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Optimization of Nitrogen Removal Rate in One-Stage Reactor through Partial Nitrification Anammox Process during Direct Treatment of Poultry Litter Wastewater

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Optimization of Nitrogen Removal Rate in One-Stage Reactor through Partial Nitrification Anammox Process during Direct Treatment of Poultry Litter Wastewater

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of the requirements for the degree of
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ABSTRACT

Anammox is an increasingly common process used for the treatment of reject water and even mainstream wastewater due to its low oxygen demand. However, anammox is not commonly utilized in the direct treatment of poultry litter because of the high organic content, which would inhibit the anammox process. Thus, this project is aimed at optimizing the nitrogen removal rate through partial nitrification anammox process (PN/A) to treat synthetic poultry litter wastewater. Nitrogen removal efficiencies will therefore be improved through optimizing the combination of three operating parameters including hydraulic retention time (HRT), dissolved oxygen (DO) concentration, and carbon to nitrogen (C/N) ratio. The results showed that 170 mg/L NH_4^+-N and 199.6 mg/L total nitrogen (TN) was removed in the continuous stirred reactor with 100% and 87.3% removal efficiency. During the operation, the relative abundance of the dominant ammonia-oxidizing bacteria (*Nitrosomonas*), the dominant anammox bacteria (*Candidatus Brocadiaceae*), and the dominant comammox bacteria (*Nitrospira*) changed from 2.75%, 2.56%, and 0% to 3.57%, 1.18%, and 0.06%, respectively.

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Chapter 1 General Introduction

1.1. Poultry litter overview

Poultry is one of the most commonly produced livestock in the United States. With the increasing number of people concerning about health risks related to red meat (McAfee et al., 2010; Wolk, 2017) and the spreading awareness of environmental pollution associated with livestock production, more consumers would want to consume carbon-light chicken meat instead of carbon-heavy beef. The growing popularity of chicken in the US is thus a result of consumers having been choosing chicken over beef due to health and environmental concerns, as evidenced by the decline in beef consumption per capita by 31% in the past 50 years. The shift of meat consumption pattern by the consumers preferring chicken to beef signals that they are worried about the environmental footprint of beef production because intensive beef farming is considered to produce more greenhouse gases than intensive chicken farming, based on a report that producing one kg of beef would emit 60 kg of greenhouse gases (CO_2-equivalents) (Al-Obadi, Kutty, Abdella, Kucukvar, & Bulak; Ritchie, 2020). In 2019, the total number of broilers produced in the U.S. was 9.18 billion, which values at $28.3 billion (*Poultry Production and Value*, 2020). In Arkansas, the poultry industry, ranked the second largest in the nation, produces more than 1 billion broilers in 2019 (*Poultry Production and Value*, 2020), which provides around 25% of agricultural jobs in the State ("Arkansas Poultry Facts," 2021). With more than 5.7 billion pounds broiler meat ("Arkansas Poultry Facts," 2021) produced, Arkansas' broiler sales reaches $3.61 billion and egg sales reaches $504 million in 2019 ("Poultry Sector at a Glance," 2021). Even though poultry accounts for the largest share in Arkansas's agricultural produce, the rapid growth of the poultry industry has also resulted in bulky litter generation. Each bird in a 42-day production cycle can generate 1.5 to 5.7 kg of litter (Bolan et al., 2010; Edwards & Daniel, 1992), which means that there are more than 1.5 million tons of poultry litter generated in Arkansas annually. Poultry litter mainly contains bedding material or litter (sawdust, wood shavings, wheat straw, peanut hulls), waste food, broken eggs, dead birds, feathers, and typically poultry manure (Edwards & Daniel, 1992; Kelleher et al., 2002), thus it is rich in nitrogen and other nutrients (Singh, Lee, Worley, Risse, & Das, 2010). But improper handling and disposal of poultry litter can have a broad unfavorable impact to the environment, such as pathogens proliferation, chemical contamination and eutrophication of water bodies due to nutrients runoff/leaching problems (Risse et al., 2006). In addition, poultry litter, if not treated properly, can also post other environmental challenges such as production of phytotoxic materials from poultry manure (Delgado, Martin, De Imperial, León-Cófreces, & García, 2010), air pollution resulting from the nuisance odors generated in production facilities, and emission of greenhouse gases (Broucek & Cermák, 2015; Kelleher et al., 2002).

