R(GCTCCACCGCTTGTGCGAGC), which amplify about 285 bp fragments from most anammox bacteria 16S rDNA (Ni *et al.* 2010).

Cloning for the standard was performed using the Thermo Scientific InstaClone PCR cloning kit according to the manufacturer's instructions. JM109 cell line was used. Plasmid was purified from selected colonies using the GeneJET Plasmid miniprep kit (Thermo Scientific). Dilutions of purified plasmid were used as standard in the qPCR reaction.

PCR amplification and detection were performed in optical 96-well reaction plates. The PCR temperature programme was initiated during 12 min at 95 °C, followed by 45 cycles of 10 s at 94 °C, 20 s at 58 °C, and 20 s at 72 °C. Each PCR mixture (10 µL) was composed of 2 µL of 5x HOT FIREPol Eva Green qPCR Supermix (Solis BioDyne, Estonia), 0.25 µL of forward and reverse primers (100 µM) and 1 µL of template DNA.

RESULTS AND DISCUSSION

Nitrite inhibition on different biomass

Batch tests were conducted during 340–490 (MBBR), 596–738 (SBR) and 173–210, 244–284 (UASB) days of the reactor operation (Figure 1). The overall results of the batch tests and the regime of nitrite spiking during both pre-batch and batch periods are presented in Table 3.

The operational conditions such as elevated total nitrogen loading rate (TNLR) and high maintained nitrite values in the reactor could enhance the biomass's tolerance towards high nitrite values. That was mostly seen for the biomass taken from UASB having both high TNLR (400–600 g N m⁻³ d⁻¹) and 50% of the measured nitrite values over the threshold. The possible effects of spiking are discussed more thoroughly in the specific chapter.

The first set of batch tests were performed with biomass carriers taken from a MBBR (Figure 2). The error bars on the graphs represent standard deviation. The highest nitrite concentration used in the batch tests was 73 mg NO₂⁻-NL⁻¹, while the IC₅₀ was calculated to be at 85 mg NO₂⁻-NL⁻¹. Due to technical problems with the MBBR, further tests with higher nitrite values could not be performed. Therefore, the calculated IC₅₀ value is used to provide comparison with the other biomasses. The biomass from MBBR achieved the highest TNRR (5 mg N g⁻¹ TSS h⁻¹) compared to other systems and the value was measured at relatively low nitrite concentration of 40 mg NO₂⁻-NL⁻¹. Similar results were described by Bettazzi et al. (2010) (highest TNRR at 37 mg NO₂⁻-NL⁻¹) with suspended biomass, while Kimura et al.

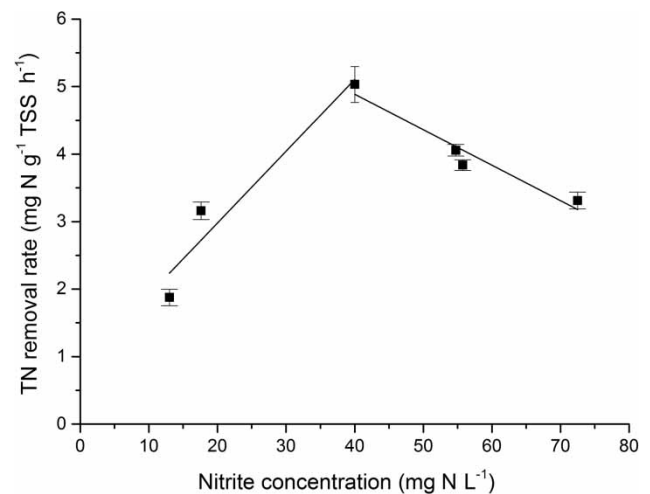


Figure 2 | The TNRR of the biomass from MBBR (biofilm carriers) on different nitrite concentrations.

(2010) have shown higher nitrite tolerance (highest TNRR at 100 mg NO₂⁻-NL⁻¹) with biofilm.

