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From freshwater anammox bacteria (FAB) to marine anammox bacteria (MAB): A stepwise salinity acclimation process

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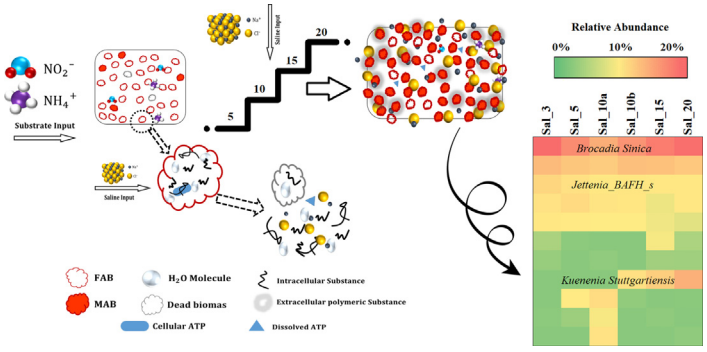
HIGHLIGHTS

- Saline induced stress correlates uniquely to the saline adaptation of anammox.
- Improvement in N-removal efficiency is observed at higher saline concentrations.
- Granules formation and bacteria aggregation tailored the saline input profile.
- MAB dominated the granules in the reactor by the latter end of 10 $g \cdot L^{-1}$ salinity.

GRAPHICAL ABSTRACT



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ABSTRACT

An investigation into the effect of stepwise saline introduction (3–20 $g \cdot L^{-1}$ NaCl) on the anaerobic ammonium oxidation (anammox) process in a lab-scale sequencing batch reactor was carried out for 252 days by evaluating the changes in influent and effluent nitrogen concentrations, conductivity, microbial extracellular polymeric substances' (EPS) ionic content, as well as stresses due to salinity, *via* microbial ATP analysis. It was observed that, effluent nitrogen concentrations remained stable at low saline levels of 3 $g \cdot L^{-1}$ to 10 $g \cdot L^{-1}$. Nonetheless, midway through 10 $g \cdot L^{-1}$ and the preliminary phase of 15 $g \cdot L^{-1}$ salinity presented a very unstable, highly fluctuating as well as deteriorating effluent nitrogen concentrations. A more satisfactory nitrogen removal efficiency of 83.7 ± 5.9% was obtained at higher saline concentrations implying that, the adaptation mechanism to tolerate increasing salinity was taking place. Saline induced stress, which measures the variation in viable anammox bacteria, was correlative to the formation of EPS and changes in its cationic contents along the increasing salinity. Although the specific anammox activity (SAA) dropped by approximately 15% from the beginning of the process to the midpoint, the drop in SAA after the midpoint was not as drastic as the initial phase. A change in microbial aggregation and dominance proved the existence of new saline-dependent species that can withstand high saline stresses. Recovery from abrupt high saline shocks in batch experiment was seen to be almost impossible.

1. Introduction

Salinity is present in the wastewaters from fish canning and seafood processing industries (Dapena-Mora et al., 2010; Ma et al., 2012), leather-processing industries and in leachate (Kartal et al., 2006; Lee

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et al., 2020; Yi et al., 2011). These wastewater sources also naturally contain high levels of nitrogen and hence the anaerobic ammonium oxidation (anammox) presents a promising and exceptional means to treat such wastewaters (Li et al., 2018; Lu et al., 2019; Strous et al., 1999). Although nitrogen removal from wastewater in itself is a simple and effective process studied by many researchers, the adoption of the anammox for this process is slightly complicated due to its many inhibitory factors and their effects less studied (Jin et al., 2012; Li et al., 2019).

There are two core categories of the anammox bacteria (freshwater anammox bacteria, FAB and marine water anammox bacteria, MAB) and their names typically comes from the environment in which they are enriched (Lu et al., 2019). Many studies have consequently stressed the importance of the seeding sludge type on the anammox process as this affects the ability of the bacteria to withstand some inhibitory factors especially salinity (Costa et al., 2014; Zhang et al., 2019). Although, Jeong et al. (2020) and Kartal et al. (2006) reported a shift in bacteria population as one of the mechanisms for adapting to saline changes, the saline introduction technique and process was affirmed to be of great importance. This implies that, various factors affect the understanding of the anammox process when treating high saline containing wastewaters. Researches in this area therefore, requires a more unique approach that will take a lot more into consideration. Gradual adaptation of anammox bacteria to saline conditions offers a superior way to address the persisting challenges and several academics continue to dive into this facet of the anammox process (Jeong et al., 2020; Lu et al., 2019).

Advances in recent anammox processes for saline wastewater treatment have shown that, the effects of salinity are measured through the basic parameters like effluent nitrogen concentrations, nitrogen removal efficiencies and specific anammox activity (SAA) (Bae et al., 2016; Jeong et al., 2020; Ma et al., 2012; Pradhan et al., 2016). These have proven to be very inadequate to account for the exact response of the anammox process and bacteria to saline changes and shocks. In addition, the low growth rate of anammox bacteria makes microbial health difficult to be considered when anammox is used in the treatment of high saline and nitrogen containing wastewaters. Therefore, in order to maximize the performance of saline enriched anammox processes, it is essential to combine these basic parameters and other correlative saline sensitivities and stresses. Also, several researchers (Fang et al., 2018; Li et al., 2018; Yi et al., 2011) have identified the significance of microbial extracellular polymeric substances (EPS) in the anammox process when treating high saline containing wastewater. Costa et al. (2018), stipulates that EPS is vital for microbial life, providing an ideal environment for chemical reactions, entrapment of nutrient, and safeguard the microbial community against saline and drought stresses in a specific environment.