1.2. Uses of Poultry litter

As a cheap source of protein and minerals, poultry litter is an age-old fertilizer. To lower the cost of growing crops and forage, producers have been using the nutrient-rich poultry litter as an economical alternative to commercial fertilizer (Evers, 1998). Studies also show extra benefits through using poultry litter (Kingery et al., 1993; Tewolde, Adeli, Rowe, & Sistani, 2011). For instance, Mitchel et al. (2006) found that the application of broiler litter can cause the increase of nutrient content in the soil without the accumulation of heavy metals. Belefant-Miller (2007) also reported that poultry litter can increase tillering in rice as well as improve the overall rice growth and yield. Despite that poultry litter has long been used as organic fertilizer, which is currently regarded as the most economical way of disposal, it was understood by many researchers that uncontrolled application of poultry litter to cropland might not be appropriate from the perspective of environmental pollution (Bolan et al., 2010; Scharbor, 2011). Soil tests demonstrated that poultry litter could be unsafe when used as fertilizer if not treated properly (Z. Chen & Jiang, 2014). Poultry litter contains not only plenty of nutrients but a significant amount of bacterial and pathogenic fungal contaminants (Kyakuwaire, Olupot, Amoding, Nkedi-Kizza, & Ateenyi Basamba, 2019). Excessive use of poultry litter in cropping systems may cause accumulation, leaching, and runoff of nitrogen in the soils, leading to nitrate (NO_3) contamination of groundwater (Bitzer & Sims, 1988).

Poultry litter has also been used as feedstuff to rabbits (Owen et al., 2009), pigs (Adesehinwa, Obi, Makanjuola, Adebayo, & Durotoye, 2010), and beef cattle (Poore, Harvey, & Crickenberger, 1995). Moreover, Oliphant et al. (1974) reported that using dried poultry manure with 30% crude protein could increase the profit of producing intensively produced beef without much differences in the performance of diet. Poultry litter can also constitute up to 32% of rabbits' diets without any side-effect on their growth (Onimisi & Omage, 2006).

However, when used as feedstock, cautions must be exercised because improper treatment of poultry litter may cause foodborne illness for animals or the accumulation of hazard chemicals in the produced meat (Haapapuro, Barnard, & Simon, 1997; Jeffrey, Kirk, Atwill, & Cullor, 1998). MuLoughlin et al. (1988) documented the first case of confirmed botulism outbreak in cattle after feeding them with ensiled poultry litter. Tokarnia et al. (2000) also reported the outbreak of copper poisoning related disease for cattle fed on poultry litter.

Other than fertilizer and animal feed, poultry litter can be a source of renewable energy. Thermochemical processes including combustion (Singh, Risse, Das, Worley, & Thompson, 2010), gasification (Jeswani, Whiting, Martin, & Azapagic, 2019), and pyrolysis (Singh, Risse, et al., 2010) and anaerobic digestion (Singh, Lee, et al., 2010) are the two major technologies to convert poultry litter to fuel source. Thermochemical technologies are promising methods to recover energy and reduce environmental impacts from biomass wastes such as poultry litter (Bora, Lei, Tester, Lehmann, & You, 2020; Cantrell, Ro, Mahajan, Anjom, & Hunt, 2007). Poultry litter contains a high energy density (14,447 kJ/kg) if fully combusted, and can be burned directly when water content is less than 9% (Dávalos, Roux, & Jiménez, 2002). Besides, the waste of combustion can still be used as fertilizer without losing much nutrients (Dagnall, 1993; MacDonald, 2009). That said, combustion/incineration of poultry litter has spawned an immense resistance from environmental groups and public health policy makers on the grounds that using poultry litter as a combustion fuel can pose a dire threat to air quality and the health of environment (Chastain, Coloma-del Valle, & Moore, 2012; Stingone & Wing, 2011). Recent research revealed that emissions from poultry litter incineration included particulate matter, dioxins, arsenic, bio-aerosols, and other toxins, the various components of which were associated with cardiovascular disease, cancer, respiratory illness, and other diseases. Furthermore, the low efficiency in recovering the energy content in poultry litter via combustion (only about 24% extracted) also makes this practice unappealing to the producers and the energy industry (Costello, 2007).

