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Effects of Oxidation Reduction Potential and Organic Compounds on Anammox Reaction in Batch Cultures

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Abstract

The present study investigates the effect of oxidation-reduction potential (ORP) and organic compounds on specific anaerobic ammonium oxidation activity (SAA) using batch experiments. The batch tests were based on the measurement of nitrogen gas production. The relationship between ORP and dissolved oxygen (DO) concentration was found to be ORP (mV) = 160.38 + 68 log [O_2], where [O_2] is the DO concentration in mg/L. The linear relationship obtained between ORP and SAA (R^2 = 0.99) clearly demonstrated that ORP can be employed as an operational parameter in the Anammox process. At ORP value of -110 mV, the SAA was 0.272 ± 0.03 g N_2-N (g VSS)$^{-1}$ d^{-1}. The investigation also revealed inhibitory effect of glucose on the SAA while acetate concentration up to 640 mg COD/L (corresponding to 10 mM) had stimulating effect on the SAA. However, acetate concentration beyond 640 mg COD/L had inhibitory effect on the Anammox activity. The results indicated that nitrogen rich wastewaters containing low level organic matter could be better treated by Anammox microorganisms in real-world conditions after some acidification process.

Keywords: Specific Anammox activity, Dissolved oxygen, Oxidation-reduction potential, Organic compounds, Wastewater

1. Introduction

Stringent effluent discharge limits for nitrogen in wastewater have led to exploration of many novel, promising and cost-effective nitrogen removal processes. Among them is the anaerobic ammonium oxidation (Anammox) process, a relatively new technological advancement in the removal of ammonia nitrogen from wastewater which is significant as it utilizes ammonium and nitrite at the same time and converts them directly into nitrogen gas.[1-4] Anammox is a lithoautotrophic biological conversion process mediated by a group of Planctomycetes bacteria including *Candidatus Brocadia anammoxidans*, *Candidatus Kuenenia stuttgartiensis*, and several species of the genus *Candidatus Scalindua*.[5] The Anammox reaction presented in Eq. (1)[6] shows the overall nitrogen balance in a ratio of 1:1.32: 0.26 for the conversion of ammonium and nitrite and the production of nitrate.

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O \quad (1)$$

Recently the combination of nitritation and denitrification, or Anammoxs, has shown interesting economical advantages for the separated treatment of ammonium-rich wastewaters such as supernatant from anaerobic sludge digesters.[3,4,7,8] In these processes, a combination of partial nitrification and Anammox require less oxygen input for the nitrification. This is because only part of the ammonium needs to be nitrified to nitrite while the remaining ammonium would combine with this nitrite in the Anammox process to produce nitrogen gas. As the Anammox reaction is an anaerobic autotrophic reaction, dissolved oxygen or organic matters are not required and are known to be inhibitors. However, during the wastewater treatment some oxygen will inevitably enter from the surrounding air and often with the influent wastewater and recirculated streams.

Recently, the growth characteristics and enrichment of Anammox microorganisms has been studied with DO concentration of the feeding solution reduced to 0.5 mg/L or lower.[9] They observed a decrease in doubling time to 1.8 days and increase in nitrogen removal from 0.4 to 0.55 Kg-N/m^3/d during a period of 7 days, from days 14 to 21. When the DO concentration in the process is below the detection limit of the commercially available DO probes (0.1 mg/L), the effects of oxygen on the Anammox process is often overlooked. Therefore, ORP can be used as an operational parameter in preference to DO in anoxic and anaerobic conditions since ORP covers anaerobic, anoxic and aero-

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bic conditions of biological wastewater treatment. However, the effect of ORP on the Anammox process has not been discussed much in the existing literature.

Though Anammox microorganisms have been described as obligate chemolithoautotrophs, many of the ammonia containing wastewaters are not free from organic matters and a purely autotrophic Anammox reaction (as shown in Eq. (1)) cannot occur in practice. However, it is reported that chemolithoautotrophs, nitrifiers for example, can grow mixotrophically and can use organic compounds as auxiliary carbon sources. This mixotrophic growth is usually beneficial as it enhances the growth rate and/or yield significantly. Particularly, it is advantageous in the Anammox process due to very low growth rate (doubling time of 10-20 days) and yield (0.066 CO_2 fixed/mol NH_4^+).[5] van de Graaf et al.[10] investigated the effects of organic compounds including glucose, fructose, cysteine, pyruvate and formate on the growth of Anammox bacteria in the concentration range 0.1-3 mM. However, in actual practice the treatment facilities often encounter wastewater from a variety of sources with a varied C/N ratio exceeding 3 mM concentration of organic compounds.

