Combined effects of dissolved oxygen and pH on nitrogen removal in a sequencing batch reactor with anammox-enriched granular sludge

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Abstract: The combined effects of dissolved oxygen (DO) and pH on nitrogen removal were investigated in lab-scale sequencing batch reactors (SBR) with anammox enriched granular sludge. The highest specific nitrogen removal rate (SNRR) was achieved under non-aerated condition ($1.1 \text{ gN g}^{-1} \text{ VSS d}^{-1}$), resulting in the nitrogen removal efficiency of 81.6%. Although the nitrogen removal in aerated conditions was easily inhibited, an increment on SNRR was observed at $\text{DO} = 0.5 \text{ mgO}_2 \text{ L}^{-1}$. This indicated that besides anammox, additional nitrogen conversion pathways were active in the biomass. The optimal SNRR was obtained for pH ranging from 6.5 to 8.5, characterized by low concentrations of free ammonia (FA) and free nitrous acid (FNA). In this pH range, nitrogen conversion ratios were similar to the stoichiometric anammox ratio (1:1.32:0.26), suggesting that anammox might be the primary process for nitrogen conversion under such conditions. Oxygen-consuming bacteria were significantly inhibited by low pH resulting in lower oxygen utilization. A DO elbow was observed after 150 min of the pH tests, which could be an ultimate indication of nitrogen removal by oxygen-consuming bacteria present in anammox-enriched granular sludge.

Keywords:

Anammox, granular sludge, dissolved oxygen, pH, nitrogen removal, combined effects.

1. Introduction

Anaerobic ammonium oxidation (anammox) has been widely recognized as a viable alternative to the conventional nitrification-denitrification in sidestream and recently mainstream reactors. Since the growth rate of anammox bacteria is very slow ($\mu=0.0027\text{ h}^{-1}$) (Strous et al., 1998; Van der Star et al., 2007), it is important to operate the anammox system at optimum conditions to support the sufficient bacteria growth rate. However, the process is inhibited by many factors, including specific substances and operating parameters (Jin et al., 2012). Among the operating parameters, dissolved oxygen (DO) and pH have been found to be crucial for the process enhancement as well as the extensive application of the anammox technology.

Previous studies have reported that the anammox process could be reversibly inhibited by DO at low concentrations ($0.16\text{ mgO}_2\text{ L}^{-1}$) in an intermittent aerated system (Strous et al., 1997). Third et al. (2005) observed anammox activity was minimal during aerobic periods and only began when the DO concentration decreased below $0.1\text{ mgO}_2\text{ L}^{-1}$ in a sequencing batch reactor (SBR) inoculated with activated sludge and operated by intermittent aeration mode. Egli et al. (2001) also observed both reversible and irreversible anammox inhibition, respectively, at a low DO concentration ($0.08\text{ mgO}_2\text{ L}^{-1}$) and a higher DO concentration ($1.44\text{ mgO}_2\text{ L}^{-1}$). Therefore, the DO concentration should be strictly controlled in anammox systems for avoiding bacteria inhibition. Recently, the anammox viability has been studied by integration with nitrification (Cema et al., 2011; Third et al. 2005) or simultaneous nitrification/denitrification (Chen et al., 2009). In this context, the DO concentration in the liquid phase was found to have other impacts on bacteria groups. Ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) could consume oxygen while anammox and/or denitrifiers metabolisms could

continuously be suppressed by high DO concentrations. In a moving bed biofilm reactor (MBBR) performing nitrification–anammox under continuous aeration, Cema et al. (2011) achieved the highest nitrogen removal efficiency of 78.3% at the $\text{DO} = 2.5 \text{ mgO}_2 \text{ L}^{-1}$. At higher DO ($4 \text{ mgO}_2 \text{ L}^{-1}$), nitrogen removal decreased, resulting in a loss of the anammox activity, which was proved by nitrite accumulation into the system. Although the DO impact on the single processes is well understood, there has been no clear comprehension until nowadays regarding the mechanisms of the DO effect on nitrogen removal and the transformation pathway, considering the coexistence of various bacteria in anammox-enriched granular sludge.

