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NaH_2PO_4 as pH buffer in an anaerobic ammonium oxidation (anammox) reactor treating high-strength livestock manure digester liquor

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ABSTRACT

In order to use livestock manure digester liquor (LMDL) as liquid fertilizer, NaH_2PO_4 was evaluated for its effectiveness as pH buffer in an anaerobic ammonium oxidation (anammox) reactor treating high-strength LMDL. The results show that influent total nitrogen (TN) concentration was increased from 500 to 2,200 mg/L with the addition of NaH_2PO_4 as a pH buffer. To the best of our knowledge, this is the highest influent TN concentration for anammox reactor in treating LMDL. An average TN removal efficiency of 85% was observed in LMDL treatment without dilution. The treated effluent may be applied directly as a terrestrial liquid fertilizer, as the effluent has a suitable balance of nitrogen and phosphorus (approximately 1:1). 16S rRNA gene analysis showed that *Kuenenia stuttgartiensis* and *Uncultured bacterium clone KIST-JJY001*, known as anammox bacteria, shared approximately 26% in genus level in the reactor. *Uncultured bacterium clone Dok53*, *Uncultured bacterium clone KIST-JJY024*, *Uncultured bacterium clone FN-11*, *Uncultured bacterium clone KIST-JJY012*, and *Uncultured bacterium clone AnSal-09*, which are often detected as co-occurring bacteria, were also found in this study.

Keywords: Anammox; PH buffer; Phosphate fertilizer; High concentration; Inorganic carbon; Biocarbonate

1. Introduction

Livestock manure digester liquor (LMDL) is generated from anaerobic methane fermentation of livestock manure. Because nutrients remain in the liquor, in most cases, it is recommended that LMDL be used as a liquid fertilizer. However, terrestrial use of LMDL is not appropriate due to the surplus of nitrogen and insufficient phosphorus. Therefore, the nitrogen surplus needs to be removed through the nitrogen removal process [1], and it is recommended that phosphorus be added.

LMDL contains a high-strength nitrogen concentration, primarily in the form of ammonia nitrogen. The biological nitrogen removal process has usually been carried out in treating LMDL by means of conventional autotrophic nitrification and heterotrophic

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denitrification systems (CND). However, the high operational costs of CND make it unaffordable.

In recent years, anaerobic ammonium oxidation (anammox) processes, in which ammonia is oxidized to nitrogen gas using nitrite as the electron acceptor under anoxic conditions [2], has been widely used in treating high-strength ammonia containing wastewater. The ammamox process has numerous advantages over CND, such as high efficiency, reduced energy use, and minimal CO_2 production [3–5]. Furukawa et al. [1] reported that the partial nitritation/anammox process (PNA) may be successfully applied to digester liquor of biogas plant with reduced operational cost. Stable nitrogen removal efficiencies of 80% were obtained with nitrogen removal rates (NRR) of 4.0 kg-N/m^3/d. Although phosphorus remained in the effluent, the treated LMDL by PNA is not suitable for use as a liquid fertilizer due to the imbalance of nitrogen and phosphorus. Beyond that, the pH of the anammox reactor will rise significantly in treating high-strength LMDL without pH adjustment, though pH was found to be critical to stable anammox activity [6]. Therefore, LMDL was usually treated with a reduced concentration in the anammox reactor [7], and a large amount of water is used to dilute the LMDL.

In light of the above, NaH_2PO_4 is considered to an appropriate buffer to control the pH of the anammox reactor when treating high-strength LMDL without dilution, and an increased ratio of phosphorus is observed in the effluent. After reducing the nitrogen concentration through anammox reactor, the effluent, with good balance of nitrogen and phosphorus, may be directly applied as a terrestrial fertilizer. Genetic analysis using the 16S rRNA gene was employed to characterize the microbial population of the anammox sludge.

