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Adaptation of anammox process for nitrogen removal from acidic nitritation effluent in a low pH moving bed biofilm reactor

Zhetai Hu, Tao Liu, Zicheng Su, Jing Zhao, Jianhua Guo, Shihu Hu, Zhiguo Yuan, Min Zheng

Abstract

Acidic partial nitritation (PN) has emerged to be a promisingly stable process in wastewater treatment, which can simultaneously achieve nitrite accumulation and about half of ammonium reduction. However, directly applying anaerobic ammonium oxidation (anammox) process to treat the acidic PN effluent (pH 4–5) is susceptible to the inhibition of anammox bacteria. Here, this study demonstrated the adaptation of anammox process to acidic pH in a moving bed biofilm reactor (MBBR). By feeding the laboratory-scale MBBR with acidic PN effluent (pH = 4.6 ± 0.2), the pH of an anammox reactor was self-sustained in the range of pH 5–6. Yet, a high total nitrogen removal efficiency of over 80% at a practical loading rate of up to 149.7 ± 3.9 mg N/L/d was achieved. Comprehensive microbial assessment, including amplicon sequencing, metagenomics, cryo-section-FISH, and qPCR, identified that Candidatus Brocadia, close to known neutrophilic members, was the dominant anammox bacteria. Anammox bacteria were found present in the inner layer of thick biofilms but barely present in the surface layer of thick biofilms and in thin biofilms. Results from batch tests also showed that the activity of anammox biofilms could be maintained when subjected to pH 5 at a nitrite concentration of 10 mg N/L, whereas the activity was completely inhibited after disturbing the biofilm structure. These results collectively indicate that the anammox bacteria enriched in the present acidic MBBR could not be inherently acid-tolerant. Instead, the achieved stable anammox performance under the acidic condition is likely due to biofilm stratification and protection. This result highlights the biofilm configuration as a useful solution to address nitrogen removal from acidic PN effluent, and also suggests that biofilm may play a critical role in protecting anammox bacteria found in many acidic nature environments.

Keywords:
- Anammox
- Low pH
- Free nitrous acid
- Wastewater treatment
- Biofilm
- Nitrogen removal

1. Introduction

Anaerobic ammonium oxidation (anammox) is a microbial process that can autotrophically convert ammonium and nitrite to dinitrogen gas (NH$_4^+$ + 1.32NO$_2^-$ → 1.02N$_2$ + 0.26NO$_3^-$). The anammox process was discovered in a denitrifying pilot plant in the Netherlands in the early 1990s (Mulder et al., 1995). After that, numerous studies have been done to apply anammox process in wastewater treatment for more efficient nitrogen removal (Ma et al., 2016; Du et al., 2017; Cao and Zhou, 2019; Liu et al., 2019; Zheng et al., 2023). Partial nitritation and anammox (PN/A, also termed deammonification) is one attractive option, as it can reduce the requirement of aeration by 60% and the demand of organic matter by 100%, compared to the conventional nitrification/denitrification process (Jetten et al., 1997). The PN/A process comprises two steps, i.e., partial nitritation (PN, NH$_4^+$→NO$_2^-$) and anammox. The developed PN/A process has been widely applied to treat high-strength ammonium wastewater but prone to failure when subjected to low-strength wastewater at low temperatures (Ma et al., 2016; Wang et al., 2022, 2021a). Adaptation of anammox process to low temperatures has attracted much attention in recent laboratory and pilot studies (Laureni et al., 2016; Liu et al., 2020; Wang et al., 2022).

The suppression of nitrite-oxidizing bacteria (NOB, NO$_2^-$→NO$_3^-$) is a well-known challenge, primarily leading to failure of PN/A process in the mainstream condition (Cao et al., 2017, 2020; Ma et al., 2016; Wang et al., 2022). Recent studies reported a stable PN process that is sustained by an acid-tolerant ammonia-oxidizing bacterium (AOB,
2. Materials and method