With regard to pH, Strous et al. (1999) reported the physiological range between 6.7 and 8.3 in a dispersed aggregated anammox system. Hence, extreme changes in this parameter should be avoided to maintain the anammox metabolism. Egli et al. (2001) observed anammox activity in a wider pH range (6.5–9.0) in a rotating biological contactor (RBC) treating leachate, in which the optimum pH was 7.5–8.0. Tao et al. (2012) also reported pH=8.1 enhanced the combined nitrification–anammox steps compared to a lower one (7.6) in a biofilter treating dairy wastewater. In a continuous-flow anammox granular sludge system, a stable anammox reaction was still maintained even at higher pHs of 8.5–9.3 (Tang et al., 2010). Ahn et al. (2004) observed that the anammox activity was not inhibited in spite of the high pH values (9.3–9.5) in an up-flow anaerobic sludge bed reactor (UASB) to integrate anaerobic digestion with anammox for piggery waste treatment. In contrary, Fux et al. (2004) reported halting the anammox activity at pH=9.3 in a fixed-bed reactor. Moreover, Jaroszynski et al. (2011) observed a higher nitrogen removal rate (60%) in a MBBR operated at pH=6.5 compared to that at a higher pH (7.5–8.1). The inconsistency in the reported pH effects on nitrogen removal in the

1 anammox process might be attributed to the differences in physical structures of sludge
2 (flocculent sludge, biofilm or granular sludge), microbial population, influent
3 characteristics, and operating conditions during the experiments. Besides the overall
4 nitrogen removal effect, actual nitrogen transformation pathway also need to be
5 investigated in anammox-enriched granular sludge.

6 Although several studies have been conducted regarding the anammox inhibition due to
7 either unfavorable DO or pH, the combined effects of these factors on nitrogen removal in
8 anammox-enriched granular sludge have not been studied so far. Based on the above
9 rationale, both effects of pH and DO on the anammox-enriched granular sludge were
10 evaluated in this study, aiming at optimizing the operational conditions for high nitrogen
11 removal by means anammox enriched granular process. For this purpose, kinetic assays
12 were conducted in lab-scale sequencing batch reactors (SBR) at different DO
13 concentrations and pH conditions.

15 **2. Material and Methods**

16 **2.1 Substrates and anammox-enriched granular sludge**

17 Ammonium chloride (NH_4Cl) and sodium nitrite (NaNO_2) were used as electron donor
18 and electron acceptor, respectively, with supplemental sodium bicarbonate (NaHCO_3) as
19 an inorganic carbon source. The biomass characteristics, initial ammonium and nitrite
20 concentrations, and operational conditions of batch experiments are summarized in Table
21 1. Anammox-enriched granular sludge was obtained from a full-scale sidestream
22 treatment system in Zurich (Switzerland) and cultivated for more than one year on
23 synthetic substrates (Sobotka et al., 2015). Besides anammox bacteria (mostly belonged
24 to *Candidatus Brocadia*), AOB such as *Nitrosomonas sp.*, NOB such as *Nitrospira sp.*

and heterotrophic bacteria such as denitrifying organisms from *Alfa* and *Betaproteobacteria* families were also identified into the anammox-enriched granular sludge by 16S rRNA gene sequences analysis (Sobotka et al., 2015).

2.2 Experimental setup and operation

Kinetic assays (Tests 1.1-1.7) at different DO concentration setups (0 to 1.0 mgO₂ L⁻¹) were carried out in a 10 L SBR with an open space and the pH was maintained at 7.6-7.8. To elucidate the biological pathways involved in nitrogen removal at 0.3, 0.5, and 2.0 mgO₂ L⁻¹, two series of tests were performed with dosing only ammonium (Tests 2.1-2.2) or nitrite (Tests 3.1-3.4) dosage in the same reactor configuration. Nitrous oxide (N₂O) production was monitored using an online nitrous oxide microsensor N₂O25 (Unisense, Aarhus, Denmark). Kinetic assays (Tests 4.1-4.10) at different initial pH values (6 to 10.5) were carried out in the SBR with a working volume of 4L of anoxic medium. The pH was adjusted by the addition of HCl (1 mol L⁻¹) or NaOH (2 mol L⁻¹), and the initial DOs were between 0.4 and 0.5 mgO₂L⁻¹. During Tests 4.1-4.10, pH and DO were not controlled, whereas Tests 5.1-5.4 were conducted in a completely sealed reactor (2.2 L of the working volume) equipped with a magnetic stirrer and a N₂ gas-tight balloon in order to maintain strictly anoxic conditions and an adequate pressure for sampling. The stirring speed was maintained as low as possible to keep the biomass in suspension. Therefore, those tests were conducted at different initial pH values (6.0 to 10.5) and initial DO = 0.03 mg O₂ L⁻¹.

