



Saline conditions effect on the performance and stress index of anaerobic ammonium oxidizing (anammox) bacteria

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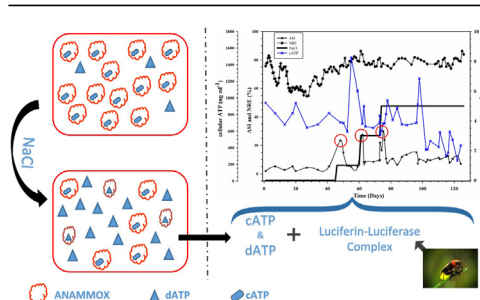
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HIGHLIGHTS

- *Candidatus Brocadia* dominated anammox process was operated for about 160 days.
- Anammox Stress Index (ASI) directly correlates with feed wastewater salinity.
- SAA decreases as Anammox Cellular ATP decreases at 5 g L⁻¹ salinity.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, a lab-scale sequencing batch reactor dominated by freshwater anammox bacteria (FAB) was used to study the performance and stress index of the anammox bacteria at various saline conditions. The reactor with an effective volume of 1.8 L was operated for about 160 days. The nitrogen-loading rate was maintained at 0.364 kg-N m⁻³ d⁻¹ throughout the operational period. At the start-up phase, the seed biomass acclimation to the lab bioreactor showed an inconsistent performance. However, a stable performance was observed after day 38. The average substrate removal efficiency was 92% during most of the operational period. Anammox stress index; a ratio of dissolved Adenosine Triphosphate (dATP_{amx}) to total Adenosine Triphosphate (tATP_{amx}) showed an irrefutable correlation between NaCl concentration, anammox stress and microbial community. A drop in the biomass cellular ATP at 5 g L⁻¹ salinity led to a significant decrease in the Specific Anammox activity. *Candidatus Brocadia* was identified as the main anammox species and its relative abundance reduced along the stepwise salinity increment.

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1. Introduction

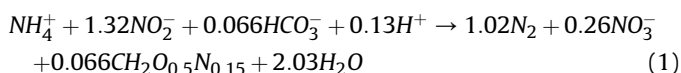
Over the last few decades anaerobic ammonium oxidation

(anammox) has gained great interest and attention as a shortcut within the nitrogen cycle for the removal of nitrogen (Mulder et al., 1995; Van de Graaf et al., 1995). Anammox can be defined as a process where nitrogen gas and nitrate are produced under anaerobic conditions using ammonium and nitrite as the major substrate in the absence of organic carbon (Strous et al., 1999). Due to this unique ability to remove nitrogen without the addition of

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organic carbon source, the anammox process has been set forth as an economical and recent substitute to treat nitrogenous compounds from wastewater (Lackner and Horn, 2012). This process consists of the anaerobic oxidation of ammonium using nitrite as electron acceptor according to the stoichiometry as follows (Strous et al., 1998):



The low growth rate of anammox bacteria being its principal disadvantage has led to numerous bioreactors designs for the treatment of high nitrogen source wastewaters as well as optimizing the enrichment of anammox processes (Strous et al., 1998; Egli et al., 2001). Amongst these, the sequencing batch reactor (SBR) has been majorly recommended for anammox start-up processes with its main advantages being; biomass retention, enable consistent and homogeneous mixture and dependability for a long period of operation despite biomass acclimation at the start-up phase usually taking longer than 90 days (Van Dongen et al., 2001; Dapena-Mora et al., 2004; Wang et al., 2009).

Several factors (including; substrate concentrations, organic matter and salinity levels) have been identified to inhibit the growth and enrichment process of the anammox bacteria (Jin et al., 2012; Lotti et al., 2012). Salinity, one major inhibitor to the anammox process has become of great interest to numerous researchers. This primarily attributes to the fact that most high nitrogen-containing wastewater sources typically have high salts contents ranging from 3 to 23 g L⁻¹, e.g. leachate, fish-canning and seafood processing industry wastewaters, leather industry wastewater, as well as wastewater from pharmaceutical and chemicals industries (Kartal et al., 2006; Dapena-Mora et al., 2010; Yi et al., 2011; Ma et al., 2012; Lee et al., 2020). Although the anammox bacteria have been considered the most resilient nitrogen removal micro-organism (Lotti et al., 2014), one relevant challenge to consider about the anammox process is the slow growth rate and low cell yield. Due to these, once inhibited, especially by salinity, recovery is very difficult and close to impossible.

Adaptation of anammox bacteria to high saline conditions presents a better way to address these challenges and a great pool of researchers have tried to dive into this aspect of the anammox process. However, the adaptation process unequivocally impacts the microbial population in many ways including; general performance and treatment capacity in terms of specific anammox activity (SAA), microbial health, total energy and active biomass ratio in the form anammox stress index (ASI) (Whalen et al., 2006a; Pistelok et al., 2016). Although researchers continue to investigate the performance and treatment capability of the anammox bacteria, there still seem to be an eminent gap that needs to be addressed when it comes to the aspect of microbial health. Adenosine Triphosphate (ATP) can be considered as the precise microbial health index since it is the energy currency of all living organisms and hence a worthwhile indicator of all cells viability (Chittock et al., 1998; Hammes et al., 2010). Typically, detecting ATP in microbial cells is divided into two main steps. The first is chemically and/or enzymatically extracting cells ATP followed by light emission measurement when the dissolved ATP reacts with the Luciferin-Luciferase complex (Whalen et al., 2006a; Greenstein and Wert, 2019; Chhuon et al., 2020). Amidst the abundant benefits of measuring microbial ATP including; being a process fast and easy to perform, affordable and able to detect for both cultured and uncultured organisms, ATP is used less often in researches and routine monitoring than projected (Venkateswaran et al., 2003; Hammes et al., 2010; Xiao et al., 2015).