2.1. MBBR set up, operation, and monitoring

A lab-scale MBBR with a working volume of 1.2 L was set up and operated for about 180 days in a temperature-controlled (22 ± 1 °C) laboratory. The K5 carriers (AnoxKaldnes AB, Sweden, specific area of 800 m²/m³) with mature anammox biofilms were taken from a pilot-scale partial nitritation and anammox (PN/A, also called deammonification) process treating anaerobic liquor as inoculum. The packing volume ratio of this pilot-scale PN/A was set as 50%, and the operating pH was maintained between 7.5 and 7.6, controlled by feeding. For the lab-scale MBBR, the packing volume ratio was 30%. The reactor was continuously fed with the effluent of a laboratory-scale acidic PN reactor, which had an average ammonium and nitrite concentrations of 24.2 ± 2.1 mg N/L and 26.8 ± 2.4 mg N/L, respectively (Meng et al., 2022) (Table 1). The pH of this acidic PN effluent (i.e., the influent of anammox process) was 4.6 ± 0.2. To test the feasibility of operating the anammox process in a lower pH condition, the pH of feeding was intentionally reduced to 4.0 during days 132–136. The hydraulic retention time (HRT) of the reactor was gradually decreased from 24 h to 8 h by periodically increasing the feeding inflow rate from 1.2 L/d to 3.6 L/d. The reactor was mixed using a magnetic stirrer at a speed of 250 rpm. The pH of the reactor was not controlled but monitored with a pH probe (Oakton, UAS) and a transmitter (mini CHEM, TPS).

Liquid samples were collected 2–3 times per week, filtered through 0.45 μm pore-size filters and then stored at 4 °C prior to analysis of ammonium, nitrite, and nitrate in the influent and effluent. Biomass samples were collected at the steady state of each HRT stage, and then stored at –80 °C condition for subsequent microbial analysis.

2.2. Batch anammox activity assays

2.2.1. The impacts of pH and nitrite on biofilm anammox activity

The impacts of pH values and nitrite concentrations on the anammox activity in biofilms were evaluated at the end of the long-term experiment through a series of batch assays in a temperature-controlled (22 ± 1 °C) laboratory. Briefly, the anammox activity was tested under the conditions of three different pH levels and seven different nitrite concentrations (pH: 5.0, 6.0, and 7.0; Nitrite: 10, 20, 30, 50, 100, 150, and 200 mg N/L). For each test, ten K5 carriers were randomly taken out from the MBBR and then added into a 200 mL glass beaker where 150 mL tap water was preloaded. After that, 1 mL of ammonia stock solution (10 g N/L of NH₄HCO₃) was dosed to the beaker to increase the ammonia nitrogen concentration to about 66 mg N/L. Subsequently, various amounts of nitrite stock solution (10 g N/L of NaN₃) were added to the glass beaker to attain the desired nitrite concentrations. Each activity test lasted for 2.5 h, during which the beaker was mixed by a magnetic stirrer at 250 rpm and the pH was manually controlled at the setpoint by dosing 0.1 M HCl and 0.1 M NaOH. In addition, nitrogen gas was supplied to ensure that dissolved oxygen (DO) was undetectable throughout the experiment. Liquid samples were taken every 0.5 h for ammonium, nitrite, and nitrate analysis. The anammox activity was determined as the volumetric ammonium consumption rate that was determined by linear regression of ammonium concentrations obtained from the batch assays.

2.2.2. The impact of pH on the anammox activities in biofilms and disrupted biofilms

This series of tests were conducted to reveal whether the biofilm structure has a protective effect for anammox bacteria against the low pH, which could be one of the reasons why the present anammox process can be operated in acidic conditions. To this aim, a total of 20 K5 carriers were collected from the MBBR and divided into two groups of 10 carriers each. One group with anammox bacteria growing in biofilms served as the control, while in the other group, biofilms were manually detached