The SBRs were equipped with an automated control system for DO, pH, and temperature. The probes/electrodes (WTWGmbH, Germany) were used for continuous monitoring of pH (SenTix21), temperature, and DO concentration (CellOx 325). The temperature was

kept constant at 30±1°C by means of a thermostatic bath. Prior to each test, anoxic conditions in the reactors were obtained by gassing the mixed liquor for approximately 5 min with N₂.

2.3 Analyses

Samples of the mixed liquor were collected every 30 minutes over a period of 2-3 hours. The samples were filtered through 0.45 µm pore-size nitrocellulose membrane (Whatman, Kent, UK) and immediately analyzed. Ammonium (NH₄⁺-N), nitrite (NO₂⁻-N), and nitrate (NO₃⁻-N) were analyzed with cuvette tests (Hach Lange, Dusseldorf, Germany) using a Xion 500 spectrophotometer (Dr Lange GmbH, Berlin, Germany). Total nitrogen (TN) was determined using a total organic carbon TOC/TN analyzer (Shimadzu, TOC-Vcan, Japan) equipped with a total nitrogen measurement unit. All conventional parameters such as pH, total suspended solids (TSS), and volatile suspended solids (VSS), were determined according to Standard Methods (APHA, 2005). The specific nitrogen removal rate (SNRR) was obtained based on TN removal measurements using the following equation:

$$SNRR = \frac{\Delta TN}{VSS \cdot \Delta t} \quad (1)$$

The inhibition of pH on the anammox activity is explicitly attributed to the pH dependency, unionized and presumably more toxic forms of the substrates, such as free nitrous acid (FNA) and free ammonia (FA) (Fernández et al., 2012). Therefore, threshold values for both FA and FNA were calculated based on the original relationships proposed by Anthonisen et al. (1976).

2.4 Models describing pH effects on nitrogen removal

1 **Michaelis model**

2 The Michaelis model could be used to predict the pH effect on enzymatic reactions (Segel,
3 1975):

$$4 \quad \frac{SNNR}{SNNR_{\max}} = \frac{1}{1 + 10^{(pK_{S1} - pH)} + 10^{(pH - pK_{S2})}} \quad (2)$$

5 where: $SNNR$ refers to the observed rate at the given pH ($\text{gN g}^{-1}\text{VSS d}^{-1}$); $SNNR_{\max}$ refers
6 to the maximum rate at the optimum pH ($\text{gN g}^{-1}\text{VSS d}^{-1}$); pK_{S1} is the logarithm of the
7 lowest pH at which $SNNR$ is equal to $\frac{1}{2} SNNR_{\max}$; pK_{S2} is the logarithm of the highest pH
8 at which $SNNR$ is equal to $\frac{1}{2} SNNR_{\max}$. The pK_{S1} and pK_{S2} values were obtained by
9 performing curve fitting with Origin software to minimize the residuals, i.e., the
10 deviations of the model prediction curves from the experimental data of $SNNR/SNNR_{\max}$.

11 The Levenberg-Marquardt (L-M) algorithm was used in the iterative procedure.

13 **Haldane model**

14 Haldane model is often used to describe the kinetics of pollutant biodegradation
15 involving inhibition of substrates at their high concentrations (Chen et al., 2011):

$$16 \quad SNNR = \frac{SNNR_{\max}}{1 + \frac{K_m}{S} + \frac{S}{K_i}} \quad (3)$$

17 where: K_m is the half saturation constant(mgL^{-1}); K_i is the substrate inhibition
18 constant(mgL^{-1}) and S is the substrate concentration (mgL^{-1}).