The objectives of the present study were to establish the relationship between ORP and DO concentration in Anammox reaction and investigate the effect of ORP on SAA in batch cultures. Further, the effects of glucose and acetate, as representative organic compounds, on the SAA have been studied to delineate the nitrogen removal in the Anammox process in the presence of organic matter.

2. Materials and Methods

2.1. Batch Test Procedure

2.1.1. Inoculation of Biomass and Composition of Feeding Substrate

The reactor and the vials of assays were inoculated with Anammox biomass belonging to the species *Candidatus B. Anammoxidans and K. stuttgartiensis* (species collected from KIST, Korea), washed and suspended in phosphate buffer (0.14 g/L KH_2PO_4 and 0.75 g/L K_2HPO_4). The experimental condition of the master culture reactor can be found elsewhere (Jung et al., 2007). The biomass concentration in all experiments was 1 g VSS/L and the mineral medium used was as described by van de Graaf et al..[10]

Table 1. Composition of feeding substrate in the batch test for Anammox bacteria

Component	Unit	Value
$KHCO_3$	mg/L	500
KH_2PO_4	mg/L	27.2
$CaCl_2 \cdot 2H_2O$	mg/L	180
$MgSO_4 \cdot 7H_2O$	mg/L	120
T. element S_1	mL/L	1
T. element S_2	mL/L	1

T. element S_1: EDTA, 5 g/L; $FeSO_4$, 5 g/L

T. element S_2: EDTA, 15 g/L; $ZnSO_4 \cdot 7H_2O$, 0.43 g/L; $CoCl_2 \cdot 6H_2O$, 0.24 g/L, $MnCl_2 \cdot 4H_2O$, 0.99 g/L; $CuSO_4 \cdot 5H_2O$, 0.25 g/L; $NaMoO_4 \cdot 2H_2O$, 0.22 g/L; $NiCl_2 \cdot 6H_2O$, 0.19 g/L; $Na_2SeO_4 \cdot 10H_2O$, 0.21 g/L; H_3BO_4, 0.014 g/L

The composition of the mineral medium for Anammox bacteria in the batch test is given in Table 1.

2.1.2. Effect of ORP on SAA

Experiments were carried out in a 1 L reactor made up of plexiglass and kept in the dark to avoid the exposure to light. The reactor contents were continuously mixed by a magnetic stirrer (150 rpm), and the reactor was closed to avoid the entry of air. The temperature was kept constant at 30°C by recirculation of water from a thermostatically controlled bath through the jacket of the reactor. pH was maintained at 7.8 throughout the experiments. The reactor was made aerobic by sparging with air and anaerobic by sparging with argon gas. To monitor both DO (Orion 850A, Thermo Electron, CO, USA) and ORP (Model 735P, Istek, Seoul, Korea) in the reactor, probes were calibrated and retained in the reactor throughout the study. During the entire study, initial concentrations of ammonia and nitrite were fixed at 70 mg N/L. The relationship between SAA and ORP was established based on nitrogen gas production in the Anammox process and calculated by stoichiometric equation (1). The SAA was calculated from the nitrogen gas production rate divided by the biomass concentration in the assays X (g VSS/L).[11]

$$SAA = \left(\frac{dN_2/dt}{X\,V_L}\right)\left(\frac{28g\ N}{mol\ N_2}\right)\left(\frac{1440\ \min}{d}\right),\ \text{g N}_2\text{-N (gVSS)}^{-1}\text{d}^{-1} \quad (2)$$

where V_L is the volume of the liquid phase (L).

2.1.3. Effects of Organic Compounds on SAA

The assays were performed in duplicate, in vials with a total volume of 30 mL and a volume of liquid of 20 mL, each closed with a gas-tight coated septum capable of withstanding about 2 bars of pressure. The initial pH was fixed and maintained at 7.8 in all experiments. The vials were placed in a thermostatic shaker, at 150 rpm and 30°C, until stable conditions were reached. Then each of the substrates $(NH_4)_2SO_4$ and $NaNO_2$ were added at 70 mg N/L and pressure was equalized to one atmosphere. When an inhibitory compound was tested, the corresponding amount of the compound was added to the substrates. The effect of organic compounds on the SAA was investigated in all the experiments based on the nitrogen gas production and their relationships were described according to the percentage of activity, calculated as[11]:

$$SAA\,(\%) = \frac{SAA}{SAA_0} \times 100 \quad (3)$$

where SAA_o is the maximum specific Anammox activity in the control assay (no presence of inhibitory compounds) and SAA is the maximum specific Anammox activity of the tests with inhibitory compounds.