2. Materials and methods

2.1. Anammox reactor

The reactor had an inner diameter of 14 cm with a total liquid volume of 10 L including a reaction zone of 8 L and a settling zone of 2 L. The reactor was made of acrylic resin and had a water jacket for temperature control. Sampling ports were located at heights of 3, 17, 20, and 25 cm above the reactor bottom. The reactor was enclosed in a black vinyl sheet to inhibit growth of photosynthetic bacteria and algae.

As shown in Fig. 1, the reactor was operated in up-flow mode, with influent introduced at the bottom using a peristaltic pump (BT100–2J, Longer Pump, China). A gas–solid separator was designed to separate the sludge and generated nitrogen gas from the treated water. A recirculation pump (BT600–2J, Longer Pump, China) was used to dilute the influent with the treated wastewater. Except for NaH_2PO_4 adding, no pH adjustment was performed in the study.

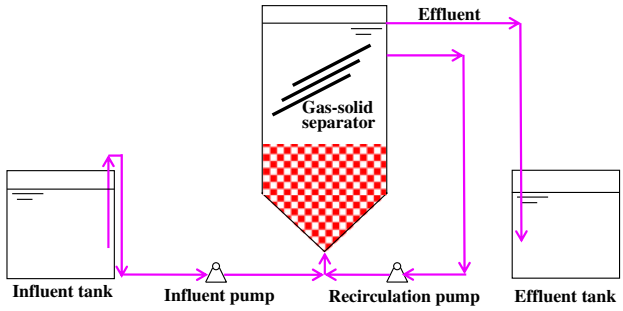

Fig. 1. Schematic view of the anammox reactor.

2.2. Anammox seed sludge

The granular activated carbon (GAC)-seeded granules [8], which have been cultivated in one 50-L reactor for 5 years in Guilin University of Technology, Guilin, Guangxi, China, were used as seed sludge in the reactor. At the start-up, 1-L GAC-seeded granules were added in the anammox reactor. The concentration of GAC-seeded granules (mass of mixed liquor suspended solids (MLSS) per liter) was 20 g MLSS/L.

2.3. Feed media

During the start-up period, the reactor was fed with synthetic wastewater with a nitrite to ammonium molar ratio of 1.0. The composition of synthetic wastewater [9] is shown in Table 1. Trace elements were added to the influent according to the reported value [10]. After the start-up period, the influent was changed to the effluent of partial nitritation system with biological selector (PNBS) [11] of LMDL, which was obtained from the digester liquor storage tank of a dairy farm in the city of Zengcheng, Guangdong, China. Characteristics of the effluent of PNBS of LMDL were pH 8.0–8.5, 200–300 mg/L SS, 500–600 mg/L total chemical oxygen demand (COD), 1,050–1,100 mg/L NH_4-N, 1,100–1,150 mg/L NO_2-N, and 30–50 mg/L NO_3-N. NaH_2PO_4 was added to adjust the pH of the effluent of PNBS of LMDL.

2.4. Batch experiments for anammox sludge activity measurement

To estimate the activity of anammox sludges, batch experiments were carried out in 250-mL conical flasks. The conical flasks were sealed with butyl rubber

Table 1
Composition of synthetic wastewater

Composition	Concentration (mg/L)
$(NH_4)_2SO_4$, $NaNO_2$ (as mg N/L)	200–1,100
$KHCO_3$	1,000
NaH_2PO_4	20–1,300
$CaCl_2{\cdot}2H_2O$	100
$MgSO_4{\cdot}7H_2O$	200
$Na_2S_2O_3$	24.81
Trace element solution 1 (g/L): $FeSO_4{\cdot}7H_2O$ 10, $C_{10}H_{14}N_2Na_2O_3$ 5.6	1 mL/L
Trace element solution 2 (g/L): $MnCl_2{\cdot}4H_2O$ 0.352, $CoCl_2{\cdot}6H_2O$ 0.096, $NiCl_2{\cdot}6H_2O$ 0.08, $CuSO_4{\cdot}5H_2O$ 0.1, $ZnSO_4{\cdot}7H_2O$ 0.172, $NaSeO_4{\cdot}10H_2O$ 0.105, $NaMoO_4{\cdot}2H_2O$ 0.11, $C_{10}H_{14}N_2Na_2O_3$ 5.0	0.5 mL/L