19 In the anammox process, ammonium and nitrite could be considered as both substrates
20 and inhibitors. Therefore, Equation 3 was expanded as follows:

$$21 \quad SNNR = \frac{SNNR_{\max}}{\left(1 + \frac{K_{mNH_4}}{S_{NH_4}} + \frac{S_{NH_4}}{K_{iNH_4}}\right) \left(1 + \frac{K_{mNO_2}}{S_{NO_2}} + \frac{S_{NO_2}}{K_{iNO_2}}\right)} \quad (4)$$

As weak acids and bases, an ionization equilibrium of free nitrous acid (FNA) and free ammonia (FA) exists in water solutions, which could be determined by pH values. Therefore, Equation 4 could be presented as follows (Yang et al., 2006):

$$\frac{SNRR}{SNRR_{\max}} = \frac{1}{\left(1 + \frac{10^{-pH} K_{mNH_4} K_b + K_{mNH_4} K_w}{10^{-pH} S_{NH_4} K_b} + \frac{10^{-pH} S_{NH_4} K_b}{10^{-pH} S_{NH_4} K_b - K_w K_{iNH_4}}\right) \left(1 + \frac{10^{-pH} K_{mNO_2}}{10^{-pH} + S_{NO_2} K_a} + \frac{10^{-pH} + S_{NO_2} K_a}{10^{-pH} K_{iNO_2}}\right)}$$

(5)

where: S_{NH_4} is the total ammonia concentration, including ionized ammonia and free un-ionized ammonia (mgN L^{-1}); S_{NO_2} is the total nitrite concentration, including ionized nitrite and free un-ionized nitrous acid (mgN L^{-1}); K_a is the dissociation constant of nitrite; K_b is the dissociation constant of ammonium, and K_w is the ionization product constant of water. The unknown parameters were estimated in a similar way to Michaelis model.

3. Results and Discussion

3.1 Effect of DO on nitrogen removal

As each test was started with different nitrogen concentrations, the $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations profiles for specific initial DO and pH were normalized. As shown in Figure 1(a-c), the highest SNRR of $1.1 \text{ gN g}^{-1} \text{ VSS d}^{-1}$ was obtained in the non-aerated reactor ($\text{DO} = 0.03 \text{ mgO}_2 \text{ L}^{-1}$), resulting in the most efficient ammonium and nitrite removal (81.6%) during 2 h measurements. In the aerated reactors, nitrogen removal was explicitly inhibited, but the actual inhibition level was different in the specified DO concentrations. When DO removed either between 0.3-0.4 or 0.6-1.0 $\text{mgO}_2 \text{ L}^{-1}$, similar trends in the effluent ammonium and nitrite concentrations were observed low SNRRs ($< 0.5 \text{ gN g}^{-1} \text{ VSS d}^{-1}$) and nitrogen removal efficiencies ($< 55\%$). On the other hand, SNRRs increased ($0.8 \text{ gN g}^{-1} \text{ VSS d}^{-1}$) at the $\text{DO} = 0.5 \text{ mgO}_2 \text{ L}^{-1}$, which resulted in the

1 nitrogen removal efficiency of 80% after 2.5 h. This finding is in contrast with the
2 directional DO suppression on nitrogen removal in the anammox process reported by Egli
3 et al. (2001). However, a similar behavior in terms of the nitrogen removal efficiency at
4 the DO range of 0.4-1.0 mgO₂ L⁻¹ was observed by Vangsgaard et al. (2012) in
5 nitrification/anammox biofilm (Figure 2). This suggests that, nitrification might be an
6 additional nitrogen transformation pathway besides anammox in the studied reactor.

7 The NO₂⁻-N removal/NH₄⁺-N removal ratio remained stable (1.3-1.5) for DO below 0.6
8 mgO₂ L⁻¹ (Figure 1d), which was similar to the theoretical anammox conversion ratio of
9 1.32 (Strous et al., 1998). In the case of higher DO (0.8 and 1.0 mgO₂ L⁻¹), the NO₂⁻-N
10 removal/NH₄⁺-N removal ratio decreased to 0.8, suggesting that AOB were much more
11 active compared to anammox bacteria. This resulted in more nitrite generation and less
12 nitrite consumption. Furthermore, with DO below 0.8 mgO₂ L⁻¹, nitrate was consumed
13 rather than produced, suggesting the possible occurrence of endogenous denitrification.
14 Only when the reactor was switched from the non-aerated to aerated phase (0.5 mg O₂
15 L⁻¹), nitrate production was observed. At the DO = 0.5 mgO₂ L⁻¹, the increase in NO₃⁻-N
16 production/NH₄⁺-N removal ratio could be attributed to the DO inhibition of endogenous
17 denitrification.