In the present work, we introduced the unprecedented and correlative effect of anammox bacteria energy production to the saline adaptation mechanism of FAB and the subsequent dominance swing to saline-friendly species at higher saline levels. The saline induced stress (SIS) serves as an effective and unique tool to accurately predict and ascertain the effect of salinity on the anammox process. We explored further the effect of other correlative parameters including, bacteria aggregation status and swing in microbial dominance in the step-wisely saline enriched bioreactor. Nitrogen removal improvements and salt tolerance mechanisms observation was plausible in a long (252 days) bioreactor operation.

2. Materials and methods

2.1. Seed sludge and feed composition

Biomass from an already existing anammox bioreactor acclimated to a nitrogen loading rate (NLR) of 0.363 kg-N·m^{-3}·d^{-1} and a salinity of 5 g·L^{-1} served as the inoculum for this study. The ratio of mixed liquor volatile suspended solids (MLVSS) to mixed liquor suspended solids (MLSS) was 0.74. *Candidatus Brocadia Sinica*, a freshwater anammox bacteria species (Li et al., 2018) was the dominant bacteria in the seed sludge. Influent composition was same as that in our prior study (Dsane et al., 2021). The major nitrogen substrate sources were $(NH_4)_2SO_4$ and $NaNO_2$ with a molar ratio of 1:1 and the total nitrogen was fixed at 200 mg·L^{-1}. Salinity, in the form of NaCl was introduced in a stepwise manner from 3 g·L^{-1} to 20 g·L^{-1}. Feed wastewater was prepared thrice weekly while maintaining a pH of 7.6 ± 0.15. The feed DO was not controlled until 52 days of operation.

2.2. Experimental set-up, instrumentation and operation strategy

A transparent plexiglass sequencing batch reactor (SBR) with effective volume of 1.8 L was used in this study. Schematics of the bioreactor was previously described by Dsane et al. (2021). Amidst the similarities in bioreactor set-up, the present work was different in terms of operational duration, strategy, nitrogen substrate and saline concentration levels. To enable the strict and effective monitoring of saline effect on microbial population shift, all other major and minor inhibitory factors were controlled at the reported optimum operating levels (Bae et al., 2016; Fang et al., 2018; Jeong et al., 2020) to prevent vague deductions. The accumulated knowledge on temperature and pH effect on the anammox process from earlier studies helped to fix these at 35.5 ± 0.5 °C and 7.8 ± 0.13 by means of a water jacket and CO_2 gas injection respectively.

The operating strategy of the bioreactor was based on the goal of gradually shifting the bacteria population from freshwater anammox bacteria (FAB) to marine anammox bacteria (MAB) population alongside creating saline adapted FAB. In doing this, it was important to prevent excessive saline shocks and hence, each salinity level was operated for at least 20 days or more, irrespective of improvement in nitrogen removal efficiency. Saline induced stress (SIS), the percentage change in the energy production of non-surviving biomass due to salinity was determined by quantifying the microbial Adenosine Triphosphate (ATP) using a LuminUltra test kit (QG21W-50, Canada). As ATP represents the basic energy currency of the bacteria, it was believed to be a clearer and more effective means to determine the response of the anammox bacteria to saline stress. SIS was therefore determined as the instantaneous dissolved ATP (dATP) contributed by the dead biomass, normalized by the total ATP (tATP) in the bioreactor.

Liquid phase ionic species (cations and anions) were analysed with Thermo Scientific's ion chromatographs (Dionex IC 5000 & 5000+ DC, USA) equipped with a CS12A as a separation column and dynamic regeneration suppressor system (DRS-600). To measure the bioreactor's influent and effluent pH, DO and conductivity, a bench meter (Star A216, Thermo-Scientific, USA) was used. MLSS and MLVSS were determined with standard methods (APHA et al., 1981).

2.3. EPS extraction for ion analysis

The thermal extraction method introduced by Brown and Lester (1980) was used for the EPS extraction. The biomass was washed thoroughly with deionized water (specific resistance: 18.3 MΩ) prior to the EPS extraction in order to eliminate the effect of bulk liquid ionic species. The measured ions in the extracted EPS included sodium (Na^+), potassium (K^+), magnesium (Mg^{2+}) and calcium (Ca^{2+}).

2.4. Batch experiments

A Lab Companion shaking incubator (IST-4075, Korea) provided the required temperature and shaking speed of 35 ± 1 °C and 100 rpm, respectively for all batch experiments. Firmly sealed 120 mL serum bottles were used for the batch tests. The bottles were filled with 100 mL of synthetic wastewater and about 12–15 mL of biomass, equivalent to approximately 0.25 ± 0.035 g-VSS·L^{-1}. The SAA, saline shock and recovery tests made up the batch experiments. For the SAA batch

experiments, total nitrogen concentration of the synthetic media was 100 mg-N·L^{-1}, pH and DO were 7.8 and 0.2 ppm respectively with no further control of these parameters. The maximum descending gradient of total effluent nitrogen concentration over time normalized by the quantity of biomass was recorded as the SAA (kg-N·kg-VSS^{-1}·d^{-1}). Short-term abrupt salinity shock loading and recovery effects were also investigated with anammox biomass at 10 g·L^{-1} saline concentration. For the purpose of obtaining accurate and consistent experimental findings, replicates of all batch experiments were conducted. The calculations for estimating the various anammox responses were as reported in previous studies (Dsane et al., 2021; Jeong et al., 2020; Kimura et al., 2011; Zhang et al., 2020). Salinity loading rate (SLR) was determined as shown in Eq. (1).

$$\mathrm{SLR}\left(kg{-}NaCl\cdot m^{-3}\cdot d^{-1}\right)=\mathrm{salinity}\left(g\cdot L^{-1}\right)\times\frac{\text{Daily Flowrate}\left(L\cdot d^{-1}\right)}{\text{Reactor Volume}\ (L)} \quad (1)$$

2.5. DNA extraction and community sequencing

DNA from the anammox biomass was extracted at each saline level as well as when there was any significant change or observation in the bioreactor. Duplicates of 25 mL biomass samples were obtained from the bioreactor at each saline concentration and DNA were isolated using QIAGEN's MoBio PowerSoil Pro DNA isolation kit (DNeasy Pro_250, USA). A Thermo-Scientific Fluorometer (Qubit™ 4, USA) was used for quantifying the yield of extracted biomass DNAs and by storing under frozen conditions (< −15 °C), the DNAs were available to be used for supplementary studies.