stoppers and purged with N_2 gas to remove oxygen. Intensive magnetic stirring was adopted to reduce mass transfer limitation [12]. The anammox nutrient medium consisted of 20 mg-N/L $(NH_4)_2SO_4$, 25 mg-N/L $NaNO_2$. The pH of the medium was adjusted at 7.3. The bottles were then incubated at 35˚C. Gas and medium samples were taken for chemical analyses at appropriate time intervals. Since nitrite might be utilized by denitrification bacteria, ammonium was preferred to evaluate the anammox sludge activity.

2.5. Analytical method

NO_2-N, NH_4-N, and total phosphorus (TP) were measured by the colorimetric method according to Standard Methods [13]. Total nitrogen (TN) was determined by the persulfate method [13] using the UV spectrophotometric screening method [13] for quantification of TN as NO_3-N (the oxidization product of the persulfate digestion). NO_3-N (of the original sample) was determined by calculation of the difference of TN and the sum of NO_2-N and NH_4-N. The pH was measured using a pH meter (9010, Jenco, USA), and dissolved oxygen (DO) was measured using a DO meter (6010, Jenco, USA). COD was measured according to the methods reported by Wenjie et al. [14].

The suspended solids (SS) content was determined according to Standard Methods [13]. The total sludge content was estimated as MLSS. For determination of MLSS, a sludge sample of known volume was washed twice by centrifuging at 1,000× g for 15 min with decanting and re-suspending in deionized water and then dried to a constant weight at 105˚C, with cooling to room temperature under desiccation prior to weighing.

2.6. DNA extraction and PCR amplification

Meta-genomic DNA was extracted using an ISOIL kit (Wako, Osaka, Japan) according to the manufacturer's instructions [13]. Amplification of the 16S rRNA gene was performed with Phusion High-Fidelity DNA polymerase (FINNZYMES, Finland) using conserved eubacterial primers 6F (forward primer: 5′-GGAGAGTTAGATCTTGGCTCAG-3′) and 1492r (reverse primer: 5′-GGTTACCTTGTTACGACT-3′). PCR was carried out according to the following thermocycling parameters: 30-s initial denaturation at 98˚C, 25 cycles of 10 s at 98˚C, 30 s at 51˚C, 20 s at 72˚C, and 5 min final elongation at 72˚C. The amplified products were electrophoresed on a 1% agarose gel and extracted fragments were purified using Wizard SV Gel and PCR Clean-Up System (Promega, USA).

2.7. Cloning and sequencing of the 16S rRNA gene

The purified fragments were ligated into the EcoRV site of pBluescript II KS+ (Stratagene, USA) and *Escherichia coli* DH10B was transformed using the constructed plasmids. White colonies including the insert were randomly chosen and the plasmids were extracted by the alkaline method. The nucleotide sequences were determined with a 3130xl genetic analyzer and a Big Dye terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). The sequences determined in this study were compared with the sequences in the nrdatabase using the basic local alignment search tool program on the NCBI website.

2.8. Denaturing gradient gel electrophoresis (DGGE)

Partial 16S rRNA gene was amplified by PCR with a eubacterial primer set, 1055F–1392R [15].

The extracted meta-genomic DNA and cloned plasmids were used as templates for the sample and markers in the DGGE, respectively. The amplified fragments were purified and combined with the GC-clamp (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCG CCGCCCCCGCCCG-3′) at the 5′ termini by a second PCR using a primer set, 357F with GC-clamp and 534R. The products were resolved by DGGE for 14 h at 90 V at 60˚C using the DCode system (Bio-Rad, Hercules, CA, USA). An 8% polyacrylamide gel with a 30–65% denaturing gradient was used, where 100% denaturant was defined as 7 Murea and 40% formamide. The gel was stained with SYBR-Gold (Invitrogen, USA) and visualized using the FLA-2000 system (Fuji Photo Film, Tokyo, Japan).