18 In order to further examine the nitrogen transformation pathway into the
19 anammox-enriched granular biomass, Tests 2.1-2.2 and Tests 3.1-3.4, were performed
20 with only ammonium or nitrite addition, respectively. Different behaviors could be
21 observed at the DO of 0.3, 0.5 and 2.0 mg O₂ L⁻¹ (Figure 3), indicating variance in the
22 combined nitrogen removal process in the anammox-enriched granular sludge. The
23 production of N₂O was influenced by DO and NO₂⁻-N concentrations, which was similar
24 to observations reported in the literature (Castro-Barros et al., 2015; Kampschreur et al.,

2008). In the tests dosing $\text{NH}_4^+\text{-N}$ (initial concentration of 80 mgN L^{-1}), when the DO was set to $0.3 \text{ mg O}_2 \text{ L}^{-1}$, 20.3 % of the initial ammonium load was removed after 3 hour's measurements, while the N_2O concentration was remaining at an undetected level (Figure 3a). At the $\text{DO} = 0.5 \text{ mg O}_2 \text{ L}^{-1}$, a small amount of N_2O was detected and then quickly consumed (Figure 3c). This indicated the possible occurrence of nitrification and subsequent removal of produced nitrite and ammonium by anammox bacteria in inner areas of the granules. In the test with nitrite dosage ($C_0 = 80 \text{ mgN L}^{-1}$), explicit N_2O peaks were detected under both tested DO conditions (Figures 3b and 3d), which might be associated with autotrophic denitrification, i.e. reduction of $\text{NO}_2^-\text{-N}$ to N_2O by AOB (Kampschreur et al., 2008). It should be noted that, unlike the test at the $\text{DO} = 0.3 \text{ mg O}_2 \text{ L}^{-1}$, a prolonged N_2O accumulation was observed when the DO was set to $0.5 \text{ mg O}_2 \text{ L}^{-1}$, indicating more active NO_2^- reduction. Furthermore, in comparison with the nitrogen removal performance at $\text{DO} = 0.3 \text{ mg O}_2 \text{ L}^{-1}$, higher $\text{NH}_4^+\text{-N}$ removal efficiencies (28.5%) and nitrite (23.6%) were obtained at $\text{DO} = 0.5 \text{ mg O}_2 \text{ L}^{-1}$, which are consistent with the findings in Test 1. It also confirmed that nitrogen removal may be performed by various processes other than only anammox. In the case of lower initial nitrite dosage (20 mgN L^{-1}), the N_2O accumulation was also observed (Figure 3e), indicating that nitrite was not an inhibitor of AOB activity in a higher concentration (80 mgN L^{-1}), as used in the last test. On the contrary, DO concentration can be an important operational parameter in order to control N_2O presence in the system, which can be easily stripped out from the liquid phase by intensive aeration (Figure 3f). Under the DO-limited conditions, AOB and NOB compete for oxygen to carry out oxidation of ammonium and nitrite, respectively. Moreover, those microorganisms share a symbiotic relationship since AOB produce nitrite required as a substrate for NOB. This

prevents the nitrite accumulation which can be toxic to anammox bacteria (Mutlu, 2015). Nevertheless, low DO concentrations favor the AOB growth over NOB, since NOB are characterized by a lower oxygen affinity (Blackburne et al., 2008). Vlaeminck et al. (2010) and Lotti et al. (2015) noted that stratification of granular or biofilm anammox systems could be observed. In studied cases, AOB and NOB act as a protective barrier to create favorable conditions for anammox development in the inner layers. Considering the findings obtained in the present study, a complex combination of different nitrogen removal processes was found to be active in the studied SBR. The actual contribution of each bioprocess was explicitly influenced by the DO concentrations. Under non-aerated conditions, anammox was the most active process in the anammox-enriched granular biomass. Endogenous denitrification could also be a possible pathway for nitrogen removal under such conditions (Dapena-Mora et al., 2004). At DO varying from 0.3 to 0.4 mg O₂ L⁻¹, inhibition of the anammox activity was observed (Figure 1c), resulting in favorable conditions to activate the AOB and NOB metabolisms. For higher DO (0.5 to 0.6 mg O₂ L⁻¹), a good balance of nitrogen removal by endogenous denitrification and nitrification was observed when AOB outcompeted NOB for oxygen. Such a situation implicitly suggests that nitrogen was removed by joint action of anammox bacteria, AOB and denitrifiers due to the occurrence of SNRR peak (Figure 1c) and similar nitrogen conversion ratios to the anammox stoichiometric values (Figure 1d). For DO higher than 0.8 mg O₂ L⁻¹, endogenous denitrification began to be inhibited and AOB are more active to use oxygen available without limitation. At high DO concentrations, an intensive consumption of NH₄⁺ by AOB, and resulting nitrite accumulation were accompanied by severe inhibition of the anammox metabolism.