Microbial sequencing and its accompanied bioinformatics analysis has become crucial in diversity studies of the anammox process (Kumwimba et al., 2020). In this work, to obtain reliable and quality analysis of all the taxonomical levels, sequencing were carried out using an Illumina MiSeq platform (iSeq™ 100, USA) ensuring that the manufacturer's protocols was keenly followed. Therefore, the 16S rRNA bacteria genes of the extracted DNA samples were targeted and with high-throughput, sequencing was achieved. By employing the bacterial universal primers (341F/805R) microbes in the lab-scale anammox bioreactor were amplified. Macrogen (Seoul, South Korea) carried out the paired-end sequencing and after obtaining the Fastq files, qiime2 software was used in processing the Fastq files. The processing included barcode deleting, quality filtering, denoising, aligning sequences, assigning taxonomies and alpha diversity assignment (Bolyen et al., 2019; Caporaso et al., 2010).

3. Results and discussion

3.1. Reactor operation and nitrogen removal

As shown in Fig. 1, the bioreactor for enriching the anammox bacteria from FAB to MAB was operated for approximately 252 days. Salinity in the form of *NaCl* was step wisely introduced into the bioreactor with the minimum and maximum injection duration being 23 and 90 days, respectively. To avoid long acclimation period in the bioreactor and enable the FAB to adequately absorb the projected stress due to high saline levels, both the NLR and salinity were assigned to 0.222 kg-N·m^{-3}·d^{-1}

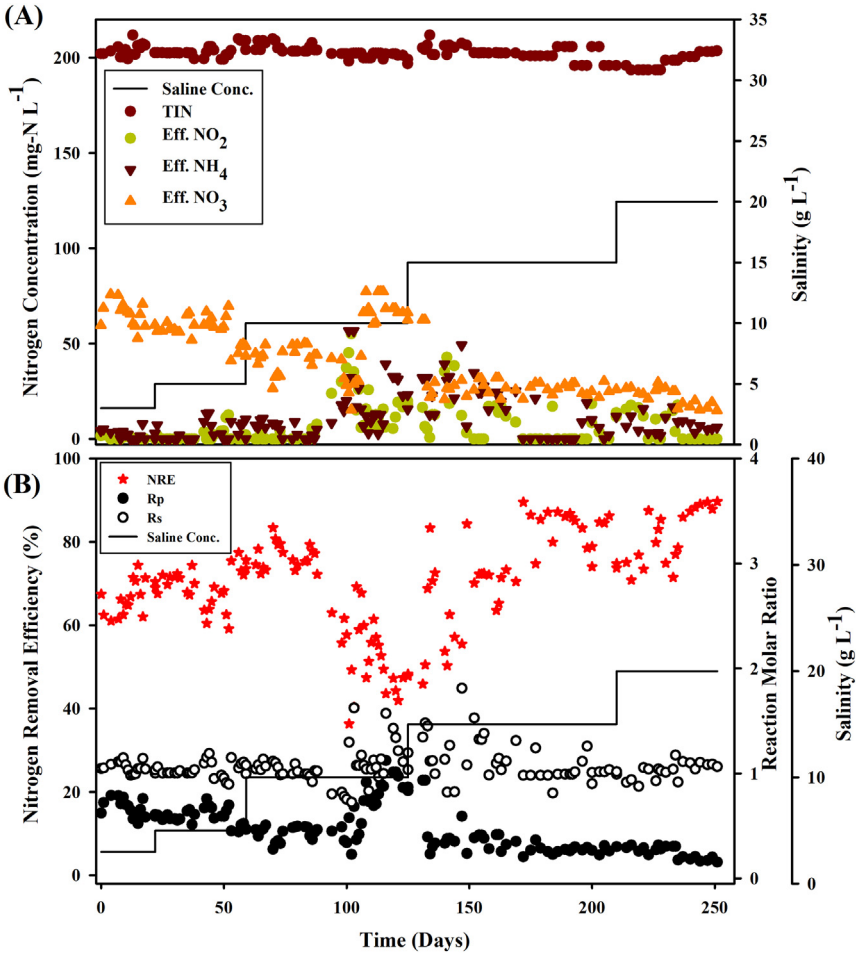

Fig. 1. Bioreactor performance and nitrogen removal.

and 3 $g \cdot L^{-1}$, respectively at start-up of this study. Based on the various reports on saline shock loads affecting the microbial performance and growth (Dsane et al., 2021; Kwak et al., 2020; Ma et al., 2012), gradual elevation at the initial stages was deemed a necessity. Throughout the operational period, the input total nitrogen substrate fluctuated only slightly.

In Fig. 1a, the daily effluent nitrogen concentrations and the stepwise saline injection are shown. The effluent concentrations until midway of the 10 $g \cdot L^{-1}$ salinity (day 92) was infinitesimal and significantly low. During this same period however, the effluent average NO_3^- concentration was 58 ± 5.2 $mg \cdot L^{-1}$. This led to a worse nitrogen removal efficiency (NRE) at the start-up phase compared with the latter part of the bioreactor operation (Fig. 1b). Wang et al. (2009) reported excessive NO_3^- production at the initial period of operation and attributed this to bioreactor acclimation and high feed DO concentration (> 3 $mg \cdot L^{-1}$). High production rate of NO_3^- in the effluent led to the stoichiometric product molar ratio (Rp), a ratio of NO_3^- produced to NH_4^+ depleted, digressing significantly from the theoretical value of 0.26 (Strous et al., 1999). In many anammox researches, emphasis was placed on achieving this theoretical molar ratio as an indication of the anammox process success (Jeong et al., 2020; Ma et al., 2012; Schubert et al., 2007).