3. Results

3.1. Effect of adding NaH_2PO_4 as pH buffer

In this study, the effects of adding NaH_2PO_4 were investigated using synthetic wastewater. Batch experiments were used to estimate the activity of GAC-seeded granules, which were used as seed sludge, and pH was recorded at the end of the batch experiments.

As shown in Fig. 2, adding more NaH2PO4 minimized the changes in pH and increased use of NaH_2PO_4 enhanced the activity of GAC-seeded granules. Average pH decreased from 8.2 to 7.2 at the highest levels of NaH_2PO_4 addition. The average activity of GAC-seeded granules increased gradually from 0.2 to 0.53 kg-N/kg-MLSS/d as the NaH_2PO_4 concentration was increased from 8.4 to 364 mg-P/L. The results show that NaH_2PO_4 have a positive effect on enhancing GAC-seeded granules activity. However, the activity of GAC-seeded granules remained stable when NaH_2PO_4 concentration was further increased to 392 mg-P/L. Therefore, the effective level of NaH_2PO_4 addition is suggested to be 364 mg-P/L. In numerous studies, pH has been shown to affect anammox activity [16]. Thus, pH management with NaH_2PO_4 as buffer enhanced the activity of GAC-seeded granules.

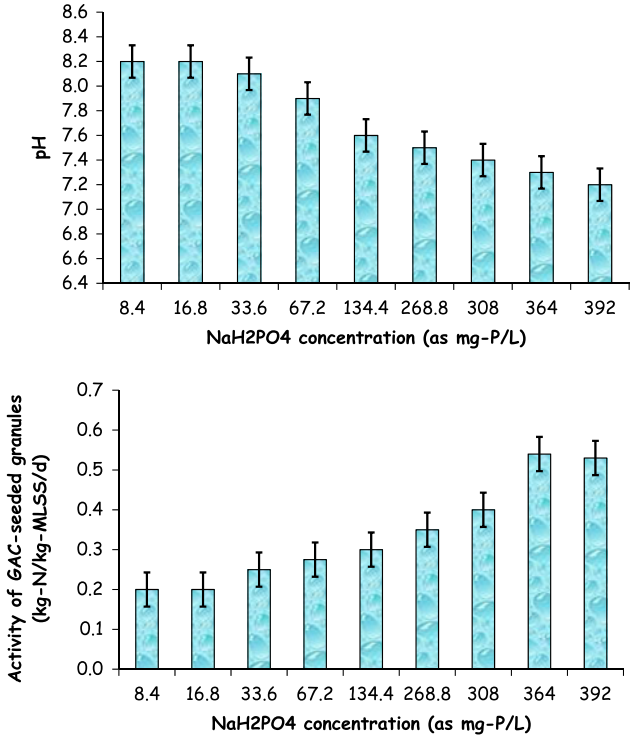

Fig. 2. Effects of adding NaH_2PO_4 as pH buffer.

3.2. Reactor performance

Using batch experiments, the activity of GAC-seeded granules was determined as 0.5 kg-N/kg-MLSS/d. Therefore, a nitrogen loading rate (NLR) of 1.0 kg-N/m^3/d was applied during the start-up period considering the dosage of GAC-seeded granules. A concentration of 364 mg-P/L of the NaH_2PO_4 buffer was added to the influent. Fig. 3 shows the course of treatment performance throughout the entire experimental period. The start-up period for the anammox reactor was considered to be from day 0 to day 60, during which time the synthetic wastewater was used. NLR was increased from 1.0 to 7.2 kg-N/m^3/d by shortening the HRT from 4.8 to 8.7 h and increasing the influent TN from 200 to 2,400 mg/L, which is higher than what was seen by Zekker et al. [17]. The corresponding NRR achieved on day 30 was 5.4 kg-N/m^3/d with TN removal rate of 82% in average. Next, NLR was maintained to investigate the stability of the anammox reactor for 30 d with HRT 8 h and influent TN 2,200 mg/L. Stable treatment performance was achieved using NaH_2PO_4 as a buffer. Starting on day 60, the influent was changed to the effluent of PNBS of LMDL. NaH_2PO_4 was still added in the influent at a level of 364 mg-P/L. The anammox reactor was operated at a HRT of 8 h with raw effluent of PNBS of LMDL. Although the effluent of PNBS of LMDL was used without any dilution, the NRR decreased on the first day, then increased, and remained at approximately 5.6 kg-N/m^3/d with influent TN concentration of 2,200 mg/L. Yamamoto et al. [7] reported that the NRR of the anammox reactor treatment was 2.0 kg-N/m^3/d with seven times dilution. In this study, NRR was almost three times higher than that value, and no dilution was performed. Except for pH adjustment using NaH_2PO_4, the conditions were similar to those described by