Anaerobic digestion is a biological process using methanogens and other anaerobic bacteria to break down organic carbons such as solid waste to produce methane (Adekunle & Okolie, 2015). Many studies have been conducted concerning the co-digestion of poultry litter with other residues from agricultural production to improve biogas production (Lohani et al., 2021; Beatriz Molinuevo-Salces, Xiomar Gómez, Antonio Morán, & Mari Cruz García-González, 2013; Valenti et al., 2018). However, the high ammonia nitrogen content in poultry litter will inhibit bacterial biomass and biogas yields (Rajagopal, Massé, & Singh, 2013; Sung & Liu, 2003; Yenigün & Demirel, 2013). Webb et al. (1985) also reported depression in gas yield caused by various ammonium-nitrogen levels during anaerobic digestion of poultry litter. To reduce the impact of high ammonia level in poultry litter on the digestion process, different strategies are extensively studied including co-digestion with a carbon rich substrate and ammonia stripping (Habiba, Hassib, & Moktar, 2009; B. Molinuevo-Salces, X. Gómez, A. Morán, & M. C. García-González, 2013). Markou (2015) observed improved biogas production from digestion of poultry litter after ammonia stripping treatment.

1.3. Biological treatment processes for removal of ammonium

Biological treatment processes are preferred by wastewater treatment plants due to the advantages such as high effectiveness and low cost (Epa, 1993). However, the biological system may be vulnerable when the microorganisms encounter unfavorable conditions such as the existence of heavy metals. So, most of the biological treatment plants are dealing with wastes, like ammonium, that can be easily biodegradable.

1.3.1 . Simple nitrification and denitrification

Nitrification and denitrification are most commonly used to remove nitrogen compounds from wastewaters. During nitrification, ammonia-oxidizing bacteria (AOB) first oxidizes ammonia to nitrite using oxygen, and then nitrite-oxidizing bacteria (NOB) oxidizes nitrite to nitrate under aerobic conditions (Ward, Arp, & Klotz, 2011). The complete reactions involved, when oxygen is present, are shown in Equation 1 (González-Cabaleiro, Curtis, & Ofiţeru, 2019) and 2 (Abeliovich, 2006).

$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + H_2O + 2\ H^+ \qquad \text{Equation 1}$$

$$NO_2^- + 0.5\ H_2O + 0.25\ O_2 \rightarrow NO_3^- + H^+ \qquad \text{Equation 2}$$

During the denitrification process, nitrate is reduced to nitrogen gas by denitrifiers, as shown in Equation 3 (Holmes, Dang, & Smith, 2019), using various carbon sources as electron donors without oxygen present.

$$2\ NO_3^- + 10\ e^- + 12\ H^+ \rightarrow N_2 + 6H_2O \qquad \text{Equation 3}$$

1.3.2. Partial nitrification/anammox process

Besides complete nitrification/denitrification, there exists another nitrogen removing process, called "partial nitrification/anammox (PN/A)". Numerous past studies have been conducted using the PN/A process, including those carried out using either synthetic (Al-Hazmi, Grubba, Majtacz, Kowal, & Makinia, 2019), pre-treated wastewater (Pedrouso, Trela, Val Del Rio, Mosquera-Corral, & Plaza, 2019), or pig manure (Pichel et al., 2019). During the PN/A process, anaerobic ammonium oxidizing bacteria (anammox) uses ammonium as its electron donor and nitrite, instead of O_2, as its electron acceptor to produce nitrogen gas. The anammox bacteria is a group of slow-growing autotrophs (Jetten et al., 2009), which oxidize nitrite to nitrate through the reduction of carbon dioxide following an overall stoichiometric reaction including cell biomass production, which is shown in Equation 4 (Strous, Heijnen, Kuenen, & Jetten, 1998).

$$1.0\ NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+$$
$$\rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O \qquad \text{Equation 4}$$

Therefore, during the PN/A process, ammonium is partially oxidized to nitrite by AOB first, and the nitrite being produced along with the residual ammonium would be consumed by anammox bacteria to generate nitrogen gas. No additional external carbon is needed to finish the whole process. Since the PA/N Process was first discovered in 1990 (Mulder, Van de Graaf, Robertson, & Kuenen, 1995), it has attracted increasing attention because it reduces about 60% oxygen demand for nitrification, which translates to savings up to 90% of the operating costs for wastewater treatment plants (Jetten et al., 2001).

However, several inhibitory substances (organic matter, salts, heavy metal and likewise), operation conditions, sludge structure, temperature, dissolved oxygen, and pH would significantly affect the anammox process performance and the treatment outcomes (Jin, Yang, Yu, & Zheng, 2012). Plus, the competition with other bacteria for space, food and oxygen can also affect the activities of the anammox bacteria. Even though this promising technique was discovered more than three decades ago, there are still many issues that need to be solved, such as the difficulties in maintaining anammox activity to a certain level and in effectively suppressing the growth of NOB (Qiu et al., 2020). Besides, as shown in Equation 4, during the PN/A process, 0.26 mol of NO_3^-, which is toxic to wildlife and human health, can be generated when 2.32 mol of NH_4^+ is consumed. Thus, there exists a possibility that a notable amount of nitrate (11.2%) may be produced during the PN/A treatment, which is then discharged to the environment if no further treatments are taken.