Although the high Rp values at the beginning of this work was similar with the findings of Wang et al. (2016), reducing the effects of all other inhibitory factors to the anammox process was essential. To this effect, Ar gas was purged directly into the feed tank after day 52 and the feed DO was maintained below 0.2 ± 0.3 $mg \cdot L^{-1}$ afterwards. Considering the fact that the DO concentration of the influent wastewater is very low in a full scale anammox plant, it was reasonable to reduce the feed DO concentration to a lower level by means of Ar purging. The subsequent low feed DO concentration, significantly reduced the effluent NO_3^- to approximately 47 ± 2.3 $mg \cdot L^{-1}$ thereby improving the Rp value. These findings indicated the importance of accurate feed wastewater and bioreactor DO levels when operating an anammox bioreactor. Around day 92, a decline in the reactor performance was observed and lasted for about 40 days. During this period, effluent nitrogen concentrations fluctuated seriously with the maximum NH_4^+ and NO_2^- being 61.3 $mg \cdot L^{-1}$ and 47.9 $mg \cdot L^{-1}$, respectively. Wang et al. (2012) & Jetten et al. (1998) confirmed that, anammox activity was somewhat occurring as long as effluent nitrogen concentrations remain lower than 70 $mg \cdot L^{-1}$. Also, the mean NRE reduced to 52 ± 7.1%. Nevertheless, the bioreactor's operating conditions was not altered because previous researches reported a plausible unstable performance due to the shift in microbial consortia and change in culture adaptation (Li et al., 2018; Lu et al., 2019; Yi et al., 2011). Around day 152, the bioreactors performance returned to normal with little or no fluctuations and the effluent nitrogen concentrations as well as the NRE improved remarkably. The NRE from this point until the end of the bioreactor operation reached a high average of 80.1 ± 6.8% and Rp quickly approached to the theoretical value of 0.26. Throughout the operation however, the stoichiometric substrate molar ratio (Rs), a ratio of NO_2^- consumed to NH_4^+ depleted remained steady with an average of 1.17 ± 0.15.

3.2. Saline inhibitory effect

In our previous work (Dsane et al., 2021), we determined the direct effect of saline inhibition through stress studies. The SIS fundamentally served as a means of understanding the direct response of the anammox bacteria to the saline injection through the microbial ATP analysis. The bioreactor, largely dominated by the anammox bacteria implied that a significant quantity of the measured ATP belonged to the anammox bacteria (Greenstein and Wert, 2019; Whalen et al., 2006). Fig. 2 shows the profile of SIS along the different saline levels. Throughout the reactor operation, the max SIS at each saline level occurred within the first 2 to 3 days of salinity elevation. At the very start of the bioreactor operation (thus at 3 $g \cdot L^{-1}$ salinity), the max SIS was 27.5 ± 1.2%. This was higher than expected as the start-up salinity was low and the manufacturer's protocol for ATP determination stipulated that, a 30% or lower

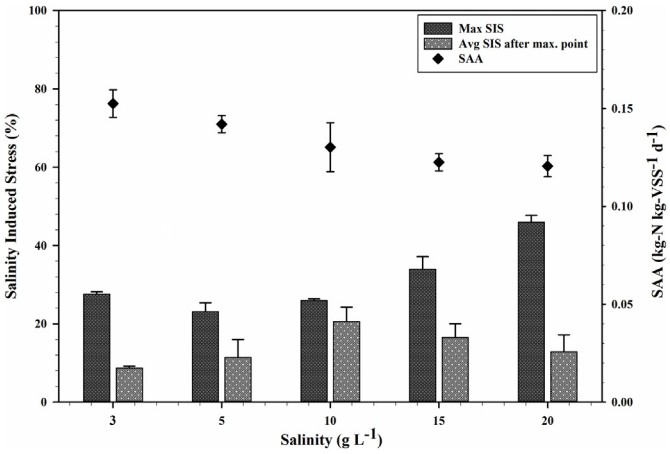

Fig. 2. Variation in specific anammox activity and saline induced stress along the different saline concentrations.

stress index in a bioreactor implied good control. Between 30 and 50% required a preventive action and a 50% or higher necessitate a prompt and rapid corrective measure peculiar to a specific reactor operation (Magic-Knezev and Van Der Kooij, 2004; Whalen et al., 2006). The SIS at this salinity level dropped drastically after 2 days and the average SIS for the remainder of 3 $g \cdot L^{-1}$ salinity was less than 10%. Although further studies may be required to identify the exact cause of the elevated max SIS at this low saline level, it was preliminarily deduced that the sudden change in feeding conditions of the biomass at the start of the reactor operation led to the max SIS observed. A direct relationship was seen between the maximum SIS and stepwise saline increment. Thus, the higher the saline concentration, the higher the max SIS measured. The average SIS after the first few cycles of each saline concentration however, increased gradually from 8.7 ± 0.5% at 3 $g \cdot L^{-1}$ to 20.6 ± 3.6% at 10 $g \cdot L^{-1}$. Although the measured max SIS for the 15 and 20 $g \cdot L^{-1}$ saline levels fell within the spot where preventive actions were required, the average SIS after the initial shock at these saline concentrations reduced by 19.8% and 37.5% respectively in comparison to the average SIS at 10 $g \cdot L^{-1}$ salinity thereby forming to a certain degree bell-shaped profile. This implied that although the initial salt introduction at each saline level presented a sudden upset to the microbial consortia, the dominant biomass had fairly acclimated to the salt levels leading to a lower average SIS as salinity increased (Li et al., 2018; Malovanyy et al., 2015).