This study focuses on developing a plausible index for

describing anammox activity variation under shock loading during a saline condition adaptation of the anammox bacteria. The presented index enables decision making in the stepwise increment of salinity in wastewater for anammox bioreactors as well as confirms the prospects of change in bacteria culture under increasing influent saline conditions.

2. Materials and methods

2.1. Inoculum

The inocula for the SBR were acquired from a two-stage anammox side stream pilot-scale reactor installed by BKT Co. Ltd at the Daejeon municipal wastewater treatment plant, Korea for rejected water treatment. The inocula were briefly enriched and adapted in a laboratory at the pilot plant premise in a continuously stirred tank reactor (CSTR) treating approximately 0.22 kg-N·m⁻³ d⁻¹ of synthetic wastewater for about 90 days, this helped to minimize the acclimation period of the anammox bacteria in the Anammox SBR. The initial total suspended solids (TSS) and volatile suspended solids (VSS) concentrations were 3.4 g L⁻¹ and 2.3 g L⁻¹, respectively.

2.2. Synthetic wastewater composition

The synthetic wastewater mainly comprising of ammonium and nitrite in the forms (NH₄)₂SO₄ and NaNO₂, respectively was prepared twice a week while maintaining a 1.045 NO₂⁻ – N to NH₄⁺ – N ratio. There was no deliberate organic carbon source supply in the wastewater as organic carbon assists in increasing the denitrification effect (Wang et al., 2017). Table 1 shows the comprehensive synthetic wastewater composition per litre of laboratory-grade deionized water as adopted from (Van de Graaf et al., 1996; Dapena-Mora et al., 2010). The influent wastewater was maintained at pH 7.6 ± 0.1 and Ar gas was purged periodically to remove dissolved oxygen from the feed solution.

2.3. Bioreactor for anammox cultivation and operation strategy

A 1.8 L operating volume lab-scale SBR was used in this study. The solution temperature of the reactor was controlled at 35.5 ± 0.5 °C with a double-layered water bath jacket. The hydraulic retention time (HRT) was 21.6 h and the daily flow rate was 2 L d⁻¹ hence cyclic treatment volume was 500 ml·cycle⁻¹. The SBR (Fig. 1(a) and (b)) was furnished with a regulative and monitoring

Table 1
Comprehensive Synthetic wastewater composition.

	Components	Concentration (mg·L ⁻¹)
Major Nutrients	NaNO ₂	788
	(NH ₄) ₂ SO ₄	754
	KHCO ₃	500
	K ₂ HPO ₄	174
	MgCl ₂	47
	CaCl ₂	55
Trace Elements Solutions	Na ₂ EDTA·2H ₂ O	30
	FeSO ₄	15
	Na ₂ EDTA·2H ₂ O	15
	ZnSO ₄ ·7H ₂ O	0.43
	CoCl ₂ ·6H ₂ O	0.24
	MnCl ₂ ·6H ₂ O	0.99
	CuSO ₄ ·5H ₂ O	0.25
	NaMoO ₄ ·2H ₂ O	0.22
	NiCl ₂ ·6H ₂ O	0.19
	Na ₂ SeO ₃	0.08
	H ₃ BO ₄	0.014

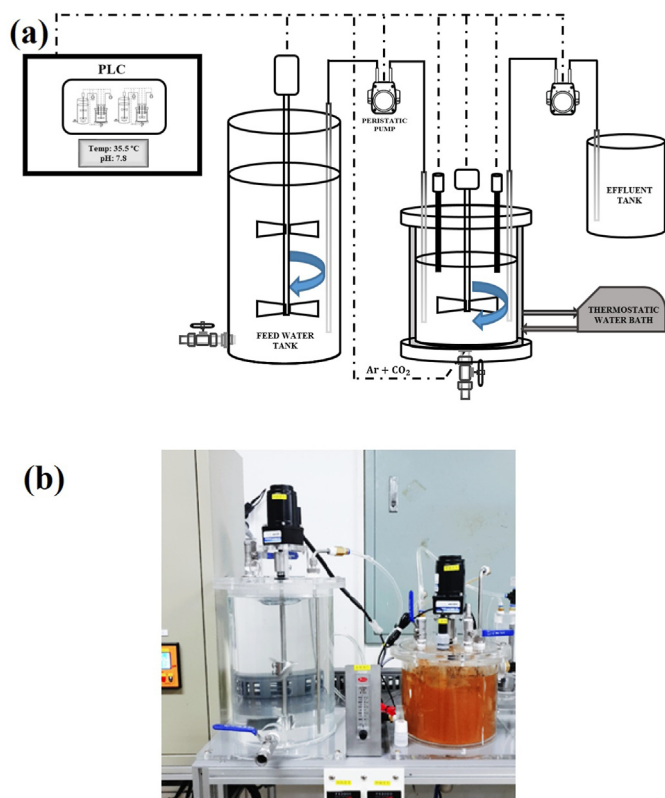


Fig. 1. (a) Schematic diagram of the lab-scale anammox cultivation bioreactor (b) Picture of the lab-scale anammox SBR.