1.3.3. Simultaneous partial nitrification, anammox, and denitrification process

The PN/A process can also be coupled with the partial nitrification process, through which nitrate is partially reduced to nitrite. This means that the nitrite being produced can simultaneously serve as "food" for the anammox bacteria, and the inhibition effect of carbon source limitation during the anammox process can be partially alleviated.

Simultaneous partial nitrification, anammox, and denitrification (SNAD) was a process first presented by Chen et al. (2009), which was found to be capable of achieving complete nitrogen removal (Lan, Kumar, Wang, & Lin, 2011). In addition, during denitrification, up to 40% of overall biological oxygen demand (BOD)/chemical oxygen demand (COD) consumption is caused by partial denitrification (Qiu et al., 2020), so the SNAD process is able to reduce part of carbon content in the wastewater. Shortly after its debut, a full-scale landfill-leachate treatment plant using the SNAD process was built in Taiwan (C.-C. Wang et al., 2010) and demonstrated the capability of treating ammonium rich, high strength wastewater, with the removal rates for COD, total nitrogen, and NH^+-N being 28, 68, and 80%, respectively. Investigations of the SNAD process for different wastewater streams using different reactor configurations continue to make headway in recent years (D. Chen et al., 2019; Lan et al., 2011; T. Liu et al., 2017).

1.3.4. Comammox process

Complete ammonia oxidation (comammox) was first observed and demonstrated to be feasible in ammonium removal by Costa et al. (2006). Then, Daims et al. (2015) discovered and cultivated a completely nitrifying bacterium that belonged to the genus *Nitrospira*, and found that it not only contained nitrite oxidoreductase (NXR), but also possessed enzymes for ammonia oxidation such as ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO), which could enable the single cell to self-transform NH_4^+ directly to NO_3^-. Moving forward, Kits et al. (2017) successfully isolated a pure culture of a comammox bacterium (*Nitrospira inopinata*) from a biofilm on the surface of a hot-water-covered-pipe and its ammonia oxidation kinetics indicated its higher ammonia affinity than AOB and ammonia-oxidizing archaea (AOA) in their study under substrate deficient condition. In the meantime, a recent study found that a comammox bacterium, *Candidatus Nitrospira nitrosa*, could be enriched in oxygen deficient systems (Camejo, Santo Domingo, McMahon, & Noguera, 2017). Since *Nitrospira*-like bacteria are among the most diverse and widespread nitrifiers in natural ecosystems and the dominant nitrite oxidizers in wastewater treatment plants, it can be postulated that the comammox process may have played a role in ammonia removal in these environments for a long time and is only discovered by the researchers most recently.

1.4. Parameters affecting the anammox process

Reviewing literature shows that there have been few investigations into reducing the nitrogen content of real poultry litter through PN/A by varying multiple operating factors and their optimization. The bulk of research in the available literature is limited to examining only one or two parameters at a time. Therefore, in this study, the focus will be placed on investigating the effect of three main operating parameters, i.e., C/N ratio, dissolved oxygen (DO), and hydraulic retention time (HRT), on the PN/A performance in removing ammonium from poultry litter.

C/N ratio is one of the crucial parameters in the PN/A process as increasing the C/N ratio would decrease the abundance of anammox while boost the growth of heterotrophic bacteria, which may inevitably lead to the simultaneous partial nitritation, anaerobic ammonium oxidation and denitritation process (SNAD) (Al-Hazmi et al., 2019). An organic carbon source is a necessity for heterotrophic denitrifiers, while the inorganic carbon (CO_2) may participate in microbial metabolisms and affect denitrification mechanisms in autotrophic denitrifiers (Xing et al., 2020). Since inorganic carbon (IC) is essential to most of autotrophic bacteria such as anammox bacteria, it is usually added to the process to balance the C/N ratio and avoid the prevalent nitrogen content in the system. Jin et al. (2014) reported that addition of IC would enhance the performance of an anammox reactor in nitrogen removal, and an optimum HCO_3^-/total nitrogen ratio of 1.20 was recommended.