The SAA on the other hand continued reducing along the increasing saline concentrations. SAA determination was crucial, as several scholars have reported significant positive and negative changes in the SAA values when salinity was introduced (Dapena-Mora et al., 2010; Jeong et al., 2020; Lu et al., 2019; Ma et al., 2012). In this work, the seed biomass' specific activity downgraded from 0.1525 kg-N·kg-$VSS^{-1} \cdot d^{-1}$ to 0.1205 kg-N·kg-$VSS^{-1} \cdot d^{-1}$ representing a 21% decrease. It must be evidently stated that the percentage variation between the SAA at the start-up and midpoint saline concentration was 14.6%, indicating that there was a slight change in SAA between the 10 $g \cdot L^{-1}$ and 20 $g \cdot L^{-1}$. Previous studies reported a more significant negative change in SAA when saline levels were as low as 2.5 $g \cdot L^{-1}$ (Jeong et al., 2020). Ma et al. (2012), on the other hand saw a case where the SAA in a UASB process drastically improved at 20 $g \cdot L^{-1}$ salinity although the biomass suspended solids reduced by 20%. We could consequently infer that change in dominant species of anammox bacteria, and biomass viability played substantial role in the anammox activity.

3.3. Variation in conductivity and EPS ion content

As salinity increases, one of the major bacteria adaptation mechanism to endure the increasing saline levels is the salt-in strategy:

where large amounts of K^+ions are pumped into the cells cytoplasm to prevent the increasing ionic gradient and stress (Li et al., 2018; Schubert et al., 2007). In Fig. 3 the relationship between the EPS cation contents and the conductivity of the bioreactor solutions is presented. While Na^+, Mg^{2+} and Ca^{2+} ions increase along the elevated salt levels, the K^+ contrastingly reduced, indicating the effect of the salt-in strategy. *NaCl* being the major saline source, explained the linear increase in Na^+ ions as salinity increased along the reactor operation. K^+ions on the other hand reduced from 29.2 ± 0.2 mg·L^{-1} (3 g·L^{-1}) to 6.7 ± 1.2 mg·L^{-1} (20 g·L^{-1}). Based on the necessity and concept of ion balance, this salt-in strategy implies that, the ionic composition of the secreted microbial EPS changes with increasing salinity in the bioreactor and this impressively communicated the adaptive nature of anammox to salinity. Though insignificant compared to the Na^+ions, the divalent cation concentrations correlated directly with the change in solution conductivity according to the salinity.

The influent wastewater conductivity reached a high of 37.2 ± 0.8 mS·cm^{-1} at 20 g·L^{-1} from 9.11 ± 0.6 mS·cm^{-1} during start-up. That of the effluent also correspondingly increased. Nevertheless, due to the accumulation of the monovalent and divalent cations in the anammox EPS, the effluent conductivity digressed gradually from that of the influent. Thus, a percentage difference in the influent and effluent conductivity intensified from 12.7% to 18.1% at the lowest and highest saline concentrations, respectively.

3.4. Granulation of the anammox bacteria

In adapting to saline effects and stresses, the anammox bacteria have been identified to shift its culture and physiology. A summary of bacteria aggregation status over the operational period in the bioreactor is described in Table 1. The highest average NRE was observed at the highest saline level and this correspondingly gave the best average Rp value. According to Zhang et al. (2020), other nitrification process could lead to higher NRE values and hence monitoring the Rp is very crucial in anammox process. The aggregation type started from finely distributed suspended biomass at 3 to 5 g·L^{-1} salinity to a few huge and distinct granule agglomerates by the 220th day of operation. Prior to granule formation, larger biological flocs which were evidently different from the suspended biomass in the first 62 days was seen between days 63 and 125. A pictorial view depicting the anammox bacteria aggregation profile in the bioreactor is illustrated in Fig. 4b. It was inferred that as performance improved and saline enriched granules were formed, the SBR reactor well known for biomass washout during the decant phase (Van Dongen et al., 2001) likely washed out most of the low settling, light weighted suspended flocs.

An annotated diagram of the saline adaptation mechanism is presented in Fig. 4a. Upon the introduction of salinity to the original FAB

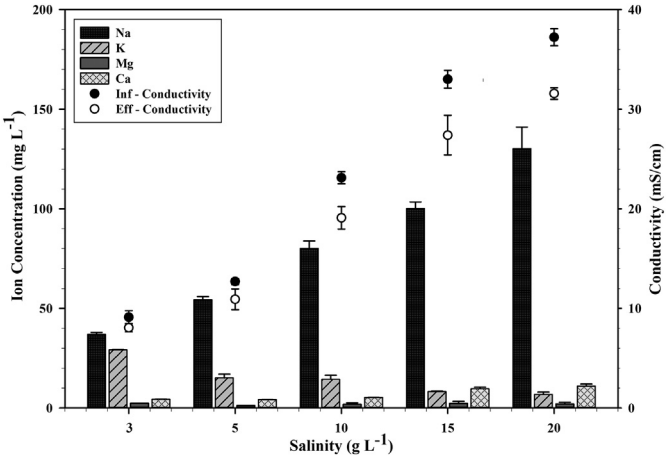

Fig. 3. Variation of conductivity and cation concentration in the anammox EPS extract.

Table 1
Summary of the anammox performance index according to the biomass aggregation status.

Aggregation status	Period (days)	Salinity (g·L^{-1})	[a]NRE (%)	[a]Rp	[a]Rs
Suspended	0–62	3–5	68.5 ± 4.6	0.61 ± 0.09	1.04 ± 0.06
Floccules	62–125	5–10	62.9 ± 13.6	0.58 ± 0.23	1.06 ± 0.18
Granules	125–220	10–20	73.5 ± 11.5	0.35 ± 0.16	1.11 ± 0.20
Agglomerated granules	220–252	20	83.7 ± 5.9	0.24 ± 0.06	1.05 ± 0.06

[a] Mean ± Std. Dev. [with period (days) as population size].

dominated bioreactor, a huge number of bacteria become extinct due to the unfavourable environmental condition presented by salinity. The dead biomass produces the dATP that leads to the SIS described earlier. On the other hand, others gradually produce antibodies (including EPS) against the introduced salinity and hence survive the stress conditions. Yet another group of salt-dependent anammox bacteria began to rapidly grow as saline level increase thereby causing a dominance shift from FAB to MAB. Therefore, the (1) salt-in strategy: where large amounts of K^+ ions are pumped into the cells cytoplasm, (2) syntheses or concentration an organic solute (compatible-solute strategy) and (3) bacteria dominance transfer, serve as the three major anammox bacteria adaptation mechanism to tolerate increased salinity (Li et al., 2018; Ma et al., 2012; Wu et al., 2019).