control system that comprised of an interface card (PCL-EXP30-TTA, XGT Panel, Korea), a real-time pH meter (APH-250V, Samsan, Korea), a solenoid valve (AV21-02-2-A-KR, SAMIN CKD, Korea) and gas flow meter (RMA-12-SSV, Dwyer, Korea). The reactor's internal temperature and dissolved oxygen (DO) concentrations were detected with an immersed temperature and DO probes. A mechanical stirrer (6SDG2-6G, DKM, Korea) provided the homogenous conditions with a set revolution per minute (rpm) of 110. The operational pH was controlled between 7.5 and 7.9 and the pH control was achieved via a mixture of Ar and CO₂ gas, which is automatically supplied when the reactor's internal pH exceeds 7.9. All probes were frequently washed and recalibrated to maintain accurate and precise reading. The operational strategy for the bioreactor was divided into two phases and is simplified in Table 2.

2.4. Analytical methods

The nitrogen concentrations ($\text{NO}_2^- - \text{N}$, $\text{NO}_3^- - \text{N}$, $\text{NH}_4^+ - \text{N}$) in both influent and effluent were analysed with an Ion chromatography (IC-5000+ DC, DIONEX). The SAA batch-test experiments were carried out in a shaking incubator (IST-4075, Lab Companion., Korea). A Thermo Scientific bench pH, DO and conductivity meter (Star A216, USA) was used to determine the pH, DO and

conductivity of the influent wastewater solution. The TSS and biomass concentration, measured as VSS was determined using the standard methods (APHA AWWA, 1998) and was repeated 3 times for each measurement. Calculations to determine the Nitrogen Loading Rate (NLR), Nitrogen Removal Rate (NRR) and Nitrogen Removal Efficiency (NRE) were established using the chemical stoichiometry and balance of nitrogen ions as shown in equations. (2-4) (Wang et al., 2017).

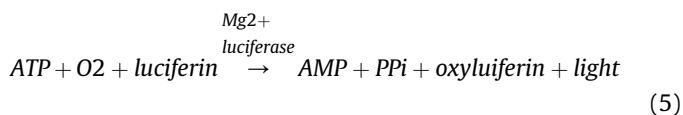
$$\text{NLR}(\text{kg} - \text{N} / \text{m}^3 / \text{d}) = [\text{TN}_{\text{inf}}(\text{mg} / \text{L})] \times \frac{24}{\text{HRT}} \times 10^{-3} \quad (2)$$

$$\text{NRR}(\text{kg} - \text{N} / \text{m}^3 / \text{d}) = \text{TN}_{\text{inf}} - (\text{TN}_{\text{eff}} - [\text{NO}_3^- - \text{N}]_{\text{eff}}) \times \frac{24}{\text{HRT}} \times 10^{-3} \quad (3)$$

$$\text{NRE}(\%) = \frac{\text{TN}_{\text{inf}} - \text{TN}_{\text{eff}}}{\text{TN}_{\text{inf}}} \times 100 \quad (4)$$

2.5. Anammox stress index (ASI)

Anammox Stress Index (ASI) was analysed in the form of ATP. ATP can be considered as the primary energy carrier for all life forms and it was measured following the product protocol of LuminUltra microbial monitoring test kit (QG21W-50, LuminUltra, Canada). ATP was determined based on the light produced when the biomass reacted with luciferase enzyme from firefly (Whalen et al., 2006a; Xiao et al., 2015). The PhotonMaster™ Luminometer (EQP-PMT) was used to measure the produced light as relative light units (RLU). The measured output light is analysed to determine the quantity of living biomass as well as biomass health in the form of stress. The luminometer was connected with an external device (mobile or computer) via a PhotonMaster Bluetooth Module (EQP-PBM). The total Adenosine Triphosphate (tATP) determination was based on both living and dead cells. The tATP extraction differed marginally to the dissolved Adenosine Triphosphate (dATP) in that; there was an initial cell lysis (Using Ultralyse30) to extract the intracellular ATP (cATP) from the living cells. ATP measuring reaction is given in equation (5) (Whalen et al., 2006a). Where ATP is Adenosine triphosphate, AMP is Adenosine monophosphate, PPI is pyrophosphate and Mg^{2+} is Magnesium ion. The ASI essentially is, a ratio of anammox dissolved Adenosine Triphosphate (dATP_{amx}) to total Adenosine Triphosphate (tATP_{amx}) expressed in percentage as seen in equation (6).



$$\text{ASI}(\%) = \frac{\text{dATP}_{\text{amx}}}{\text{tATP}_{\text{amx}}} \times 100 \quad (6)$$

Table 2
Bioreactor operational strategy.

Phase	Operational Period (days)	Composition of SBR Stages	Salt Concentration ($\text{g} \cdot \text{L}^{-1}$)
1	0–42	Concurrent Fill & Mix ^a (300 min) – Settling (30 min) – Decant (10min) – Idle (20min)	0
2	43–126	Fill (20 min) – Mix & React (280 min) – Settling (30 min) – Decant (10min) – Idle (20min)	1–5