2.2. Sampling and Analysis

Samples of liquid solution were prepared by filtering through

0.45 μm filter papers (GF/C-Whatman®). NH_4^+-N was measured by spectrophotometric method at λ_{max} of 425 nm (DR 2000, Hach, CO, USA). NO_2^--N and NO_3^--N were measured by ion chromatograph (DX-80, Dionex, CA, USA). The production of CO_2 gas was analyzed with a gas chromatograph (DS 6200, Donam instruments, Daejon, South Korea) by measuring the overpressure in the headspace with a time frequency depending on the biomass activity in each vial and reactor test, while N_2 gas was measured and/or calculated by GC and Eq. (1), respectively. The ORP and DO of the reactor were recorded more frequently (eight times at 0, 1st, 3rd, 5th, 9th, 16th, 22nd and at the 24th hour) during the first day of assay and twice a day (at 10 AM and 10 PM) for the remainder of the study period.

3. Results and Discussion

3.1. Relationship between DO Concentration and ORP in Anammox Reaction

The relationship between ORP and DO at low concentrations was established in the batch Anammox reaction (Fig. 1). The ORP was found to be linearly related to the logarithm of DO concentration (R^2 = 0.913). This type of linear relationships between ORP and logarithm of DO are well documented. For instance, Peddie et al.[12] related ORP with low levels of DO (<0.1 mg/L) obtained from pilot-scale batch study data where aerobic sludge digesters were undergoing alternating aerated and non-aerated conditions. The relationship between ORP and logarithm of DO concentration indicated with a stronger linearity that ORP could be used to measure very low DO concentrations.

The linear relationship obtained in this study between the ORP and DO concentration is in accordance with previous investigations[13,14] and is mentioned in Eq. (4):

$$ORP\ (mV) = 160.38 + 68 \log [O_2], \quad (4)$$

where $[O_2]$ is the DO concentration in mg/L. In this study the slope of straight line, defining the relationship between ORP and DO, was 68.0 mV and was in accordance to the previously reported results for activated sludge treatment processes.[13,15,16]

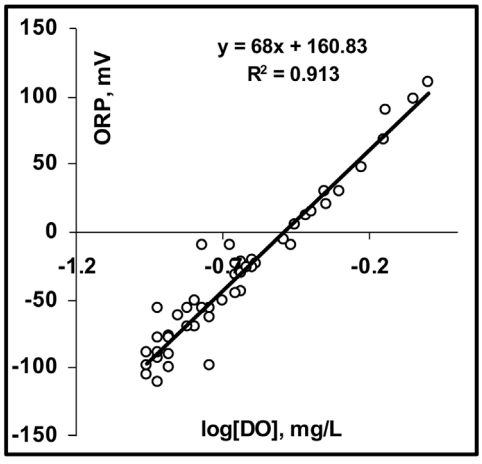


Fig. 1. Relationship between DO and ORP in Anammox reaction.

Corresponding to ORP values -110 to 110 mV, the DO concentrations were obtained in the range of 0.0001-0.18 mg/L (Eq. (4)). In the study by Lie and Welander,[15] the DO concentration of 0.01 mg/L was reported during the denitrification activity of activated sludge at an ORP of 110 mV. Peddie et al.[12] studied the use of ORP for the control of aerobic sludge digestion and observed 0.43 mg/L of DO concentration corresponding to ORP value 110 mV during the nonaerated conditions in aerobic sludge digester. Therefore, in this study DO concentration of 0.0001-0.18 mg/L corresponding to ORP value -110 to 110 mV appears relevant and that this extremely low DO concentration is not possible to measure accurately by commercially available DO probes.[16] Under such circumstances, ORP can be used as a strong operational parameter in estimating the DO concentrations.