3.5. Shift in microbial abundance

The phylum level of the microbial community abundance in the anammox bioreactor is presented in Fig. 5. A total of six microbial samples representing all the different saline levels were analysed and samples were taken on days 6, 45, 104, 124, 182, and 227. At 10 g·L^{-1} salinity however, samples were obtained twice (Sal_10a & 10b) based on the extreme fluctuation observed in the reactors performance during this period. In all samples, uncultured bacteria formed approximately 11 to 13.7% of the total microbial abundance. *Planctomycetes*, which is known to be the phylum of the anammox bacteria (Jetten et al., 1998; Strous et al., 1998) reduced from 36.20% in the seed sludge (Sal_3) to 25.09% by day 104 (Sal_10a) indicating the observed effect of saline stress (ref. Fig. 2). Nonetheless, substantial improvement in the relative abundance of the *Planctomycetes* was detected by the latter part of the reactor operation. The improved abundance percentage of the *Planctomycetes* as saline level increased was contrary to the findings of Jeong et al. (2020), who saw a perpetual reduction in the anammox phylum as salinity increased. The relative abundances of the other phyla making up the bacteria community included; *Proteobacteria* (10.89–18.59%), *Nitrospirae* (4.08–29.46%), *Chloroflexi* (9.02–18.93%), *Chlorobi* (3.93–9.17%), *Verrucomicrobia* (0.22–1.39%), *Acidobacteira* (0.46–3.05%), *Bacteroidetes* (0.77–2.81%), *Firmicutes* (0.0–1.34%), and *Armatimonadetes* (2.78–3.82%), just to state a few. Various anammox researches have reported similar detections in the observed microbial community structures with slight variations in the percentage abundances (Gilbert et al., 2014; Jeong et al., 2020; Li et al., 2009). Generally, the *Planctomycetes* phylum was dominant in all samples except for Sal_10a, in which the *Nitrospirae* phylum skyrocketed to about 30%. The anammox process typically does not require an organic carbon source attributed for the relatively low abundance of potential organic carbon degraders such as *Firmicutes* and *Bacteroidetes* (Pradhan et al., 2016). From Fig. 5, it can be seen that the overall effect of salinity on the *Proteobacteria*, *Chlorobi* and a few other phyla was minimal except for the point where *Nitrospirae* skyrocketed and drastically suppressed the phylum of the anammox bacteria as well as other phyla. The presence of *Nitrospirae* phylum in anammox processes have been reported by numerous scholars with diverse relative abundances (Costa et al., 2014; Costa et al., 2018; Egli et al., 2003; Jeong et al., 2020).

Fig. 6 summarises as a heat-map, the abundance of the total *Planctomycetes* identified in the bioreactor at the different saline