3.2 Effect of pH on nitrogen removal

Profiles of the normalized concentrations of $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ for specific initial pH values are compared in Figure 4 (a-b). The observed trends of ammonium and nitrite concentrations were similar with the initial pH varying from 6.5 to 8.5. As shown in Figure 4c, the maximum SNRR (around $0.8 \text{ gNg}^{-1}\text{VSS d}^{-1}$) was also reached at pH in the same range of 6.5-8.5, resulting in high nitrogen removal efficiencies (61.8-78.1%). The optimal pH range in the present study is consistent with the optimal range (6.7-8.3) for anammox reported by Strous et al. (1999). In addition, the SNNRs and nitrogen removal efficiencies varied in terms of the different intensities of pH shocks. A decline in the SNRR occurred once the pH deviated from the optimum range and the nitrogen removal activity was hampered. Only minor nitrogen removal efficiency (<7%) was observed for the extreme initial pH values (6.0 and 10.5).

The pH can affect directly the bacterial growth and its enzymatic activities (Campos and Flotats, 2003). Indirectly, the pH affects the anammox activity through changes in the concentration of pH-dependent, unionized and presumably more toxic forms of FA and FNA substrates (Fernández et al., 2012). It should be noted that the optimal SNRR in the present study occurred only when the FA and FNA concentrations were also maintained at low levels (Figure 4c). A significant decline in the SNRR occurred when the initial pH decreased from 6.5 to 6.0, with the FNA concentrations increasing from 0.02 to 0.08 mg N L^{-1} . A similar behavior was observed when the initial pH raised from 8.5 to 10.5, with FA concentrations increasing from 6.8 to 31.3 mg N L^{-1} . In both cases, nitrogen removal efficiencies deteriorated, which confirmed that high SNRRs were correlated with low FA or FNA concentrations. FA and FNA have been previously described as strong anammox inhibitors regardless of the pH in the medium (Jaroszynski et al. 2012; Fernández et al.

2012). Jung et al. (2007) and Jaroszynski et al. (2012) reported that FA would inhibit anammox at concentrations as low as 1.7 mg L⁻¹. Fernandez et al. (2009) reported that 0.0015 mgFNA L⁻¹ induced a loss in the nitrogen removal efficiency and disabled the biofilm system in a continuous feeding test. The bacterial inhibition by FA has been related with its diffusion through the cell membrane into the cytoplasm and the subsequent change of the inner pH (Wittmann et al. 1995; Martinelle et al. 1996), which could results in neutralization of the membrane potential, thereby ultimately leading to cell death.

However, some recent studies showed that FA is not an important inhibitory factor when its concentration is below 17 mg L⁻¹ (Tang et al., 2010; Fernández et al. 2012). Puyol et al. (2014) also suggested that the anammox process is mainly inhibited by pH itself rather than by FA in mildly alkaline solutions. Anammox bacteria were previously characterized by the presence of the *Anammoxosome*, a membrane-bound organelle, where ammonium and nitrite are converted to N₂ via hydrazine intermediate. Between the *Anammoxosome* and the riboplasm (equivalent to cytoplasm in anammox bacteria), there is an intracellular proton gradient responsible for energy production (van der Star et al. 2010). Therefore, extreme pHs may affect the proton transfer and alter metabolic processes which are dependent of pH gradients, such as energy generation by adenosine triphosphatases (ATP) and active transport of proteins (some of which are NO₂⁻ transporters) (Luet al., 2013). Long-term pH alterations in the medium could result in disruption of the proton motive force, thus inhibiting the associated energy generation for enzymes reaction which requests strict pH condition (Carvajal-Arroyo et al., 2014). In addition, exposure to extreme pH medium may cause denaturation of cellular proteins,

original bond cleavage and other biological structures destruction, and ultimately leading to enzymes inactivation.