<table>
<thead>
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<th>Parameters</th>
<th>Unit</th>
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<th>Number of samples</th>
</tr>
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<tbody>
<tr>
<td>NH₄ concentration</td>
<td>mg N/L</td>
<td>24.2 ± 2.1</td>
<td>23</td>
</tr>
<tr>
<td>NO₂⁻ concentration</td>
<td>mg N/L</td>
<td>26.8 ± 2.4</td>
<td>23</td>
</tr>
<tr>
<td>NH₄ concentration</td>
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<td>COD concentration</td>
<td>mg/L</td>
<td>62.3 ± 5.7</td>
<td>16</td>
</tr>
<tr>
<td>pH</td>
<td>–</td>
<td>4.6 ± 0.2</td>
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from the carrier and then exposed to sonication for 5 min in a 70 W sonicator to produce equally distributed the disrupted biofilm. The effect of sonication on the loss of anammox activity was negligible (Fig. S1). To initiate each test, 0.2 mL of ammonia stock solution (10 g N/L of NH$_4$HCO$_3$) and 0.25 mL of nitrite stock solution (10 g N/L of NaNO$_2$) was added to the reactor (with a working volume of 150 mL) to increase the ammonium and nitrite concentration to 20 mg N/L and 17 mg N/L, respectively. After that, the anammox activity was measured at seven different pH levels, namely 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5, according to the method detailed in Section 2.2.1.

2.3. Chemical analysis

The concentrations of ammonium, nitrite, and nitrate were measured by a Lachat QuickChem800 flow injection analyzer (Lachat Instrument, Milwaukee, Wisconsin). FNA concentration was calculated according to the method detailed in Section 2.2.1.

2.4. Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) analysis was performed on biofilm cryosections of the K5 carrier taken from the MBBR according to a modified method reported in literature (Zhao et al., 2023). Briefly, the carriers were taken from the MBBR and immediately submerged in 4% w/v paraformaldehyde at 4 °C for 8 h for fixation. Next, the biofilm was embedded, frozen, and cut into slices that were 16–20 µm in thickness. These slices were placed on slides for FISH analysis, targeting anammox bacteria using the probes detailed in Table S1. The FISH-targeted cells were visualized using ZEISS LSM 510 inverted META confocal laser scanning microscope.

2.5. Microbial assessments

2.5.1. Amplicon sequencing

Biofilm samples were collected from thin and thick carriers on Day 170 from the MBBR for analysis of the microbial community by amplicon sequencing in the Australian center for Ecoinformatics at The University of Queensland. The microbial DNA was extracted using Qiagen DNeasy Powersoil Pro-Kit (cat #7016) following the manufacturer’s protocol, and the quality was checked with gel electrophoresis. The 16S rRNA gene-targeted amplicons were analyzed via a high-throughput sequencing method. The 16S rRNA gene encompassing the V6 to V8 regions was targeted using the 926F (5′-ACA CTY AAA KGA ATT GRC GG-3′) and 1392wR (5′-ACG GCC GTG GWG TRC-3′) primers. Raw sequencing data was processed by Quantitative Insights Microbial Ecology II (QIME II) in multiple steps, including the removal of poor-quality sequences. Finally, the sequences were clustered into operational taxonomic units (OTUs) at a 97% identity threshold.

2.5.2. qPCR

Real-time qPCR was also performed to quantify the anammox 16S rRNA genes in the biofilm of MBBR, with the targeted genes and detailed procedure summarized in Table S2 (Zheng et al., 2020). The qPCR amplification reactions were conducted with a 25 µL mixture, consisting of 1 µL (10–20 ng/µL) DNA, 12.5 µL Platinum Green Hot Start PCR Master Mix (2X, ThermoFisher Scientific), 10.5 µL nuclease-free water, and 1 µL primers (20 µm), in an Applied Biosystems Veriti™ 96-Well Thermal Cycler (Model 9902) using programs specified in Table S2.

2.5.3. Metagenomic assessment

DNA was extracted and purified following the standard manufacturing procedure (Qiagen DNeasy PowerSoil Kit). To construct the paired-end library, the extracted DNA was fragmented using Covaris M220 (Gene Company Limited, China) to an average size of approximately 400 bp. The NEXTFLEX Rapid DNA-Seq (Bioo Scientific, Austin, TX, USA) was then utilized for the paired-end library construction. Metagenomic sequencing of the extracted DNA was conducted on the Illumina NovaSeq PE150 (Illumina Inc., San Diego, CA, USA) platform at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) using the NovaSeq Reagent Kits in accordance with the manufacturer’s instructions.