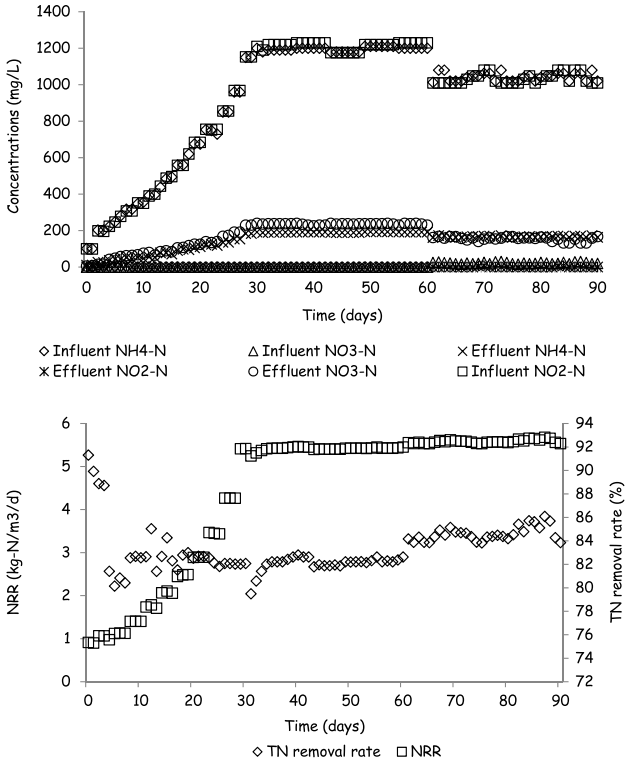

Fig. 3. Reactor performance during the study.

Yamamoto et al. [7]. Therefore, it is recommended that NaH_2PO_4 be used as a pH buffer in treating high-strength LMDL.

Table 2 shows the composition of the effluent treated by the anammox reactor. The effluent COD was approximately the same as the influent COD. Effluent TP concentration was near to the effluent TN concentration, which was mainly composed of NH_4-N and NO_3-N. The treated effluent, with good balance of nitrogen and phosphorus (about 2:1), could be applied directly as a terrestrial liquid fertilizer.

3.3. Bacteria community analysis

The GAC-seeded granules used was inoculated from one 50-L reactor [18], which was known to be clustered with *Uncultured bacterium clone KIST-JJY024, Uncultured bacterium clone FN-11, Uncultured bacterium clone KIST-JJY012, and Uncultured bacterium clone AnSal-09*. After 30 d of treatment of the LMDL, a phylogenetic analysis showed that approximately 26% in genus level was identified as *Kuenenia stuttgartiensis* and *Uncultured bacterium clone KIST-JJY001* (Table 3). It can be concluded that *Kuenenia stuttgartiensis* and *Uncultured bacterium clone KIST-JJY001* are effective in treating high-strength ammonia containing wastewater in the anammox reactor. In addition, *Uncultured bacterium clone Dok53*, *Uncultured bacterium clone KIST-JJY024*, *Uncultured bacterium clone FN-11*, *Uncultured bacterium clone KIST-JJY012*, and *Uncultured bacterium clone AnSal-09*, which are often detected as co-occurring bacteria, were also found to be abundant in this study. The function of the co-occurring bacteria remains largely unknown.