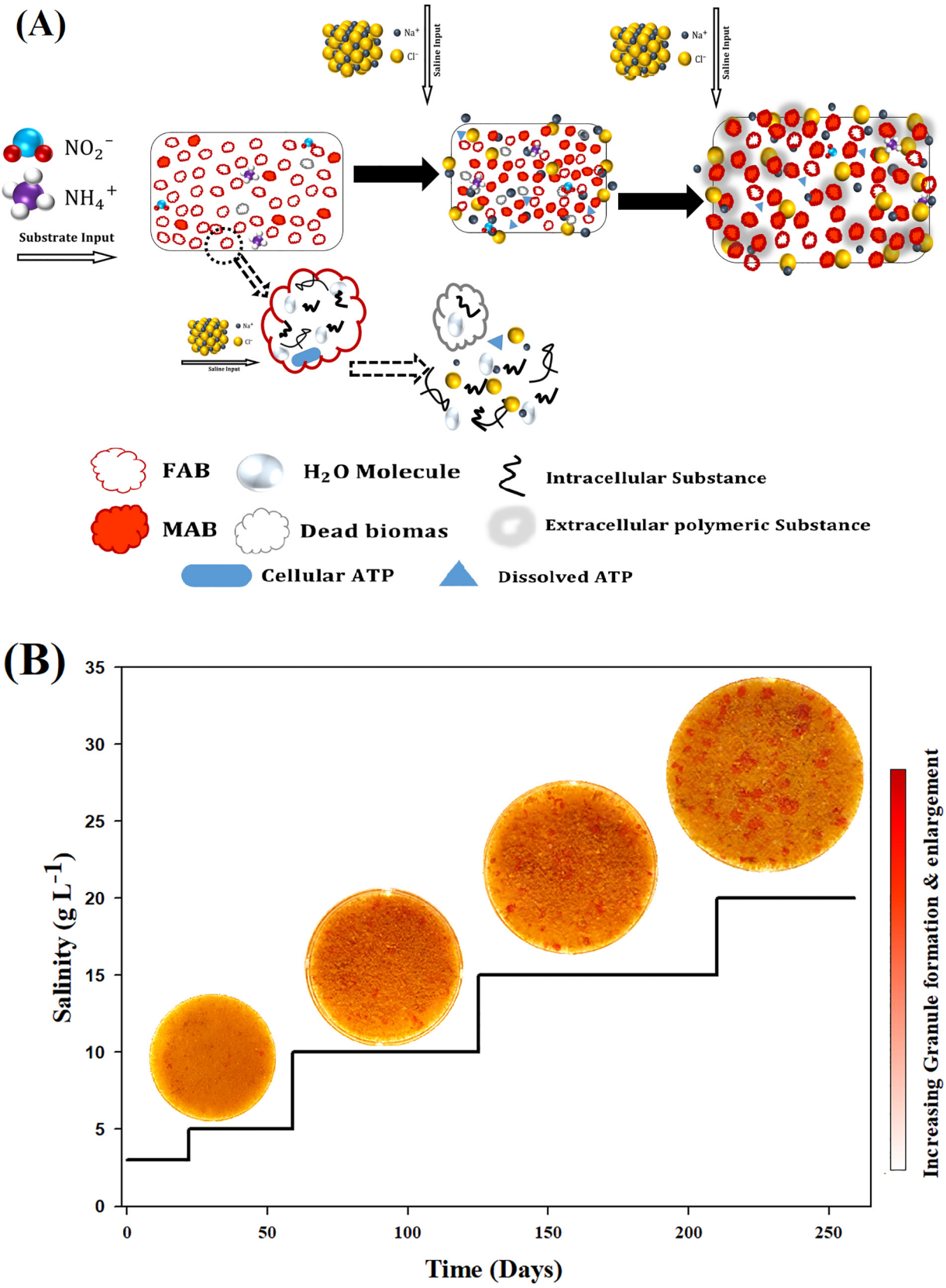

Fig. 4. (a) Annotated diagram of the anammox bacteria saline adaptation mechanism (b) Pictorial view of the changes in biomass aggregation status in the bioreactor.

concentrations and collected samples. The four major anammox species forming the bulk of the *Planctomycetes* phylum were identified to be *Brocadia Sinica*, *Kuenenia Stuttgartiensis*, *Jettenia caeni and Brocadia_AM285341_s* (a basonym of *Brocadia Sapporensis*) with similarity percentages of 99.9%, 99.9%, 98.6%, and 98.8%, respectively (Yoon et al., 2017). Among these, only the *Kuenenia Stuttgartiensis* is originally known to survive in both FAB and MAB environmental conditions (Li et al., 2018). With the exception of the known anammox species

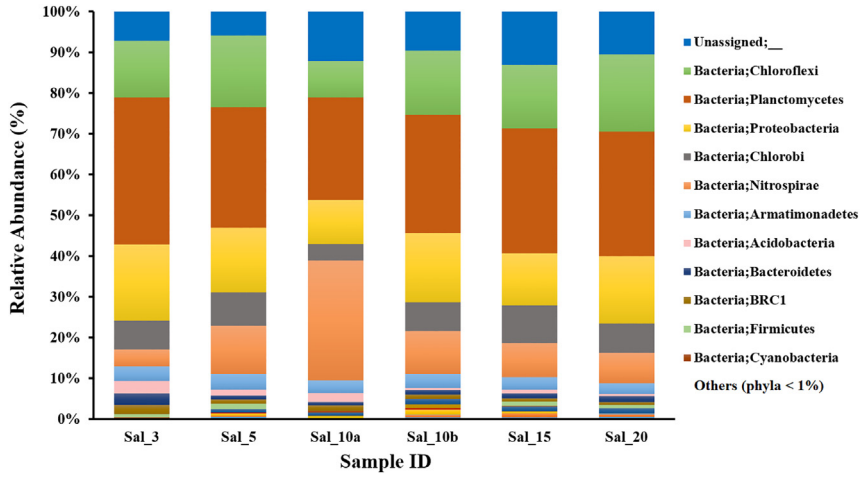

Fig. 5. Variation in the phylum level of the microbial community structure along the different saline concentrations.

identified, there were numerous strains of the *Phycisphaeraceae* family found in the *Planctomycetes* phyla. The *Phycisphaeraceae* family is well-known to be majorly found in marine ecosystems and identified to display fascinating cell biological features which include formation of outer membrane vesicles (Kallscheuer et al., 2020).

The seed biomass (Sal_3) contained only three of the major anammox species with the relative abundances of 20.63%, 8.15% and 3.94%, for *Brocadia Sinica*, *Brocadia_AM285341_s* and *Jettenia caeni* respectively. Overall, the relative abundance of the *Jettenia caeni* species throughout the collected samples did not significantly increase along the different saline concentrations. Meng et al. (2018), also reported a similar situation where the *Candidatus Jettenia* species reduced seriously at 5 $g \cdot L^{-1}$ salinity and kept reducing continuously as saline levels increased. The presence of *Kuenenia Stuttgartiensis* was first seen in sample Sal_10b, and its abundance increased over 3 times by 20 $g \cdot L^{-1}$ salinity (Sal_20). The gradual upsurge of the *Brocadia Sinica* species from day 124 amidst the initial decline in the first three samples confirms the findings by Li et al. (2018), that FAB can be adapted to higher saline conditions if gradually, carefully and systematically introduced to salinity. The *Brocadia Sapporensis* species somewhat behaved similarly. *Gemmataceae* family of the class *Planctomycetia* was only present in sample Sal_10a. According to Kulichevskaya et al. (2020), *Gemmataceae* belongs to the order *Gemmatales* and accommodates strictly aerobic, chemo-organotrophic planctomycetes with spherical or ellipsoidal cells. The substantial presence of this family in sample Sal_10a could explain the favourable conditions that caused the steep increase in *Nitrospirae* and some strains of the *Phycisphaeraceae* family.