Quality control using Trimmomatic was performed on raw reads to remove adapters and filter reads with low quality (PHRED < 30). We then conducted an assembly of the remaining high-quality reads using MetaSPAdes with a range of k-mer lengths (k = 21, 33, 55, 77, 99, and 127). The resulting assembly was then processed through two different binners, MaxBin2 (v2.2.4) and metaBAT2 (v2.12.1), for optimal binning. CheckM (v1.0.12) and GTDB-tk (v2.2.3) were used for quality assessment and taxonomy classification on the bins.

To assess the sublineage-level phylogeny of anammox, we obtained 45 representative anammox metagenome-assembled genomes (MAGs) from the NCBI database as references. Additionally, we included the only 2 bins that belonged to anammox Ca. Brocadia and constructed the phylogenetic trees using *Nitrospira defluvii* as the outgroup. 120 single-copy marker genes for bacteria from the 47 anammox genomes were aligned using Mafft. The alignment contained 29 species, including the outgroup, and was truncated from 5013 to 4362 amino acid positions (87%). ModelFinder, embedded in IQ-TREE v2.1.2, was employed and JTT+F+R3 was determined to be the best-fit phylogenetic model. We reconstructed the corresponding phylogenetic tree with 1000 bootstrap iterations and visualized it in iTOL v6 (https://itol.embl.de).

3. Results and discussion

3.1. Anammox bacteria functioned in acidic conditions

Fig. 1 presents the nitrogen loading rate (NLR), nitrogen removal rate (NRR), pH, and effluent nitrogenous compounds (ammonium, nitrite, nitrate, and total nitrogen) concentrations of the anammox process. With the feeding of acidic wastewater, the pH of the anammox process gradually decreased from approximately 7.5 to below 6.0 during the first 25 days (Fig. 1a). Thereafter, the pH was self-sustained between 5.0 and 6.0, except for a further stage between Days 132 and 136 during which pH decreased to less than 5.0. The average pH of the reactor was 5.2 ± 0.2, slightly higher than that in the feeding (4.6 ± 0.2).

During the transition period of pH (i.e., days 1–25), the NLR and NRR remained relatively stable (Fig. 1a). With the decrease of HRT from 24 h to 8 h, the NLR of the anammox reactor gradually increased from 53.6 ± 2.7 mg N/L/d to 149.7 ± 3.9 mg N/L/d. Correspondently, the NLR of the reactor raised from 44.8 ± 3.4 mg N/L/h at the HRT of 24 h to 124.1 ± 3.9 mg N/L/h at the HRT of 8 h (Fig. 1a). The average nitrogen removal efficiency (NRE) of the anammox reactor was 81.3 ± 2.4% throughout the experimental period (Fig. S2a), which was slightly lower than the theoretical value (i.e., 89%) of the anammox reaction. This discrepancy was due to the lower molar ratio of nitrite to ammonia of 1.0 in the influent (Table 1), as opposed to the theoretical ratio of 1.32 for the anammox process. Specifically, the effluent anammox, nitrite, and nitrate concentrations were 5.1 ± 0.8 mg N/L, 0.5 ± 0.5 mg N/L, and 4.0 ± 0.8 mg N/L, respectively (Fig. 1b). The ratios of NO$_3$$_{removed}$/NH$_4$$_{removed}$ and NO$_3$$_{produced}$/NH$_4$$_{removed}$, which are two commonly used indicators of the anammox reaction stoichiometry, were 1.24 ± 0.06 and 0.20 ± 0.04, respectively (Fig. S1b). These values were within the ranges reported in the anammox process, with ratios of 1.15–1.32 for NO$_3$$_{removed}$/NH$_4$$_{removed}$ and 0.16–0.26 for NO$_3$$_{produced}$/NH$_4$$_{removed}$ (Lotti et al., 2014; Strous et al., 1999), implying that the majority of ammonia and nitrite in the influent was removed by anammox bacteria in the MBBR.