^a Input flowrate = 100 ml h⁻¹

$$cATP_{amx} = tATP_{amx} - dATP_{amx} \quad (7)$$

2.6. Specific anammox activity (SAA) & shock loading batch test

Batch experiments were carried out in tightly closed 120 ml serum bottles filled with 100 ml of batch synthetic media, 10 ml of biomass, corresponding to approximately $0.25 \text{ g-VSS} \cdot \text{L}^{-1}$ and placed in a shaking incubator (Lab Companion IST-3075, JEIO TECH). The incubator's temperature and rotating speed were set to $35 \pm 1^\circ \text{C}$ and 120 rpm, respectively. To achieve the maximum plausible SAA, all essential factors that affect the SAA were set to the reported optimum points (Tang et al., 2009; Bae et al., 2016). The SAA determination batch assay's substrate concentration was fixed at $100 \text{ mg-N} \cdot \text{L}^{-1}$. The initial pH and DO of the batch assay were 7.8 and 0.2 ppm respectively. There was no pH control during batch-test experiments. The maximum specific anammox activity was estimated as the maximum descending slope of total substrate concentration over a period of time normalized by the quantity of biomass (Bae et al., 2016). In addition to the SAA, short term batch salinity shock loading experiments were conducted at 0.5% and 1% salinity input concentrations for 12 h as a preliminary test to determine the effect on biomass health as ASI and performance as NRE prior to implementation in the SBR for long term continuous operation. Three distinct input TN concentrations (100, 320 and $480 \text{ mg-N} \cdot \text{L}^{-1}$) of anammox batch assay were used for the salinity shock loading experiments. All batch test experiments were conducted in triplicates.

2.7. Microbial consortia analysis

After acclimation was fairly achieved at each salinity level, 25 mL of the biomass sample was obtained from the bioreactor and DNA extraction was carried out using the MoBio PowerSoil DNA isolation kit (Solana Beach, CA, USA) following the manufacturers' protocol. To determine the yield and quality of the extracted DNA, Qubit Fluorometric Quantification was done using Qubit™ 4 Fluorometer (Thermo Scientific, USA). All samples were duplicated for accuracy and stored under frozen conditions ($<-15^\circ \text{C}$) for further studies. From the extracted DNA, the 16S rRNA bacteria genes were targeted and sequenced using high-throughput sequencing. (341F: 5'-CCTACGGGNGGCWGCAG-3' and 805R: 5'-GACTACHVGGGTATCTAATCC-3') bacterial universal primers were employed to amplify the community of microbes in the lab-scale anammox SBR. The paired-end sequencing was carried out at Macrogen (Seoul, South Korea), using an Illumina MiSeq platform (Illumina, San Diego, CA, USA) and following the manufacturer's protocols.

3. Results and discussion

3.1. Initial start-up performance and nitrogen removal

The anammox SBR was operated for 126 days. The entire operation can be divided into two phases (Table 2). In both phases, the total input nitrogen, number of cycle per day and operational pH range were maintained at $320 \text{ mg-N} \cdot \text{L}^{-1}$, 4 cycles per day, and between 7.5 and 7.9 respectively. During phase 1 (0–42 days), the SBR operation was split into 4 stages, a concurrent 300 min of filling and mixing stage, 30 min settling, 10 min decant and 20 min of idle stage. The average NRE in this phase was 66.5%. With no significant improvement by day 41, phase 2 (42–126 days) was initiated. In the second phase, there were 5 stages per cycle. 20 min filling, 280 min of reacting and mixing, 30 min settling, 10 min decant and 20 min

of idle stage. This led to an instantaneous improvement of NRR and NRE. It was acknowledged that the nitrite accumulation observed in the bioreactor when the solution was sampled, filtered and analysed prior to each consecutive feeding during phase 1 led to the low reactor performance in this phase (Jin et al., 2012). The effluent nitrite concentration was, however, consistently lower than $70 \text{ mg} \cdot \text{L}^{-1}$ and hence the anammox activity was not completely lost (Jetten et al., 1998; Wang et al., 2012). Fig. 2 shows the nitrogen concentrations during the entire experimental period. NaCl was step-wisely introduced into the reactor on days 48, 62 and 75 at concentrations of $1 \text{ g} \cdot \text{L}^{-1}$, $3 \text{ g} \cdot \text{L}^{-1}$ and $5 \text{ g} \cdot \text{L}^{-1}$ respectively.

In phase 1, effluent ammonium concentration (closed inverted triangles) lingered around $28.8 \pm 5.5 \text{ mg N} \cdot \text{L}^{-1}$, effluent nitrite (opened triangles) on the other hand fluctuated rapidly in the very initial phase and steeped to the highest concentration of $57 \text{ mg N} \cdot \text{L}^{-1}$ on the 27th day. Effluent nitrite concentration dropped significantly by the end of phase 1 and remained infinitesimal and barely detected during the entire period of phase 2. Conversely, nitrate produced (closed squares) in the bioreactor increased steadily from day four and plateaued around $62.3 \pm 9 \text{ mg N} \cdot \text{L}^{-1}$ through the entire phase 2. During phase 2 however, the effluent substrate concentrations maintained a significantly low level amidst the stepwise salinity increment in the feed. Previous researches reported similarly low effluent substrate concentrations even when NaCl was increased beyond $20 \text{ g N} \cdot \text{L}^{-1}$ (Lu et al., 2019; Jeong et al., 2020). In Fig. 3, the nitrogen loading and removal rates are shown over the reactor operational period. The NLR (closed circles) was maintained at $0.36 \text{ kg-N m}^{-3} \text{d}^{-1}$ throughout the entire operational period. The substrate utilization rate or NRR (open crossed circles) was distinctive between phases 1 and 2. While the NRR averaged approximately $0.301 \pm 0.03 \text{ kg-N m}^{-3} \text{d}^{-1}$ during the entire phase 1 period, it rarely fluctuated during phase 2 and obtained a high average of $0.356 \pm 0.01 \text{ kg-N m}^{-3} \text{d}^{-1}$; corresponding to over 98.5% of influent substrate removal. The bioreactor's performance at the same input conditions started deteriorating by day 158 and this was considered as the breakpoint of the biomass to saline effect. To prevent complete biomass activity loss, numerous researches reseed their reactors or change the operating conditions (Van Dongen et al., 2001; Jeong et al., 2020; Kwak et al., 2020). The unexpected biomass deteriorating at the later part of $5 \text{ g} \cdot \text{L}^{-1}$ salinity level informed the possibility of the sludge being dominated by Freshwater Anammox Bacteria (FAB) (Kartal et al., 2006; Wu et al., 2019). The product and substrate reaction molar ratios of the anammox process, R_p (NO_3^- production/ NH_4^+ depletion) and R_s (NO_2^- conversion/ NH_4^+ depletion) were seen to marginally