3.2. Effect of ORP on Anammox Reaction

3.2.1. Effect of DO Concentration on Anammox Activity

The response of Anammox microorganisms, to varied DO concentrations was investigated in the 1 L batch reactor over a period of 48 hours. The influent DO concentration was varied with time depending on the performance of the reactor in converting NH_4^+ and NO_2^- to nitrogen gas. During every step decrease in the DO concentration, it was observed that the biomass took few hours to attain steady state in the NH_4^+ and NO_2^- conversion. The DO concentration was decreased gradually in five steps (2.0, 1.5, 1.0, 0.5 and 0.1 mg/L). It is clear from Fig. 2 that Anammox activity remained almost constant up to DO concentration of 0.5 mg/L and further, there was a significant increase in Anammox activity with decrease in DO concentration to 0.1 mg/L. No ammonium was oxidized in the presence of 2.0, 1.5, 1.0 mg DO/L. However, at DO concentration of 0.5 mg/L there was an apparent conversion of NH_4^+ and NO_2^- and further decrease in DO concentration to around 0.1 mg/L abruptly increased the conversion of NH_4^+ and NO_2^-. During the entire period, NH_4^+ and NO_2^- were consumed in the ratio of 1:1.05 and the NO_3^- accumulation was in the range of 1.2 -15.6 mg NO_3^--N/L . At DO concentration of 0.1 mg/L, NH_4^+-N conversion efficiency was around 85%, which showed the activity of the Anammox microorganisms at this concentration.

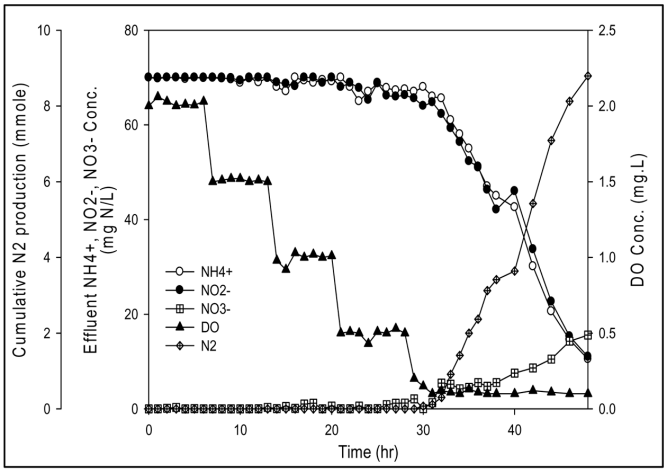


Fig. 2. Effect of DO on Anammox activity.

Gong et al.[17] observed a total nitrogen removal efficiency of 83.77% at influent NH_4^+-N concentration of 200 mg/L and DO concentration of 0.02 mg/L using two types of seed sludge (aerobic nitrifying sludge and Anammox sludge) in a membrane-aerated biofilm reactor operated for completely autotrophic nitrogen removal. Contrary to this, Hao et al.[18] reported that the Anammox activity is very much sensitive to oxygen and that DO concentrations of 0.03 mg/L could inhibit the Anammox process. Recently, Jung et al.[19] elucidated that in a continuous culture with nitrite and ammonium concentration of 5 mM each, influent DO concentration at less than 0.2 ppm plays an important role in the Anammox activity. For example, in their study they observed a rapid increase in the ammonium removal rate from 10 to 30 g N/m^3.day when DO concentration in the continuous reactor was reduced to less than 0.2 ppm. Therefore, taking into account the result from the present investigation and previous findings, it can be generalized that in realistic situation Anammox microorganisms would perform well with sustained nitrogen removal provided a DO concentration of $\leq$0.1 mg/L is maintained throughout the process.

3.2.2. Effect of ORP on SAA

The relationship between ORP and the SAA was determined based on nitrogen gas production in the Anammox reaction (Fig. 3). The obtained relationship with stronger linearity between SAA and ORP was indicated by high correlation coefficient of 0.99. When the ORP value decreased below 0 mV, SAA increased sinewy and was 0.15 ± 0.03 g N_2-N (g VSS)$^{-1}$ d^{-1}. At ORP value $\leq$ -50 mV, the SAA was $\geq$ 0.23 ± 0.03 g N_2-N (g VSS)$^{-1}$ d^{-1}. During this period of reaction, the overall nitrogen balance showed a ratio of 1:1.30:0.25 for the conversion of NH_4^+ and NO_2^- and the production of NO_3^-, respectively (data not shown). This overall nitrogen balance ratio is similar to that reported by Strous et al.[6] The linear relationships between DO and the ORP (Fig. 1) and between the SAA and ORP (Fig. 3) suggest that SAA increases linearly with the decrease in DO concentration within the ORP interval (-110 ~110 mV) and an ORP value of $\leq$ -50 mV is found appropriate for continued nitrogen removal in the Anammox process. Being an important parameter in controlling the Anammox reaction, DO can be measured by ORP as a surrogate indicator to maintain uninterrupted nitrogen removal in the Anammox process.