The average NRE of the reactor plunged from 80.4 ± 1.9% in days...
30–129 to 9.9 ± 4.3% in days 132–136 (Fig. S2a). The extremely low NRE in this period (i.e., days 132–136) can be attributed to the decrease in pH in the reactor to 4.6 ± 0.1 and the corresponding increase in the FNA concentration to above 0.43 mg N/L. The effects of pH, nitrite, and FNA concentrations on anammox activity in the MBBR were explored, and the results are presented in Section 3.2. After the pH of reactor was restored to the range of 5–6, the activity of anammox recovered rapidly, resulting in the jump of NRE from 8.1% to 76.4% in only four days. These results indicate that the anammox activity can recover after being subjected to short-term exposure to low pH (i.e., pH < 5.0) and high FNA (i.e., >1 mg N/L). Nevertheless, the anammox activity restoration following more prolonged exposure is unclear.

3.2. Effects of pH and nitrite on the anammox activity in biofilms

To shed light on the response of anammox biofilms to the changes in pH and nitrite concentrations, a series of batch assays were performed at the end of reactor operation. The results of the batch tests were presented and summarized in Fig. 2, according to the pH, nitrite concentration, and FNA concentration used in the tests. The anammox activity obtained at the condition of pH 7.0 and nitrite of 10 mg N/L was set as 100% relative anammox activity (RAA) which was used to compare with the anammox activity determined in other conditions. This generated a value of RAA for each condition, as shown in Table 2.

It is evident that a lower pH generally led to a lower RAA (Fig. 2). Nevertheless, the correlation between the pH and the extent of inhibition was not strong. For instance, the RAA of greater than 95% was observed at pH from 5 to 7 when the nitrite concentration was 10 mg N/L, which agreed with the performance in the long-term reactor operation (Fig. 1). This also means that FNA at about 0.22 mg N/L (calculated at a condition of pH 5.0, nitrite of 10 mg N/L, and temperature of 22°C) only had minor impact on anammox activity in biofilms in this study. Still, the RAA could vary in a broad range at the same pH, suggesting that other factors should also influence the anammox activity.

High nitrite concentrations are also known as a crucial inhibitor of anammox bacteria at neutral pH conditions with negligible FNA (Lotti et al., 2012). At the pH of 7.0, a nitrite concentration of 200 mg N/L reduced the RAA of anammox biofilms by close to 40% (Fig. 2b). However, the correlation between RAA and nitrite concentration suggests that the nitrite concentration was not the main inhibitory factor either, as different levels of inhibition were observed at the same concentration of nitrite. These observations suggest that the FNA concentration appears to play a more important role in RAA reduction for the anammox biofilm in this study (Fig. 2c). The activity of anammox biofilm decreased sharply with the increase in FNA concentration, which followed an exponential model (98.1 × e−2.1x). The model estimated a reduction of RAA of about 50% at an FNA concentration of approximately 0.33 mg N/L, as opposed to a previous study reporting a reduction of anammox activity of 50% even at an extremely low FNA concentration of 0.01 mg N/L (Fernández et al., 2012).

3.3. Identifying the anammox bacteria

Microbial community composition of the biofilm in MBBR was revealed by 16S rRNA gene-targeted amplicon sequencing. A total of 274 OTUs were identified in the biofilm sample. The Good’s coverage value was 0.999, suggesting that the libraries were of sufficient size. The results of phylum and genus-level classifications of the obtained OTUs were shown in Fig. 3. More than 90.0% of the reads were represented by the top ten phyla, among which Planctomycetota accounted for 29.3% followed by Chloroflexi (21.2%), Proteobacteria (14.4%), Bacteroidota (12.8%), Acidobacteriota (5.6%), Patescibacteria (4.6%), Cercozoa (3.3%), Nitrospirata (1.7%), Firmicutes (1.0%) and Verrucomicrobiota (0.9%). By now, all known anammox species belong to Planctomycetota (Pradhan et al., 2020). At the genus level, Ca. Brocadia was the only detected anammox bacteria, with a relative abundance of 16.3%. Ca. Brocadia was widely detected in anammox process for wastewater treatment, especially in the biofilm process (Oshiki et al., 2016). The abundance of Ca. Brocadia in the thin and thick biofilm was also compared, and the results showed that Ca. Brocadia only had a relative abundance of 0.5% in the thin biofilm, considerably lower than the thick biofilm (i.e., 16.9%) (Fig. S3). Both higher relative abundance and
biomass content suggest that the majority of anammox bacteria were enriched in the thick biofilm. The overall microbial community composition exhibited notable distinctions between the thick and thin biofilms, although denitrifiers such as *Denitratisoma* and *Pseudomonas* were identified in both thick and thin biofilms with comparable relative abundances (Fig. S3).