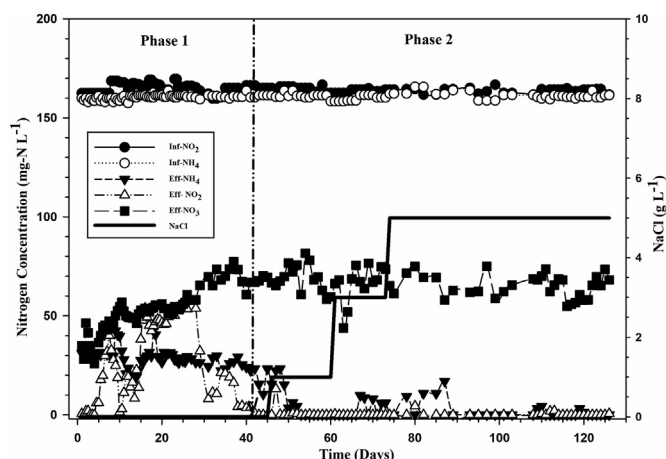


Fig. 2. The performance of anammox bioreactor under different saline conditions.

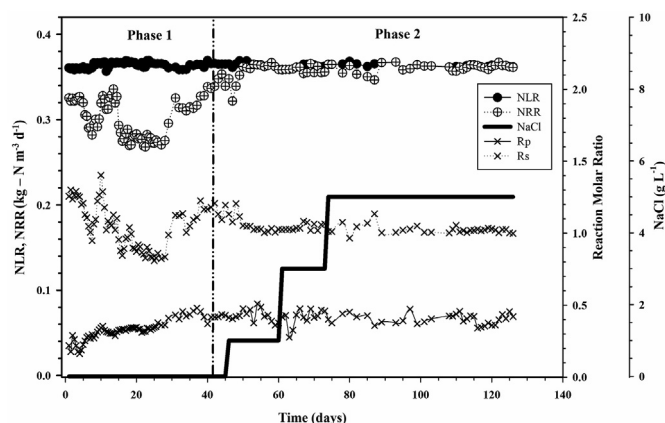


Fig. 3. Nitrogen loading/removal rates and reaction molar ratios of the anammox bioreactor.

deviate from the stoichiometric values in equation (1) especially during most of phase 1. This could be associated with the low NRE within this phase and the general acclimation time requirement of the anammox process. At the process immediate start-up, however, nitrate production to ammonium depletion ratio, R_p was approximately 0.271 somewhat comparable to the stoichiometric value. In previous studies, this was a clear indication of the anammox process being achieved in the study (Zhang et al., 2018; Jeong et al., 2020). Nitrite conversion to ammonium depletion ratio, R_s behaved likewise.

3.2. Specific anammox activity (SAA)

To validate the study on anammox ATP variation (cellular ATP for that matter), investigation of SAA was essential and appropriate. The biomass production in terms of suspended solids concentration and specific activity during the reactor operational period is shown in Fig. 4. Overall, the TSS reduced from an initial of $3530 \pm 10 \text{ mg L}^{-1}$ to $3253 \pm 105 \text{ mg L}^{-1}$ by day 122. The VSS however, fluctuated only slightly over the entire period and hence there was no apparent need for biomass re-inoculation as salinity increased step-wisely. The highest and lowest VSS concentrations were obtained on days 24 and 122, which corresponded to $2560 \pm 80 \text{ mg-VSS} \cdot \text{L}^{-1}$ and $2267 \pm 94 \text{ mg-VSS} \cdot \text{L}^{-1}$, respectively. Although the stepwise salinity increment in this study appeared to have had some visible but minimal effect on the TSS and VSS in general, some previous studies reported that significant variations

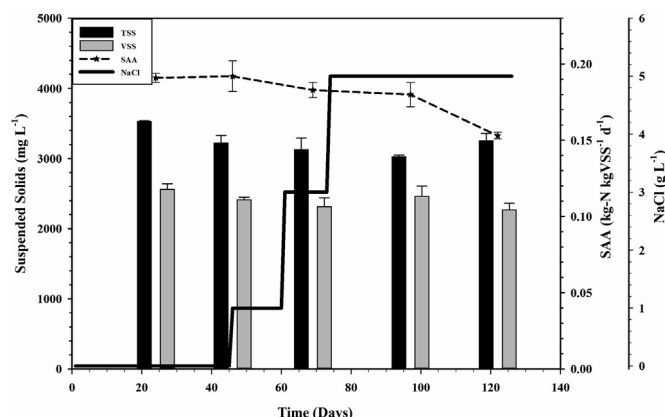


Fig. 4. Suspended Solids concentration and specific anammox activity profile.