In order to further explain the inhibition effects of pH, FA or FNA, two pH models were applied to describe the SNRRs observed in the studied reactor. As shown in Figure 5, both Michaelis model ($R^2 = 0.84$) and Haldane model ($R^2 = 0.93$) accurately predicted the SNRRs under a wide range of pH. Considering the substrate inhibitory effects onto the biomass, the Haldane model fitted the experimental data better compared to the Michaelis model, which only considered pH effects. Consequently, the Haldane model is more suitable to predict the kinetic characteristics of the granular anammox-enriched biomass at different pH. As the actual presence of FA or FNA is pH-dependent, the direct inhibitory effects of pH are masked. Further studies are needed to elucidate and quantify the potential contribution of these factors to inhibition on anammox process.

The observed nitrogen conversion ratios in terms of the initial pH are shown in Figure 4d.

It should be noted that under the optimal pH conditions (6.5-8.5), the nitrogen conversion ratios were similar to the stoichiometric anammox ratio, i.e. 1:1.32:0.26 ($\text{NH}_4^+\text{-N}:\text{NO}_2^-\text{-N}:\text{NO}_3^-\text{-N}$), suggesting that anammox might be the primary process for nitrogen conversions under such conditions. With pH values outside the optimal pH range, the nitrogen conversion ratios deviated from the stoichiometric anammox ratio, resulting in fluctuations in the nitrogen transformation pathways. With the lowest initial pH (6.0), the $\text{NO}_3^-\text{-N}$ production/ $\text{NH}_4^+\text{-N}$ removal ratio was around 1, which was much higher than the anammox stoichiometric value (0.26). This indicated that in low pH conditions NOB were much more active in comparison with other bacteria, resulting in a relatively high production of nitrate. With the high initial pH (9.5), nitrate utilization by heterotrophic denitrifiers was faster than its production by anammox and/or NOB. In addition, an

increase in the NO_2^- -N removal/ NH_4^+ -N removal ratio was observed at pH 6.5 and 9.5. This proves that anammox bacteria, in comparison with AOB, were more sensitive to pH shocks. As a result, when pH started to deviate from the optimum range, AOB appeared to be more active, producing more nitrite.

3.3 Combined effects of DO and pH on the nitrogen removal activity

As presented above, both DO and pH as sole factors, had certain impacts on nitrogen removal. Hence, two-dimensional contour plots were constructed to get better visualization of the relationship between SNNR and experimental levels of the two tested variables (Figure 6). The contour plots showed a double-peak phenomenon, which indicated a significant interaction between the two variables and SNNR. The maximum SNNRs ($> 0.9 \text{ gN g}^{-1}\text{VSS d}^{-1}$) were observed at pH ranging from 7 to 8 and DO concentration varying from 0.0 to 0.1 $\text{mg O}_2 \text{ L}^{-1}$. Moreover, another local SNRR peaks ($0.75\text{-}0.9 \text{ gN g}^{-1}\text{VSS d}^{-1}$) were found at pH and DO in the range of 7.5-8.5 and 0.5-0.6 $\text{mg O}_2 \text{ L}^{-1}$, respectively. This suggested that not only anammox bacteria but also other microbial groups (e.g. AOB, NOB and denitrifiers) were responsible for nitrogen removal in the anammox-enriched granules. The cross sectional view further confirmed that the combined nitrogen removal reached a relatively higher level at 0.5-0.6 $\text{mgO}_2 \text{ L}^{-1}$ with the optimal pH between 7.5-8.0.

According to the theoretical anammox reaction, H^+ is consumed when nitrite is utilized as an electron acceptor to oxidize ammonium, resulting in an increase (approximately by 0.5) in the pH value (Figure 7a). The DO concentration was also temporarily changed. As indicated in Figure 7b, the DO significantly raised from 0.5 to 4.5 $\text{mg O}_2 \text{ L}^{-1}$ in the abiotic assay. In this case, the DO profile can be represented as a reoxygenation curve due

to O₂ dissolution from the air to liquid phase by mixing operation. In the case of bioassays, the DO concentrations increased to a lesser extent in comparison with the abiotic assay, revealing that a certain amount of O₂ was consumed by some microbial groups in the anammox-enriched granules. Furthermore, with a higher initial pH (varying from 8.5 to 10.5), the DO concentrations maintained in a low level (approximately 0.4-0.5 mg O₂ L⁻¹) during the tested period, whereas the values increased to approximately 2.7 mg O₂ L⁻¹ at the lowest pH (6.0). It further proved that oxygen-consuming bacteria (AOB or NOB) could be inhibited by low pH and thereby less oxygen was utilized. An explicit DO elbow can be observed after approximately 150 min in the physiological pH range, when ammonium and nitrite were almost completely removed in the reactor. This is an ultimate indication of nitrogen removal by oxygen-consuming bacteria present in the anammox-enriched granular sludge.