Dissolved oxygen (DO) level in the liquid is another key factor in the anammox process. Low DO concentration ($< 1.0\ mg \cdot L^{-1}$) is widely used in anammox applications, which can inhibit nitrite oxidizing bacteria (NOB) and prevent nitrate accumulation (Miao et al., 2016). Thus, the ammonia oxidizing bacteria (AOB) can compete with NOB for low oxygen affinity in the anaerobic process (Li et al., 2018; Sin et al., 2008). Although various experiments have been conducted to identify the most favorable DO level for the anammox process (Kwak, McCarty, Bae, Huang, & Lee, 2012; Pichel et al., 2019; W. Wang, Wang, Wang, Zhang, & Yan, 2019), there is no agreement on the best DO range because of the differences in the experimental environment such as the wastewater categories, reactor types, temperature and/or other parameters.

Hydraulic retention time (HRT) is a principal parameter in many wastewater treatment processes. Shorter HRTs with higher TN removal is the optimal condition that the operators want to obtain. However, the TN removal efficiency is related to the bacterial composition, bacterial activity, and other environmental and operating factors. The optimal HRT range must be determined on a case-by-case basis. In this study, pretrials of HRT will be conducted according to the data from the start-up performance of the anammox reactor to determine the appropriate HRT range for the subsequent experiments.

1.5. Objectives of this research

This project is aimed at finding a proper treatment scheme of a one-stage partial nitrification and anammox reactor for poultry litter wastewater containing high content of ammonium nitrogen. DO, HRT, and C/N ratio were three parameters investigated in this study. Central Composite Design (CCD) coupled with Response Surface Methodology (RSM) were used to optimize the removal rates for ammonium nitrogen (NH_4^+-N) and total nitrogen (TN). A quadratic regression model was generated by the CCD/RSM experimental design using software JMP and the experimental data, which appropriately described the performance of the reactor in ammonium removal under different combinations of the three controlling parameters. Besides, uncertainty analysis was also performed to determine the system error of the model in accurately predicting the changes in response variables when varying the controlling parameters. To understand the bacterial makeup in the anammox sludge, 16S ribosomal ribonucleic acid (rRNA) high-throughput sequencing approach was employed to reveal the bacteria composition by targeting Variable region 4 (V4). The sequencing reads was processed by software Mothur and Microsoft Excel to delineate bacterial structures. Comparisons of bacterial sequencing results between this project and those from previous studies were also presented.

Chapter 2 Material and Methods

2.1 Synthetic wastewater

Raw chicken litter was collected from a chicken farm at the University of Arkansas. The experimental substrate with 0.2% total solid content was prepared by adding 0.2 g poultry litter to one liter tap water. The average nutrient content of poultry litter used in this study was shown in **Table 1**. The prepared substrate was stored in a feeding tank (8L).

Table 1 . Average nutrient content of poultry litter sample.

C (%)	N (%)	P (%)	C/N ratio
29.77	3.08	1	9.72

The concentrations of NH_4^+–N and total carbon in the poultry litter wastewater were adjusted through adding ammonium chloride (NH_4Cl_2) and sodium bicarbonate ($NaHCO_3$), respectively. The mineral medium added to the synthetic wastewater consisted of (per L): 0.027 g KH_2PO_4, 0.009 g $FeSO_4 \times 7H_2O$, 0.005 g EDTA, 0.24 g $MgSO_4 \times 7H_2O$, 0.143 g $CaCl_2 \times 2H_2O$ and 0.3 mL trace elements solution. The trace element solution was composed of (per L): 1.247 g $ZnSO_4 \times 7H_2O$, 1.119 g $MnCl_2 \times 4H_2O$, 0.044 g $CuSO_4 \times 5H_2O$, 0.2015 g $Al_2(SO_4)_3 \times 14H_2O$, 0.03 g $CoCl_2 \times 6H_2O$, and 0.1 g KCl, and 0.975 g EDTA. The mineral medium and trace element solution were prepared according to Magrí et al. (2012).

2.2 Analytical methods

The elemental composition (C, N, O, H, P, S) of poultry litter was analyzed using an elemental analyzer. The pH and oxidation-reduction potential (ORP) were determined potentiometrically using a digital pH meter (Luoyang Guantuo Electronic Technology Co., Ltd., pH-101, China) and an ORP meter (Jinan Advantech Analytical Instrument Co. Ltd., pH-501, China). The concentration of DO was measured with a digital, portable DO meter (Remondaoto, RMD-ISDT10, Utah), and the monitored data was collected by a datalogger (Campbell Scientific, CR1000X, USA). The synthetic wastewater was prepared every two days and analyzed for nitrogen and COD before use. Effluent samples were taken daily for ammonium, nitrite, nitrate, total nitrogen (TN) and total COD analyses. All the nitrogen species (NH_4^+-N, NO_2^--N, NO_3^--N), TN and COD were measured using Hach vials (catalog#: TNT 830, TNT 835, TNT 839, and TNT 822) with a Hach DR 3900 spectrophotometer (Hach Lange GmbH) according to the manufacturer's protocol. The removal efficiencies of ammonium nitrogen and total nitrogen were obtained using the following equation:

$$\text{N removal efficiency (\%)} = \frac{Ci-Cf}{Ci} * 100 \qquad \text{Equation 5}$$

where C_i and C_f are the initial and final concentrations of ammonium or total nitrogen (mg L^{-1}), respectively.