were observed in VSS only after salinity was increased beyond 10 g L^{-1} (Ma et al., 2012). The ratio of VSS to TSS during the reactor operational period was 0.74 ± 0.12 and throughout the operational period, effluent TSS and VSS were infinitesimal and hence neglected. Consequently, the relatively lower VSS to TSS ratio of 0.69 by day 122 was assumed to be as a result of microbial loss and/or gradual consortium shift as salinity levels increased (Ma et al., 2012; Jeong et al., 2020). SAA at the beginning and midpoints of phase 2 was approximately 5.3% lower than that of phase 1. This was somewhat contradictory from reports in earlier studies (Jeong et al., 2020), who testified to the possibility of a 50% reduction in SAA after salt concentration was increased to 2.5 g L^{-1} . Dapena-Mora et al. (2007) (Dapena-Mora et al., 2007) also reported a case where SAA was barely reduced even after salinity was increased to 11.7 g L^{-1} NaCl. It was therefore deduced that, the prior bacteria enrichment conditions, viable biomass, as well as the anammox species, plays a significant role in the microbial activity.

3.3. Variation of cellular ATP and ASI of anammox bacteria

With numerous reports given about the inaccuracies of the MLSS and MLVSS in predicting the exact amount of viable biomass, a combination of this and other techniques such as cellular ATP determination could help give a more concise representation of the amount of viable biomass in a bioreactor (Archibald et al., 2001). In order to accurately assess the performance of the anammox process, the ATP of the biomass was determined frequently and especially when the salinity levels were raised. The cellular Adenosine Triphosphate (cATP) which gives a direct indication of the microbial living population was determined by subtracting the dATP from the obtained tATP. (Equation (7)). It can be seen from Fig. 5 that, the ASI of the bioreactor maintained a low value of about $3.72 \pm 1.3\%$ throughout the most part of phase 1 (0–42 days). When salinity was step-wisely introduced into the bioreactor at days 48, 62 and 75 at concentrations of 1 g L^{-1} , 3 g L^{-1} and 5 g L^{-1} correspondingly, the ASI elevated instantaneously to 23.44%, 27.56% and 29.85% respectively. Based on the guidelines given by the LumiUltra technologies Ltd., a 30% or lower stress index in a bioreactor implied good control. Between 30 and 50% required a preventive action and a prompt and rapid corrective measure is to be taken when the stress index exceeds 50% within a bioreactor (Whalen et al., 2006b). While the ASI response in the bioreactor tailored the pattern of NaCl input (circled areas in Fig. 5), the NRE was seen to have no apparent effect from the input salinity up to about 5 g L^{-1} . Although the high-level ASI values remained within the

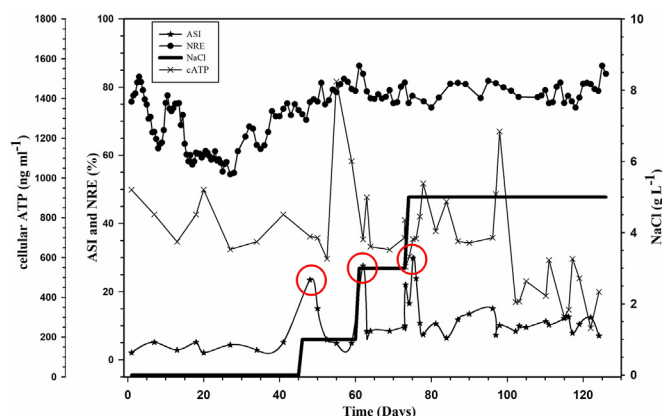


Fig. 5. The effect of salinity of the cellular ATP, anammox stress index (ASI) and nitrogen removal efficiency (NRE).

good control boundary, it also implied increased death rates of microbes as dATP values increased. This, therefore, justifies the hypothesis of a shift in bacteria cultures as salinity increase (Jeong et al., 2020). At 1 g L⁻¹ feed salinity the ASI level reduced to the initial levels after about 5 days of operation. Once reduced, the ASI remained at a level slightly higher as that in phase 1 for several days and this informed the decision to instantly raise the salinity levels. As expected, the feed salinity increment to 3 g L⁻¹ rapidly raised the ASI to a point 17.7% higher than what was seen at 1 g L⁻¹ salinity. The further 8.2% increase of ASI between 3 and 5 g L⁻¹ salinity indicated a conclusive effect of salinity on anammox stress and health. It must be stated that at each salinity level, the average ASI value after the peak points was not exactly as it was at a salinity of 0 g L⁻¹. Though infinitesimally different, the average ASI was after each peak point was 7.67 ± 4.3%, 8.95 ± 0.7%, and 11.08 ± 3.7% respectively. This indicated that there was less shock as compared to the peak points but the biomass was not completely recovered to the normal level.

The cATP generally fluctuated through the entire reactor operational period. However, the average cATP during the initial start-up phase prior to the obvious decrease after day 100 hovered around 788.25 ± 152.79 ng L⁻¹. This average cATP value reduced by

32.1% during the latter part of phase 2. Since the cATP gives to some extent a more accurate information of the actual available viable cells and the living microbes play the main role in the determination of microbial activity, it was also hypothesized that the lower cATP levels after day 100 could accurately account for the lower SAA value obtained on day 122 (ref. Fig. 4). This correlation helped render these two factors complementary.