The qPCR results showed that the abundance of the investigated anammox gene (Table S2) increased as the HRT decreased and the nitrogen loading increased (Fig. S4). This result also implies that the anammox bacteria were able to grow in the acidic conditions. In line with the jeopardized nitrogen removal performance, the copy number of anammox gene decreased on day 138, potentially caused by the adverse effects of low pH and high FNA applied during this period (days 132–136).

To investigate whether the anammox bacteria enriched in acidic environments are novel, metagenomic sequencing was performed and two high-quality anammox bins were recovered (Table S3). Based on the phylogenetic analysis using a concatenated alignment of 120 conserved single-copy bacterial marker genes, we identified the placement of the two recovered anammox bins within two clades of anammox *Ca. Brocadia* (Fig. 4). The maximum average nucleotide identity (ANI) of two recovered anammox bins and public anammox genomes was 96.42% and 98.67%, respectively, suggesting that they are very similar to the previously known anammox strains (Ali et al., 2016; Oshiki et al., 2015).

According to read mapping against the assembled metagenomes, the total relative abundance of anammox bacteria in the MBBR reached up to 34.2% (bin1 of 33.1% and bin2 of 1.1%), further indicating the dominance of anammox *Ca. Brocadia* in present MBBR. Collectively, results from the metagenomic assessment suggest that the enriched anammox bacteria in the acidic MBBR were unlikely to tolerate low pH.

### 3.4. Investigating the mechanism of acidic tolerance of the anammox biofilm

#### 3.4.1. Effect of pH on the activity of anammox in biofilms and disrupted biofilms

To further confirm if the enriched anammox bacteria can tolerate low pH and reveal the actual reason behind the robust anammox biofilms in acidic conditions, independent batch tests were carried out on the biofilms and the disrupted biofilms, respectively, at the end of the long-term experiment.

In this series of tests, the anammox activity at pH 7.0 was set as 100% RAA. Fig. 5 presents the RAA of anammox biofilms and the disrupted biofilms in different pH conditions (4.5–7.5). In the case of anammox biofilms, the anammox activity maintained stable at pH values ranging from 6.0 to 7.5. However, as the pH decreased further, the anammox activity declined sharply, showing an activity loss exceeding 80% at pH 4.5.
4.5. Compared to the biofilm culture, the anammox in disrupted biofilms were more sensitive to pH, with more than 70% and almost 100% of activity lost at pH 6.0 and pH 5.0, respectively. This observation was in line with previous studies, showing that anammox bacteria in the disrupted biofilms are barely active at pH of 6.0 (Carvajal-Arroyo et al., 2013; Fernández et al., 2012; Yin et al., 2016). In contrast to the stable anammox activity observed at pH 5.5 during the long-term operation, the activity of anammox in disrupted biofilms decreased by 90% from pH 7.0 to pH 5.5. Together these results indicate that the anammox bacteria in the present biofilm were not inherently acid-tolerant.

3.4.2. Spatial distribution of anammox bacteria in biofilms

Another potential reason for the observed stability of anammox biofilm under low pH is associated with the protective effect of the multi-layered biofilm structure. To provide supportive evidence for this hypothesis, FISH was carried out for the cryosections of intact biofilms to reveal the spatial distribution of anammox bacteria in biofilms. The biofilm attached to the carrier surface unevenly, exhibiting a thickness in the range of 188–1794 µm, with an average value of 884.4 µm. Notably, the majority of anammox bacteria occupied the inner layer of biofilms near the carrier surface, with a depth of approximately 450 µm (Fig. 6). This result suggests that biofilms might act as a shield protecting anammox bacteria from the acidic bulk liquid outside. Together with the results mentioned in Section 3.4.1, it can be concluded that the protective effect of the thick biofilms in this MBBR enabled the long-term stable operation of anammox biofilm in acidic conditions.