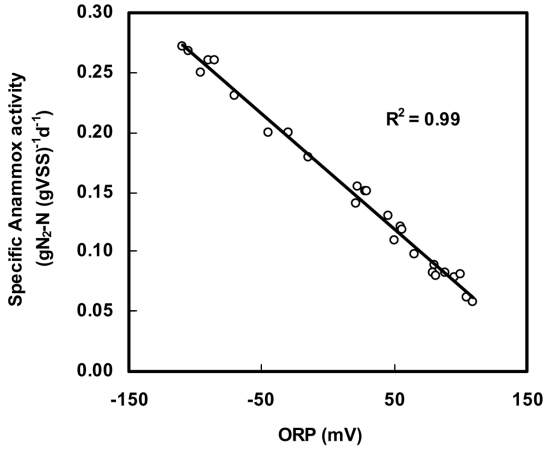


Fig. 3. Relationship between ORP and SAA in Anammox reaction.

3.3. Effects of Organic Compounds on SAA

3.3.1. Effect of Glucose on SAA

In all assays, the %SAA was found to decrease significantly with an increase in glucose concentration (Fig. 4(a)). At glucose concentration <3 mM (~580 mg COD/L), the %SAA was inhibited by ~5%. This is similar to the results obtained by previous researchers[5,10] at same glucose concentration. At glucose concentrations of 20 and 40 mM (~3,840 and 7,680 mg COD/L), SAA was inhibited by ~ 40% and 70%, respectively. This can be ascribed to the structural characteristics of Anammox microorganism and chemical nature of glucose molecule.

Production of CO_2 was observed in the presence of organic compounds (Fig. 5). The CO_2 production was found to increase slowly with increase in glucose concentration from 1~3 mM and decreased with an increase in concentration >5 mM (Fig. 5(a)). The %SAA was found to decrease significantly with increase in glucose concentration (Fig. 4(a)). The maximum removal efficiency of NH_4^+ and NO_2^- were less than 10% and the NO_3^- production was decreased from 5.25 to 0.45 mg/L (data not shown). These results indicated that denitrifiers were present in the inoculum together with Anammox microorganisms and were competing for the organic compound feed at lower concentrations.

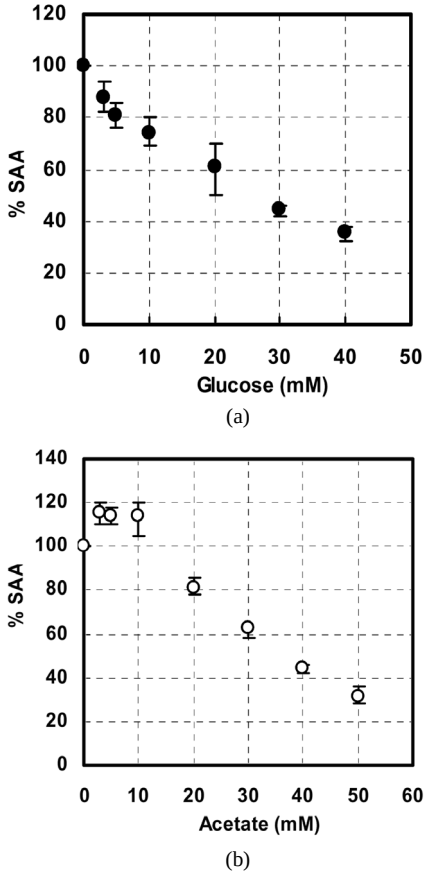
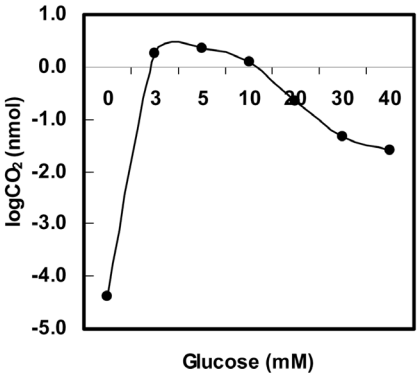
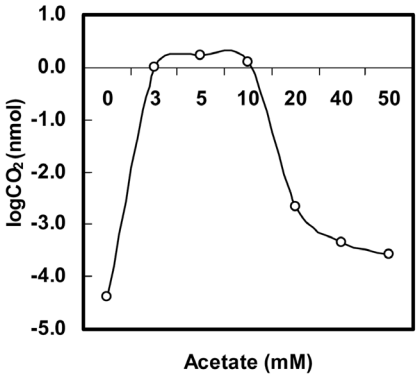


Fig. 4. Effects of organic compounds (a) glucose and (b) acetate on SAA.