3.4. Influence of saline stress on microbial consortia

The anammox stress index as a measuring factor or index of dead anammox bacteria as a result of saline stress will be incomplete without a keen consideration of the microbial consortium. To evaluate the variation of microbial community diversity in response to saline changes, high-throughput sequence analyses was performed using the microbial community DNA collected from the inoculum after acclimation in the laboratory scale bioreactor to the saline levels of 0 g L⁻¹, 3 g L⁻¹ and 5 g L⁻¹ on days 40, 64 and 105 respectively. All microbial samples for DNA extraction was taken when; (1) the reactor effluent concentrations was maintained at low levels (2) the biomass had somewhat acclimated to the different salinity levels and (3) ASI has reduced relative to the peak

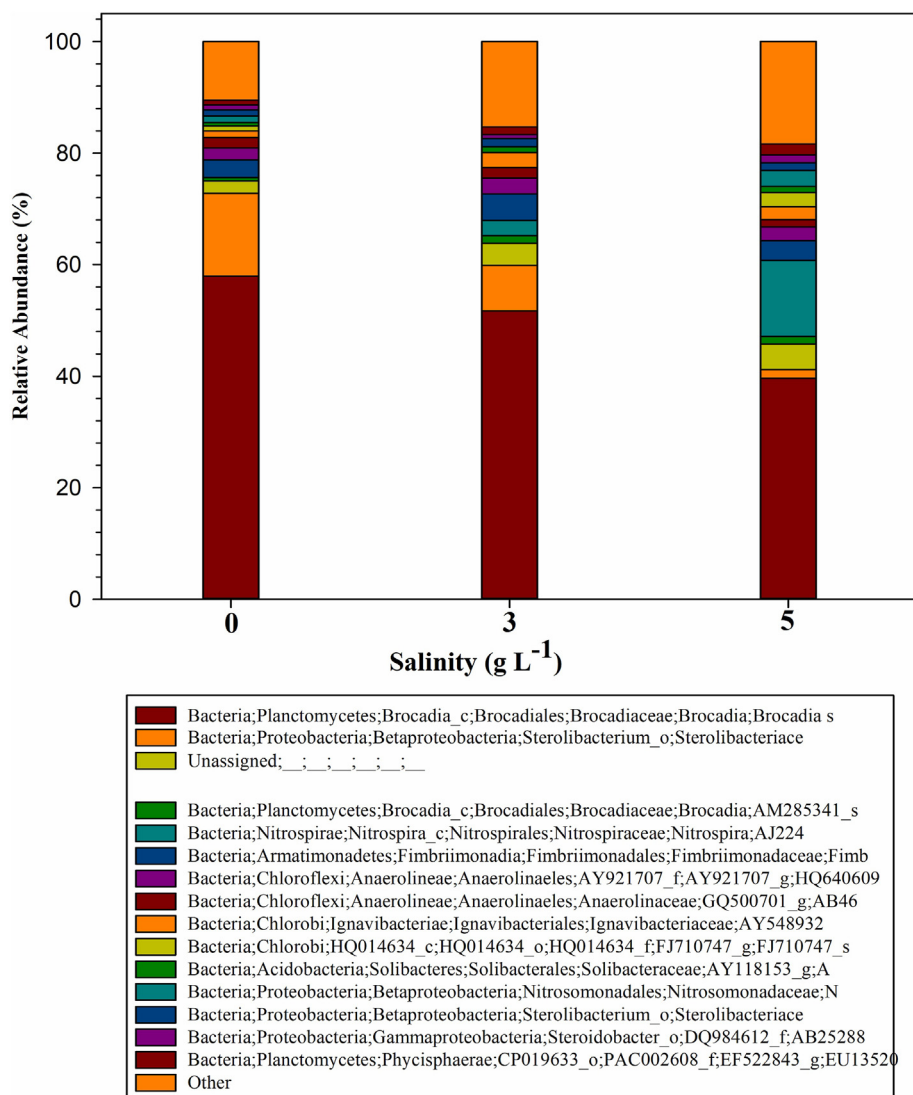


Fig. 6. Microbial community analysis of the bacteria consortia in the lab-scale bioreactor at different saline levels.

points. It was believed that sampling at the points where ASI was at the peak point could not give a consistent information since it represented a shock point for the biomass and not a point of acclimation. To observe preliminary microbial changes as well as the assertion of ASI being strictly or majorly an anammox bacteria index, the microbial relative abundance was considered. The microbial community structure of the anammox system at the species level is illustrated in Fig. 6. When the saline conditions were applied (even at minimal levels), a significant decrease in the relative abundance of the anammox bacteria is observed. *Candidatus Brocadia*, the main anammox species in the lab-scale bioreactor is a freshwater water anammox species and this species have been reported to significantly be inhibited at saline levels above 3 g L^{-1} (Wu et al., 2019). The dominant anammox species reduced by 10.7% between 0 g L^{-1} and 3 g L^{-1} salinity. A further decrease from 51.7% at 3 g L^{-1} to 39.6% at 5 g L^{-1} indicated that the anammox stress index is a direct measure of the anammox bacteria health. A total of 31.6% declining change was seen between days 40 and 105 for the *Candidatus Brocadia* species and the growth of *Nitrospira* sp. from 0 to 13% can be attributed to DO and $\text{NH}_4^+ - \text{N}$ substrate being at favourable levels (Mehrani et al., 2020). According to Daims et al. (2015) and van Kessel et al. (2015) (Daims et al., 2015; van Kessel et al., 2015) the *Nitrospira* sp. could account for performing the complete oxidation of ammonia to nitrate and hence higher NRE even by day 105.