Fig. 4. Phylogenetic tree of anammox bacteria based on genomes calculated with 1000 bootstrap replications, with all displayed nodes supported by bootstrap $\geq 70\%$.

Fig. 5. Response of different types of anammox biomass to different pH conditions.
3.4.3. Possible reasons for achieving the acidic anammox process

There are two potential reasons for achieving the acidic anammox process in the biofilm configuration reactor. One reason could be the limitation of proton diffusion. For example, Hou et al. (2017) found that pH inside biofilm increased from 4.8 to 6.8 with biofilm thickness increasing from 0 to 1000 µm. Hu et al. (2023a) also revealed that proton diffusion was limited inside biofilm, resulting in a pH of approximately 6.1 at the surface and 6.7 at a depth of 300 µm. In the presented MBBR system, the averaged biofilm thickness was 884.4 µm, indicating the occurrence of proton limitation. The second reason might be related to alkalinity regenerated through denitrification, which creates a relatively neutral environment within the biofilm. The evidence of denitrification included the presence of denitrifiers in the biofilm, the presence of organics in the feed (Table 1), and a relatively low NO$_3^{–}$ produced/NH$_4^{+}$ removed ratio (0.20) compared to the theoretical value of 0.26. Therefore, proton diffusion limitation and alkalinity regeneration could both play a role in maintaining the performance of acidic anammox process.

3.5. Implications

In the present study, the feasibility of using anammox process to treat the effluent from a mainstream acidic PN process was demonstrated. The long-term operation of the anammox process successfully achieved a significant removal of nitrogen at pH of 5.2 ± 0.2, with the effluent TN concentration remaining stable below 10 mg N/L and a total NRR of 124.1 ± 3.9 mg N/L/d.

Indeed, the feasibility of the anammox process in handling acidic wastewater reinforces its potential application in wastewater treatment plants where acidic wastewater streams or wastewater with low alkalinity are prevalent. Wastewater with low alkalinity is common, such as the digester supernatant (Wang et al., 2021b), domestic sewage with organics removed by CEPT (Hu et al., 2023b), source-separated urine (Li et al., 2020), and landfill leachate (Ren et al., 2022). This study offers a promising avenue for the development of sustainable and cost-effective wastewater treatment systems that can effectively handle low-alkalinity wastewater and achieve efficient nitrogen removal.

Despite recent reports on the presence of anammox bacteria in various acidic natural environments, such as red soils with a pH range of 4.4 – 6.0 (Wu et al., 2018) and freshwater environments with a pH below 4.0 (Zhu et al., 2015), no acidophilic anammox bacteria have been identified to date. Anammox activity has been shown to contribute to over 50% of N$_2$ production in some acidic natural environments (Wu et al., 2018), suggesting the potential existence of acid-tolerant anammox bacteria. Thus, from an ecological perspective, further research efforts should be directed towards investigating the presence of acidophilic anammox bacteria in acidic environments.

4. Conclusions

The anammox process is generally thought to be active only in neutral conditions, which was challenged by the findings of this study. Here, the anammox process received the effluent from a mainstream acidic PN process. Under the acidic condition (i.e., pH 5 – 6), the anammox process showed stable performance for a long-term, with an average NRE of higher than 80%. Results of batch tests showed that, at a condition of pH 5.0 and nitrite of 10 mg N/L, higher than 95% of RAA in biofilm can be retained. This result indicates the reliability of the two-stage PN/A process with the use of acidic bioprocess to achieve PN, as the anammox process can handle the acidic feeding with a satisfactory efficiency. Microbial assessment reported the majority of anammox bacteria were enriched in the carriers with thicker biofilm. Further batch tests and the FISH assessment revealed that the anammox bacteria in present process was not inherent acid tolerant. Instead, the multi-layer structure of biofilm plays a crucial role in preventing the anammox bacteria from the acidic condition, thereby enabling anammox activity in acidic condition.

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.

Data availability

Data will be made available on request.

Acknowledgments

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Supplementary materials


Reference