2.3 Seed Sludge and Reactor start-up

The inoculated sludge included the ANAMMOX biomass, Brocadia caroliniensis (NRRL B-50286) (**Vanotti Matias B**., 2013), and the high performance nitrifying sludge (HPNS) (NRRL B-50298) (Vanotti, Szogi, & Ducey, 2013), which were obtained from the USDA/ARS Coastal Plains Soil, Water, and Plant Research Center at Florence, SC. The start-up performance of the reactor was presented in **Figure 1**. The sequencing batch biofilm reactor (SBBR) was first seeded with 500 mL anammox sludge in phase I and was cultured using synthetic wastewater with equal amounts of ammonium and nitrite (100 mg/L). The reactor was maintained at a constant temperature of 35 °C by a magnetic hot plate with a stirrer, and the pH was maintained between 7.8 and 8.5. Nitrogen gas was passed through the reactor to maintain anoxic condition. As shown in phase I in **Figure 1**, the reactor entered a stable condition after two weeks of operation. The effluent concentrations of NH_4^+-N and NO_2^--N gradually decreased from 77.5 and 77.2 mg L^{-1} to 23.25 and 27.4 mg L^{-1}, respectively. The effluent NO_3^--N concentration was lower than 12 mg L^{-1}.

In phase II (**Fig. 1**), 170 mL of HPNS was mixed with the anammox sludge. The reactor was fed with the adjusted influent (140 mg/L ammonium and 20 mg/L nitrite) continuously at a hydraulic retention time (HRT) of 48 hours under an aerobic/anaerobic intermittent cycle (min : min) and a constant dissolved oxygen (DO) level (14 : 46 and 0.2 mg L^{-1}), respectively. It took 2 weeks to achieve the stable partial nitrification anammox process. During phase II, the effluent NH_4^+-N concentration decreased from 47.2 to 20.3 mg L^{-1} and the average effluent NO_2^--N and NO_3^--N concentrations were lower than 13 mg L^{-1}.

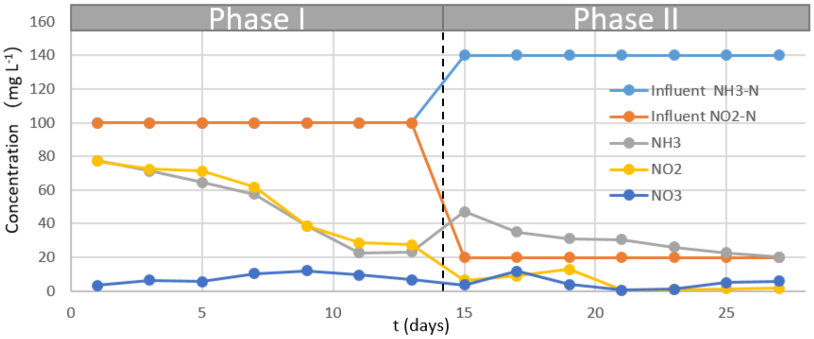

Figure 1. The Start-up performance of the reactor

2.4 Operation strategy

As shown in **Figure 2 (b)(c)**, a 7 L lab-scale sequencing batch biofilm reactor (SBBR) with a working volume of 5 L was used in this study. The SBBR was equipped with influent/effluent discharging and an air supply subsystem. After 4 weeks of stabilization period, experiments were run according to the experimental design (see below) to obtain the responses, i.e., the TN and NH_4^+-N removal rates under room temperature (~25 °C). The pH was maintained from 7.5 to 8.5

by dosing sodium hydroxide solution. Two peristaltic pumps (Cole-Parmer, UX-77921-65, USA) were operated at a timed dispensing mode for feeding and discharging, respectively. Mixers were installed in the influent tank and SBBR for complete mixing at a rate of around 160 rpm during the feeding and reaction periods to ensure the whole content was homogeneous. Timers (**Figure 2 (a)**) were connected to the wires of mixers to control the working time. The timed dispensing mode and timers were adjusted according to the selected HRT of experimental runs.