3.5. Short term salinity shock loading effect

Salinity inhibition is considered as one of the major inhibitory factors to the anammox bacteria and numerous reports have shown that recovery from salinity shocks is close to impossible or may take longer than 5–25 days depending on the saline concentration (Ma et al., 2012). Increasing salinity loads in the anammox process must be controlled as much as possible. Short term batch-test shock loading presents the needed preliminary knowledge to understand the specific sensitivities and effect that salinity poses to the anammox process prior to long term elevated salinity levels in the bioreactor. Fig. 7, shows the response of anammox to 0.5% and 1% batch salinity shock loads. The batch experiments were conducted for a maximum of 12 h. The inocula for the short term batch experiments were prior enriched and adapted in a bioreactor with TN concentration of $480 \text{ mg-N} \cdot \text{L}^{-1}$ with no salinity. After 12 h in a shaking incubator, the biomass health was instantaneously

measured as ASI. It was observed that, at a TN concentration of $100 \text{ mg-N} \cdot \text{L}^{-1}$, the effect of 0.5% and 1% salinity levels on the NRE is minimal while this effect increases as TN concentrations increase. The difference in NRE between the two saline levels at a TN of $100 \text{ mg-N} \cdot \text{L}^{-1}$, was about 2% with the lower saline level being higher. (Ma et al., 2012; Jeong et al., 2020), reported a similar NRE at these TN and saline levels. At $320 \text{ mg-N} \cdot \text{L}^{-1}$, a more distinctive difference was observed in the removal efficiencies. While the 0.5% saline concentration obtained an average of NRE of 80%, the 1% saline level only removed 67% of the initial TN supplied. Although the inocula for the batch test were enriched at a $480 \text{ mg-N} \cdot \text{L}^{-1}$ TN concentration, the significant effect of both saline levels on the NRE was extreme. The NRE at 1% salinity concentration was 39% and this was approximately 26% lower than that obtained at 0.5% saline concentration. These NRE values were inconsistent with a previous study (Lu et al., 2019) who reported close a double these NREs at a similar TN concentration and salinity of 1%. However, the inoculum for that study was enriched in high saline conditions. It was therefore concluded that the lower NRE attained in this study was as a result of the inocula previously enriched with no salinity compared to the other study.

Microbial health was also seen to be generally affected more by the 1% saline condition than it was by a 0.5% salinity level. There was a wide percentile change in ASI for the two saline conditions at all input TN concentrations except for $480 \text{ mg-N} \cdot \text{L}^{-1}$ which showed just a 1% change ASI between the two salinity levels. It was acknowledged that, although salinity plays a major role in deteriorating anammox bacteria health, a combination of salinity as higher TN concentration tends to accelerate the declining performance.

4. Conclusion

In this study, the adaptation of the anammox bacteria in varying input saline conditions was investigated and a plausible and reliable index for describing anammox activity as input salinity change was introduced. Experimental information obtained from a lab-scale SBR and a series of batch experiments aided to deduce that; Anammox adaptation to saline conditions is a robust area of research and hence efforts to obtain meaningful and enlightening information is possible and necessary. Also, microbial health is an essential factor to be considered in saline adaption as it provides an index that correlates directly to saline changes in the bioreactor. Midway through the 0.5% feed salinity adaptation, the biomass cATP drastically reduced indicating a lower microbial cell viability leading to a SAA of $0.153 \text{ kg-N kg-VSS}^{-1} \text{ d}^{-1}$ (20.1% lower than was obtained at lower salinities). dATP of the anammox bacteria increased direct proportionally to the input salinity and hence ASI response was used to determine next salinity level increment. Although most high nitrogenous wastewater sources contain high levels of salinity, adaptation and enrichment in the lab-scale settings require reasonable input TN levels to prevent complete biomass activity loss due to inhibition.

Credit author statement

Victory Fiifi Dsane: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. Sumin An: Data curation, Methodology, Project administration, Writing – review & editing. Taesok Oh: Investigation, Technical support, Writing – review & editing. Jiyun Hwang: Methodology, Validation, Writing – review & editing. Yuri Choi: Project administration, Validation. Younggyun Choi: Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing.

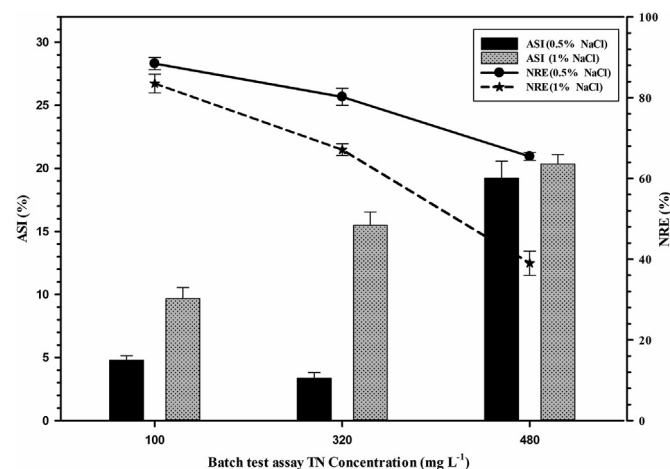


Fig. 7. The performance and response of Anammox under different salinity shock loads.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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