TNRR's dependence on nitrite concentration for the suspended biomass from the SBR is shown on Figure 3. The overall resistance of suspended biomass to nitrite inhibition measured as IC₅₀ was 15% higher (98 mg NO₂⁻-NL⁻¹) than for biofilm carriers. The highest TNRR (2.6 mg N g⁻¹ TSS h⁻¹) was achieved at 81 mg NO₂⁻-NL⁻¹ (*p*-value < 0.05), which is two times higher than the respective concentration found for MBBR. The results differ greatly from the inhibitory nitrite values reported by Wett (2007), who found nitrite to be inhibitory in concentrations as low as 9 mg NO₂⁻-NL⁻¹. Although a similar deammonification SBR system as ours was used in that study, no nitrite spiking was carried out. Furthermore, based on the results published

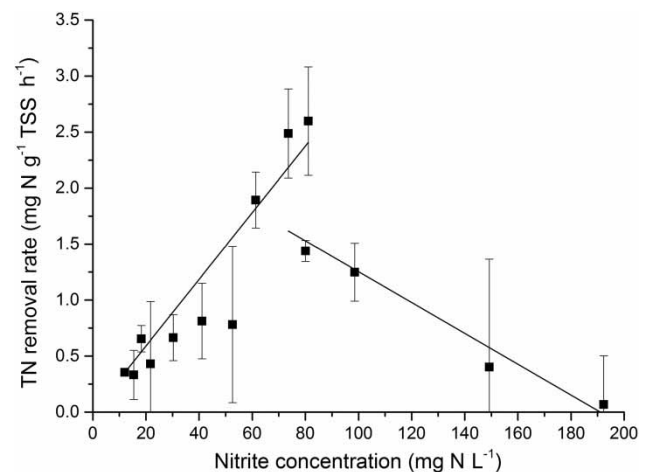


Figure 3 | The TNRR of the biomass from SBR at different nitrite concentrations.

in Wett (2007), the low nitrite values reported might be the result of anammox efficiency loss not the cause of it (Lotti et al. 2012).

In the batch tests with granules from the UASB, the highest TNRR was achieved at $83 \text{ mg NO}_2^- \text{-NL}^{-1}$ (p -value < 0.05) (Figure 4), which was comparable to the respective value in the SBR. The IC_{50} value for the UASB biomass was calculated to be at $240 \text{ mg NO}_2^- \text{-NL}^{-1}$ (p -value < 0.05), which was by far the highest among three different biomasses used. Similarly, very high IC_{50} values were acquired by both Dapena-Mora et al. (2007) (at $350 \text{ mg NO}_2^- \text{-NL}^{-1}$) and Lotti et al. (2012) (at $400 \text{ mg NO}_2^- \text{-NL}^{-1}$, granular biomass). As the UASB was originally inoculated with biomass acquired from Lotti, the similar results were to be expected.

Nitrite limitation

Various other authors have achieved the highest TNRRs at nitrite concentrations from 40 to $120 \text{ mg NO}_2^- \text{-NL}^{-1}$ (Strous et al. 1999b; Dapena-Mora et al. 2007; Bettazzi et al. 2010). The fact that until first signs of inhibition, the TNRR in the system rises with the increase of nitrite concentration (no plateau is usually reached), may refer to nitrite limitation in the anammox process.

During the course of nitrite inhibition research, we also studied whether nitrite spiking has an effect on nitrite limitation. As shown in Table 3, no apparent effect from spiking on nitrite limitation was observed. Despite being spiked with different frequency, the batch tests with either SBR or UASB biomass showed similar results to low nitrite concentrations (peak TNRRs at 81 and $83 \text{ mg NO}_2^- \text{-NL}^{-1}$, respectively).

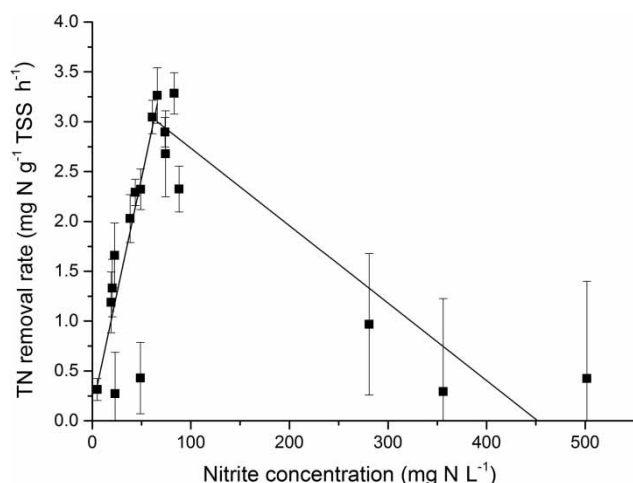


Figure 4 | The TNRR of the biomass from UASB at different nitrite concentrations.