(a)



(b)

Fig. 5. CO_2 production by oxidation of (a) glucose and (b) acetate.

The organic compounds act as electron donor and nitrate and/or nitrite as the electron acceptor in these cases. These observations substantiated the fact that the presence of glucose had antagonistic effect on the Anammox activity during the removal of nitrogen from wastewater.

3.3.2. Effect of Acetate on SAA

To investigate the effect of acetate on the SAA, it was added in vials at concentrations up to 50 mM (3,200 mg COD/L). Acetate concentration up to 10 mM (640 mg COD/L) did not affect the Anammox activity and there was no decline in the SAA (Fig. 4(b)). Besides, the overall nitrogen balance showed a ratio of 1:1.30 for the conversion of NH_4^+ and NO_2^-, which confirmed the continual nitrogen removal by Anammox reaction. Concentration of 20 mM (1,280 mg COD/L) acetate resulted in 20% inhibition in the SAA. With acetate concentration < 20 mM, increase in N_2 gas production was observed with gradual increase in acetate concentration. This result is similar to previously reported results of the effect of acetate on Anammox activity.[5,10,11] Güven et al.[5] observed the consumption of > 95% ammonium and nitrite at acetate concentration <10 mM. van de Graaf et al.[10] observed an increase in the Anammox activity in batch tests when acetate was added to the inoculum. They carried out assays using ammonia and nitrate as substrates and observed that acetate was used by one species of Anammox microorganisms for the reduction of nitrate to nitrite. This was very much advantageous to decrease the nitrate concentration during the Anammox reaction.

Gas chromatograph analysis of CO_2 concentration in the headspace of the vials during the experiments showed that acetate oxidation led to CO_2 production (Fig. 5(b)). The presence of CO_2 could not be observed without the addition of acetate and there was around 99% N_2 in the vials. However, CO_2 production was found to increase significantly from a minimum of 1.02 to a maximum of 1.62 nmol with increase in acetate concentration from 3 to 10 mM. During this period, nitrate production decreased from 15.47 to 6.72 mg/L. This result indicated that acetate was oxidized mainly to CO_2 with nitrate and/or nitrite as the electron acceptor. The N_2 and CO_2 production decreased with an increase in acetate concentration beyond 10 mM. These observations also demonstrated that acetate-consuming denitrifiers were present in the inoculum but apparently they were not able to compete with Anammox bacteria at lower concentrations of acetate in the feed. The direct use of nitrate, as electron acceptor, by Anammox microorganisms for wastewater treatment has been described by Güven et al.[5] Generally, in wastewater nitrate is more frequently and sufficiently available than nitrite and Anammox microorganisms are believed to be dependent on other bacteria to reduce nitrate to nitrite.

4. Conclusions

Results based on batch Anammox experiments showed the relationship between ORP and DO concentration as: ORP (mV) = 160.38 + 68 log [O_2], where [O_2] is the DO concentration in mg/L. The SAA was observed to increase linearly with the decrease in DO concentration within the investigated ORP interval (-110 ~ 110 mV). The high determination coefficient (R^2 = 0.99) obtained from the linear relationship between ORP and SAA demonstrated that ORP can be employed as an operational parameter, which can ensure sustained nitrogen removal in the Anammox process. The SAA corresponding to the ORP value of -110 mV was found to be 0.272 ± 0.03 g N_2-N (g VSS)$^{-1}$ d^{-1}. With regard to the effect of organic compounds on SAA, the investigation revealed inhibitory effect of glucose on the SAA, while an acetate concentration up to 640 mg COD/L (corresponding to 10 mM) had a stimulating effect on the Anammox activity. However, acetate concentration beyond 640 mg COD/L had an inhibitory effect on the Anammox activity. This result was significant from the application view point of nitrogen rich wastewaters containing low levels of organic matter, which could be better treated by Anammox microorganisms after an acidification process.

Acknowledgements

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