Table 2
Composition of the effluent treated by anammox reactor

Parameters	pH	TN	TP	COD
Effluent	7.3 ± 0.1	330 ± 20	350 ± 15	530 ± 20

4. Discussion

As shown in Fig. 3, the influent TN concentration was increased to 2,200 mg/L with a nitrogen removal efficiency of 85% near the end of the study. To the best of our knowledge, this is the highest TN concentration to be directly treated to date (Table 4) in treating LMDL. High NO_2-N concentrations are inhibitory to anammox bacteria [19,20]; thus, highly concentrated nitrogen wastewater is usually diluted prior to treatment in anammox reactors [19]. However, this practice is illegal for wastewater treatment in most cases, as in China. Accordingly, adding NaH_2PO_4 as demonstrated here could be a highly effective option for high-strength LMDL wastewater treatment.

Numerous research results, including results from the current study, show that pH influences anammox activity dramatically [21,22]. As observed in this study, to prevent a pH increase in the anammox reactor, NaH_2PO_4 provides H^+, smoothing the anammox reaction. As shown in Fig. 2, the addition of NaH_2PO_4 enhances anammox activity. At the end of the study, NaH_2PO_4 showed no adverse effect on anammox activity, which is optional as pH buffer in anammox reactor.

Furukawa et al. [1] used 0.2-M HCl to control pH. However, Cl^- is not useful if the treated LMDL is to be recycled and used as liquid fertilizer. On the other hand, the treated effluent reported by Furukawa et al. [1] is insufficient due to the low TP. Thus, the application of treated wastewater as liquid fertilizer is limited. In this study, the treatment of LMDL by anammox reactor has a suitable balance of nitrogen and phosphorus (approximately a 1:1 ratio), which is preferred in terrestrial fertilizer use [23]. Therefore, the treated LMDL, using NaH_2PO_4, has strong potential for application as liquid fertilizer. In a further study, the potential of NaH_2PO_4 as a terrestrial fertilizer will be examined.

Table 3
Homology search results for 16S rRNA gene sequences of the main bacterial members in the community

Taxon	Identity (%)	Number of clones	Accession
Uncultured bacterium clone KIST-JJY030	97	1	EF654699
Kuenenia stuttgartiensis	98–99	4	CT573071
Uncultured bacterium clone KIST-JJY001	96–100	2	EF515083
Uncultured bacterium clone Dok53	99	1	FJ710771
Uncultured bacterium clone KIST-JJY024	99	5	EF594056
Uncultured bacterium clone FN-11	91	1	FJ917566
Uncultured bacterium clone KIST-JJY012	99–100	7	EF584532
Uncultured bacterium clone delph2B11	96	1	FM209162
Uncultured bacterium clone AnSal-09	99	1	AB434261

Table 4
Comparison of possible TN concentration for different reactors in wastewater treatment

Reactor type	Reactor volume (L)	NRR (kg-N/m^3/d)	TN concentration (mg/L)	Refs.
Polyethylene glycol (PEG) gel carrier	1.0	4.0	1,350	[1]
Granules	3.0	2.0	262	[7]
Porous polyester non-woven fabric carrier	2.85	0.22	198	[19]
GAC-seeded granules Present work	10	5.6	2,200	

5. Conclusions

The addition of NaH_2PO_4 effectively enhanced the activityof GAC-seeded granules in treating high-strength wastewater. $NaH_2PO_4$of 364 mg-P/L could control the pH at about 7.3 in treating high TN containing wastewater of 2,400 mg/L. NRR of 5.6 kg-N/m^3/d was achieved in treating raw effluent of PNBS of LMDL with influent TN of 2,100 mg/L. The treated LMDL may be suitable for use as a terrestrial liquid fertilizer.

Acknowledgments

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