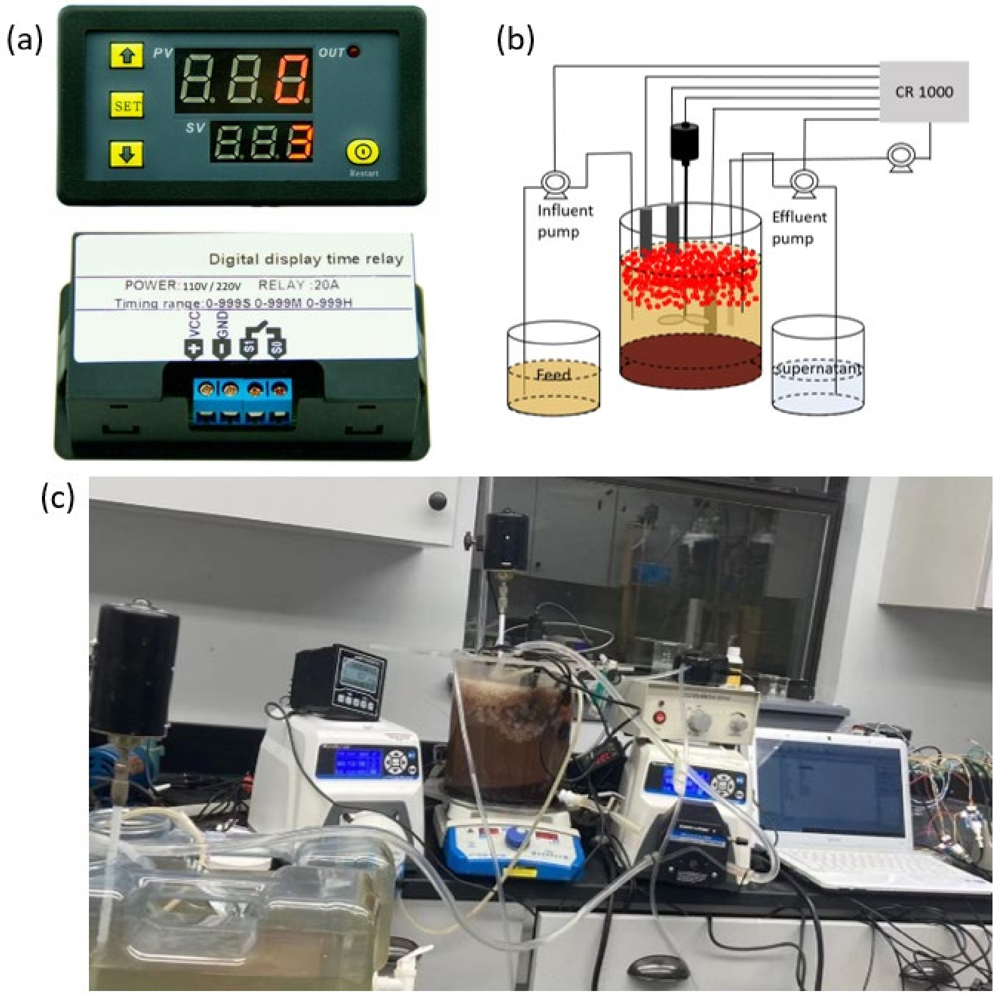

Figure 2. (a) Time relay, (b) reactor schematic and (c) complete system setup in the laboratory

The intermittent aeration cycle was controlled by a timer, and the aeration rate was measured by a gas flowmeter. The timer was connected to the wires on the air pump. As shown in **Figure 3**, there were four recurring cycles within each HRT, and the number of aerobic/anaerobic intermittent cycles (aeration on for 14 min and off for 46 min) within recurring cycles were changed according to the preset HRT of experiments. During each intermittent cycle, aeration was achieved by dispersing air through the liquid via an aeration stone, which was connected to an air pump and located at the bottom of the reactor.