While MBBR and UASB were spiked with similar frequency, the nitrite concentrations at which the peak TNRR was achieved differed twofold. This indicates varying limiting concentrations for different types of reactors and biofilm. While in this research the peak TNRRs for suspended biomass were observed at around $80 \text{ mg NO}_2^- \text{-NL}^{-1}$, the peak TNRRs from other authors with suspended biomass have been at 37, 50 and $120 \text{ mg NO}_2^- \text{-NL}^{-1}$ (Strous et al. (1999b), Dapena-Mora et al. (2007) and Bettazzi et al. (2010), respectively).

The results showed that while nitrite spiking does not have a significant effect on nitrite limitation, the type of biomass might be the most important factor concerning nitrite limitation. The calculated TNRRs for all reactors on different nitrite values is shown in Table 4. When MBBR was operated within the threshold of $20 \text{ mg NO}_2^- \text{-NL}^{-1}$, the achieved TNRR was 4 times higher than in SBR and 2.5 times higher than in UASB, while the peak TNRR values only differed 2 and 1.5 times, respectively. These results suggest that an anammox reactor with biofilm carriers would be the easiest to operate, as sufficient rates can be achieved in both under and over the strict threshold (Wett 2006).

The effect of nitrite spiking on nitrite inhibition can be observed when comparing the TNRR at peak nitrite concentrations with IC_{50} values. The biomass from SBR, which was not spiked showed the peak TNRR values at $81 \text{ mg NO}_2^- \text{-NL}^{-1}$ and IC_{50} at $98 \text{ mg NO}_2^- \text{-NL}^{-1}$, the slope of inhibition was steep (nitrite IC_{50} concentration only 1.2 times higher than the most efficient concentration). This means operating the reactor near the most efficient nitrite concentrations would be risky and difficult as even a slight increase in nitrite concentration could bring on significant inhibition. The inhibition slopes for both MBBR and UASB were gentler. MBBR was spiked 38% of the time and the difference between IC_{50} and peak TNRR nitrite concentrations was 2.1 times, while the respective values for UASB were 50% and 2.9 times.

Table 4 | Calculated TNRRs at different NO_2^- concentrations in reactors

$\text{NO}_2^- \text{-N}$ concentration (mg L^{-1})	TNRR ($\text{mg N g}^{-1} \text{TSS h}^{-1}$)		
	MBBR	SBR	UASB
20	2.50	0.60	1.05
40	5.03 ^a	1.18	1.97
60	3.84	1.78	2.90
80	2.79	2.37 ^a	3.83 ^a

^aPeak TNRR values.

Based on the results of this study, nitrite spiking frequency did not have any visible effect on nitrite limitation. However, by adapting the anammox reactor to periodical high nitrite concentrations, the slope of inhibition can be decreased significantly, which makes the system more tolerable to even higher nitrite concentrations. This effect can be used to operate anammox reactors on higher stable nitrite concentrations in order to maximise TNRR without risking strong inhibition in the system.

qPCR studies

The results of the quantitative anammox 16S rRNA analysis showed that the highest abundance of anammox gene copies per a gram of TSS were determined in MBBR (1.04×10^9 copies g^{-1} TSS) (Figure 5). The amount of anammox gene copies in the biomass taken from SBR and UASB systems were similar (3.98×10^8 and 4.72×10^8 copies g^{-1} TSS, respectively, p -value < 0.05), which are, respectively, 2.6 and 2.2 times lower than in the biomass taken from MBBR. This result was expected as SBR was a deammonification system, a significant part of the biomass could belong to other bacteria, which do not carry the anammox gene. As the UASB granular biomass contains a considerable mineral part, the lower amount of gene copies per a gram of suspended solids was expected as well.

In order to provide better comparability between the influent and biomass from the reactors, the quantitative anammox 16S rRNA analysis was also carried out from the reject water from Tallinn WWTP anaerobic tank. The amount of anammox 16S rRNA in the reject water was 2.26×10^6 copies g^{-1} TSS, which was a hundredfold lower than in the reactors.

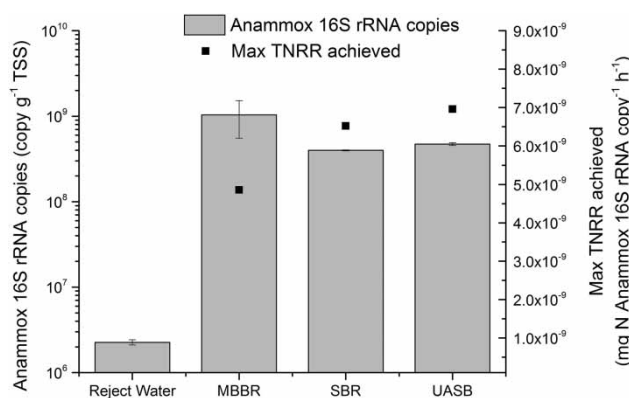


Figure 5 | Gene copies of anammox 16S rRNA and the maximum TNRR per a gene copy in reject water and in tested biomasses.