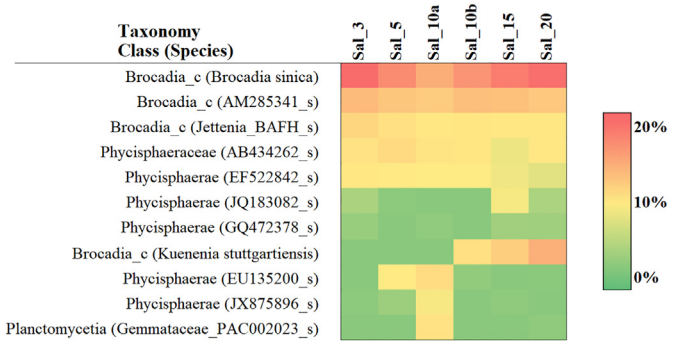

Fig. 6. Specific relative abundance of the total *Planctomycetes* identified in the bioreactor at the different saline concentrations.

3.6. Recovery from saline shock

A short-term semi-batch test was performed to ascertain the feasibility of direct enriching of MAB by shock load to a reactor with high saline levels. The 10 $g \cdot L^{-1}$ saline level that led to the formation MAB in the continues operated lab-scale SBR served as the saline concentration for the shock enrichment. Prior to the abrupt saline shock and subsequent recovery study, the selected biomass sample was enriched in a 0.667 kg-N$\cdot m^{-3} \cdot d^{-1}$ NLR synthetic wastewater for 19 to 21 days producing an average NRE of 92 $\pm$ 3.21%. When 10 $g \cdot L^{-1}$ NaCl was introduced at once, substrate removal and biomass performance began to diminish and microbial activity was completely lost after 9 days of exposure. The experience of reactor performance fluctuation preceding a significant dominance shift and hence, MAB formation (ref. Fig. 1) encouraged the decision to continue operating the batch tests for another 14–18 days. With no improvement whatsoever by the 27th day of saline exposure, the recovery phase of the biomass was activated. The recovery process was primarily a complete elimination of the introduced salinity while maintaining all other operational factors. Although Meng et al. (2018) reported a recovery period range of 7 to 18 days for gradual saline inhibited anammox processes, there was no recovery in the semi-batch experiment even after 25–30 days of operation with no salinity.

Table 2 compares the SLR of diverse anammox saline treatment processes and correlates this to the total inhibition duration and consequent recoverability from saline inhibition and shocks. The many researches presented in Table 2 show that the possibility of ultimate recovery from saline shocks is mainly dependent on the saline adaptation process (gradual or abrupt) as well as the instantaneous NLR variation during saline loading. Generally, relatively low NLR anammox process with gradual saline exposure are likely to recover from saline shocks by producing the necessary adaptation mechanisms (Ali et al., 2020; Dsane et al., 2021; Jeong et al., 2020; Li et al., 2018; Lu et al., 2019). Consequently, the recovery from the saline shock can be achieved not only by the defensive mechanism of FAB but also by the bacteria dominance shift to MAB.

4. Conclusion

In this study, anammox bacteria was successfully enrichment from an FAB dominance to MAB. Although the stepwise saline acclimation process of anammox bacteria is a time consuming process and perfectly negating all other anammox inhibitory factors is a difficult task, the obtained information from the effluent nitrogen concentrations, reaction molar ratios, saline induced stresses and EPS ion content helped to infer these conclusions. Firstly, the anammox bacteria is a resilient

Table 2
Comparison of Saline loading rates, Inhibition durations and the Ultimate recoverability of some anammox processes.

Reactor operation type	Dominant anammox species	[a]NLR ($kg\text{-}N \cdot m^{-3} \cdot d^{-1}$)	Salinity ($g \cdot L^{-1}$)	[b]SLR ($kg\text{-}NaCl \cdot m^{-3} \cdot d^{-1}$)	Total inhibition duration (days)	Ultimate recoverability	Reference
Biofilm (non-Woven)	*Ca. Jettenia & Ca. Kuenenia*	0.696	0–20	0–0.60	250	Yes	Meng et al. (2018)
[c]RBC	[d]N.M	0.70–1.1	0–30	0–38.9	180	Yes	Windey et al. (2005)
Airlift	*Brocadia & Kuenenia stuttgartiensis*	1.0	0–30	0–60	150	No	Guo et al. (2020)
SBR	N.M	0.30–0.60	0–20	0–20	160	Yes	Dapena-Mora et al. (2010)
UASB	N.M	5.04	5–60	60–720	0.5	No	Ma et al. (2012)
SBR	*Jettenia & Kuenenia*	0.28–1.11	6.7–30.2	13.2–60.4	275	Yes	Huang et al. (2021)
UASB	*Unclassified Brocadiaceae & Ca. Kuenenia*	10.4	0–35	0–380	400	No	(Lu et al., 2019)
SBR	*Brocadia Sinica & Ca. Kuenenia*	0.222	3–20	3.3–22.2	252	Yes	This Study
SBR	*Brocadia Sapporensis*	0.667	10	11.1	27	No	This Study

[a] NLR (Nitrogen Loading Rate).
[b] SLR (Salinity Loading Rate).
[c] RBC (Rotating Biological Contactor).
[d] N.M (Not Mentioned).

microorganism and with favourable conditions, adaptation to saline environments can be achieved. Also, the essential saline adaptation mechanism of could be observed. The changes in bacteria aggregation status at higher saline levels, led to an enhanced bioreactor performance, improved average NRE of 83.7 ± 5.9% and a product molar ratio (Rp) value, marginally close to the theoretical value of 0.26. A subsequent semi-batch test of salinity shock loads indicated that these MAB and saline adapted FAB can only be formed through a deliberate gradual acclimation process. The comprehensive knowledge amassed in the area of saline anammox enrichment has provided the potential for future research into the granulation behaviour of MAB and how this differs from other microbial granulation pattern. Studies into the quantification of specific bacterial genes and proteins of saline-friendly anammox should also be considered in the future.

CRediT authorship contribution statement

Victory Fiifi Dsane: Methodology, Investigation, Formal analysis, Writing - original draft. **Sumin An**: Methodology, Validation, Project administration. **Muhammad Kashif Shahid**: Technical advice, Writing - review & editing. **Younggyun Choi**: Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

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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