4. Conclusions

Nitrogen removal by the anammox process resulted in the highest SNRR in a granular anammox-enriched biomass subjected to non-aerated conditions. In aerated-conditions, nitrification and endogenous denitrification were also found to be active in the biomass, resulting in significant SNRR values. With pH ranging from 6.5 to 8.5, optimal SNRR was achieved in the performed tests, guaranteed by the maintenance of low FA and FNA concentrations in the reactor. While considering the combined effects of DO and pH on SNRR, proper pH and DO concentration should be maintained to enhance nitrogen removal by combined processes in a anammox-enriched granular sludge.

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Figures caption:

Figure 1. Profiles of normalized concentrations of $\text{NH}_4^+\text{-N}$ (A) and $\text{NO}_2^-\text{-N}$ (B); SNRRs (C) and nitrogen conversion ratios in anammox-enriched granular sludge at different DO concentrations (D).

Figure 2. Comparison of nitrogen removal in anammox-enriched granular sludge (this study) and biofilm (Vangsgaard et al., 2012) at different DO concentrations.

Figure 3. Behavior of nitrogen compounds in the studied reactor at the DO concentrations of 0.3 (A-B), 0.5 (C-E) and 2.0 (F) $\text{mgO}_2 \text{ L}^{-1}$.

Figure 4. Profiles of normalized concentrations of $\text{NH}_4^+\text{-N}$ (A) and $\text{NO}_2^-\text{-N}$ (B); specific nitrogen removal rates (SNRRs) (C); nitrogen conversion ratios in anammox-enriched granular sludge under different initial pH conditions (D).

Figure 5. Effects of pH on the $\text{SNRR}/\text{SNRR}_{\text{max}}$ ratio. Fitting of the experimental data by the Michaelis model ($R^2 = 0.84$) and Handale model ($R^2 = 0.93$).

Figure 6. The interactive effects of pH and DO on the specific nitrogen removal rates (SNRRs).

Figure 7. Profiles of pH (A) and DO concentrations (B) in the studied reactors under different initial pH conditions.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Table 1. Initial conditions of the performed experiments in terms of biomass, substrates, DO and pH.

Tests No.	Aim of investigation	Biomass (gVSS L ⁻¹)	NH ₄ ⁺ (mg N L ⁻¹)	NO ₂ ⁻ (mg N L ⁻¹)	DO (mgO ₂ L ⁻¹)	pH	Volume(L)
1.1-1.7	Effects of DO concentration on nitrogen removal	3.0±0.5	103.9±10.3	127.0±17.4	0.03-1.0	7.7 ±0.1	10
2.1-2.2	NH ₄ ⁺ utilization pathways under different DO concentrations	3.0±0.5	87.3±2.0	-	0.3 and 0.5	7.7 ±0.1	10
3.1-3.4	NO ₂ ⁻ utilization pathways under different DO concentrations	3.0±0.5	-	80.5±0.9, 22.3±1.2	0.3 and 0.5 0.5 and 2	7.7 ±0.1 7.7 ±0.1	10 10
4.1-4.10	Effects of initial pH on nitrogen removal at DO=0.5 mgO ₂ L ⁻¹	1.3±0.3	33.1±2.4	43.0±1.5	0.5±0.1 ^a	6.0-10.5 ^b	4
5.1-5.4	Effects of initial pH on nitrogen removal at DO=0.03 mgO ₂ L ⁻¹	1.3±0.3	39.1±2.7	50.7±3.9	0.03 ^a	6.0-10.5 ^b	2.2

a. initial DO concentration;

b. initial pH.

Table 2. Values of the model parameters in the examined pH models.

Michaelis model			Haldane model		
Parameters	Unit	Values	Parameters	Unit	Values
pK_{S1}	-	6.47	K_a	-	4.6×10^{-4}
pK_{S2}	-	9.41	K_b	-	1.57×10^{-5}
			K_w	-	10^{-14}
			K_{mNH_4}	mg/L	48.4
			K_{iNH_4}	mg/L	1123.2
			K_{mNO_2}	mg/L	6.6
			K_{iNO_2}	mg/L	720.6