To compare the maximal TNRRs of different biomass, the qPCR results were used to calculate the maximum nitrogen removal rate per an anammox 16S rRNA gene copy (Figure 5). The biomass with the highest maximum TNRR per a gene copy was from the UASB (6.96×10^{-9} mg N anammox 16S rRNA $copy^{-1}\ h^{-1}$), while the biomass from the SBR and MBBR achieved 6.52×10^{-12} and 4.86×10^{-12} mg N anammox 16S rRNA $copy^{-1}\ h^{-1}$, respectively.

Based on the results acquired by qPCR analysis, the higher limiting nitrite concentrations for anammox process could also mean higher V_{max} values for well-functioning anammox biomass. For the MBBR at $40\ mg\ NO_2^- - NL^{-1}$ the highest TNRR per a gene copy was about 1.4 times lower than for the UASB. The peak TNRR was achieved on similar nitrite concentrations for both UASB and SBR and the maximum TNRRs per gene copy were similar as well (6.96×10^{-9} mg N anammox 16S $copy^{-1}\ h^{-1}$ and 6.52×10^{-9} mg N anammox 16S $copy^{-1}\ h^{-1}$, respectively).

In the biomass from the MBBR, *Candidatus Brocadia fulgidia* and *Candidatus Kuenia stuttgartiensis* (Zekker et al. 2012) and in the biomass taken from the SBR anammox clones closest to *Candidatus Brocadia fulgidia* (Zekker et al. 2014b) were found as the most abundant ones. In the biomass from Rotterdam pilot plant, the inoculum for the UASB, *Candidatus Brocadia anammoxidans* was with relative abundance of 50–60% out of all anammox bacteria (van der Star et al. 2007). Although characterisation of different anammox cultures has been carried out in the recent years (Awata et al. 2013; Ali et al. 2015), no information was available for the characteristics and tolerance of *Candidatus Brocadia fulgidia*. For that reason, giving objective conclusions based on the microbiological data from the MBBR and SBR are difficult. Although *Candidatus Brocadia anammoxidans* has been reported to be less tolerant to nitrite than other cultures (Oshiki et al. 2011), in current research this anammox bacteria showed the highest nitrite tolerance. This could either be due to the type of biomass (granular) or the effect of nitrite spiking, which could prove the importance of both in operating a stable anammox system.

CONCLUSION

Nitrite inhibition and limitation for anammox process were studied with biomass taken from three different reactors: MBBR, SBR and UASB. Batch tests showed that while the response to nitrite inhibition can be lessened with nitrite spiking, nitrite limitation is primarily affected by the type

of biomass. Nitrite spiking was carried out in the MBBR and UASB and both biomasses were less susceptible to nitrite inhibition – the difference between the highest TNRR (inhibition threshold) and IC_{50} values was two times in the MBBR (40 and 85 mg NO_2^- -NL $^{-1}$) and three times in the UASB (83 and 240 mg NO_2^- -NL $^{-1}$). The SBR in which no nitrite spiking was carried out before batch tests, had a stronger and steeper response to nitrite inhibition (highest TNRR at 81 and IC_{50} at 98 mg NO_2^- -NL $^{-1}$).

The biomass from the MBBR achieved both the highest maximum TNRR (5.03 mg N g $^{-1}$ TSS h $^{-1}$) and highest abundance in 16S rRNA gene copies (1.04×10^9 copies g $^{-1}$ TSS). The reactor also showed the highest TNRRs at low nitrite concentrations, which could make operating a biofilm reactor cheaper and technologically easier than suspended anammox biomass reactors.

The highest TNRR per 16S rRNA anammox gene copies was achieved with biomass from the UASB at 83 mg NO_2^- -NL $^{-1}$. Contrary to earlier research on anammox reactors, reactors working on suspended or granular may function better at relatively high nitrite concentrations around 60–80 mg NO_2^- -NL $^{-1}$, indicating a strong limiting effect of nitrite on anammox process, which should be researched even further.

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