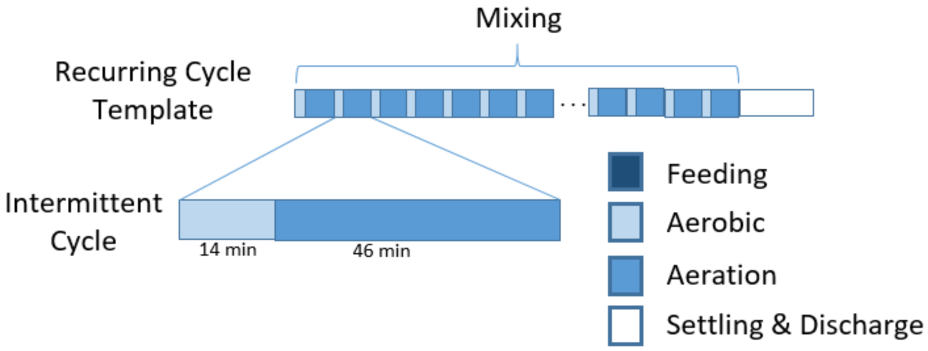

Figure 3. Operation logic diagram

The air pump was connected to a relay switch (SparkFun Electronics, COM-14236-MD, USA), which was connected to the datalogger. During the aerobic period, when the DO concentration reached the programed upper limit, the datalogger would control the relay switch and cut off the power of the air pump. Likewise, as the aerobic bacteria kept on consuming the oxygen in the system, the DO concentration would gradually decrease to the programmed lower limit, and the air pump would be turned on.

2.5 Experimental design

In this study, C/N ratio, DO, and HRT were selected as the three independent variables to determine their effects on the performance of the SBBR reactor in nitrogen removal. As shown in

Table 2, each independent variable was varied over three levels between - 1 and + 1, i.e., 1, 2, and 3 for C/N ratio, 0.2, 0.35, and 0.5 for DO (mg L^{-1}), and 24, 48, and 72 for HRT (h). These levels were chosen based on start-up performance of the reactor. Experimental design was carried out using the response surface methodology (RSM) through Central Composite Design (CCD) in the statistical software JMP to determine the optimal combination of these three independent variables. The software JMP pro 15 (SAS Institute, Cary, NC, USA) was used to generate the experimental runs of CCD/RSM, and a total of 16 experiments for the three factors were conducted (= 2k + 2k+1), where k is the number of factors (k = 3).

Table 2. Independent Variables and their levels for the central composite design

Factors	Unit	Code	Levels		
			Low (-1)	Center (0)	High (+1)
DO	mg/L	X_1	0.2	0.35	0.5
HRT	h	X_2	24	48	72
C/N ratio	-	X_3	1	2	3

2.6 Microbial activity tests

Samples used for microbial community analysis were collected from the seed sludge and the biomass in the reactor (day1 and day 96). DNA extractions were performed using the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Germantown, MD, USA) as per the manufacturer's protocol. The extracted DNA was diluted to 10 ng/µL and quantified by a NanoDrop One Microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA). The fourth hypervariable (V4) region of 16S ribosomal RNA (rRNA) was amplified from each sample using forward primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′) tailed with the Illumina sequencing primer and barcode sequence, respectively. The cycling conditions consisted of 35 cycles of denaturation at 95 °C for

30 s, annealing at 55 °C for 30 s, and extension for 60 s at 72 °C, followed by a final extension step at 72 °C for 5 min. Polymerase chain reaction (PCR) amplicons were purified and normalized to be equimolar with the SequalPrep Normalization Plate Kit (Invitrogen, Carlsbad, CA, USA). The quantity and quality of the library were determined by KAPA Illumina Library Quantification Kits (Roche, Indianapolis, IN, USA) and an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), respectively. Then, the library was sequenced on an Illumina MiSeq sequencer using the MiSeq Reagent Kit v2 (Illumina, San Diego, CA, USA) to obtain 2 × 250 bp paired-end sequences.

Mothur v1.39.5 was used to process the sequencing reads, which clustered the sequences into Operational taxonomic units (OTUs) at 97% sequence identity. The OTUs were then classified against the RDP (Ribosomal Database Project) database, and the analysis of similarity (ANOSIM) was performed through Mothur to evaluate the dissimilarity between groups.

Chapter 3 Results and Discussions

Total nitrogen removal rate is the vital parameter in evaluating the anammox process performance because it can directly reflect the efficiency of anammox bacteria in the system. To investigate the most favorable condition of PN/A to remove the nitrogen in the poultry litter wastewater, no COD was introduced in the reactor except the small amount of organic carbon brought in from the poultry litter. Besides, the organic carbon needs to be broken down by other heterotrophic bacteria first before it can be used by denitrifying bacteria. Therefore, the total nitrogen removal rate caused by denitrifiers can be ignored.

3.1 Analysis of response surface methodology

Table 3 presented the NH_4^+-N and TN removal rates of the random experimental runs generated by Design of Experiments (DOE) in JMP, and the results were rearranged in a chronological order. During the whole experimental runs, influent NH_4^+-N and TN were constantly kept around 170 mg/L and 200 mg/L, respectively. The optimal running conditions for the anammox reactor found in the experimental runs for NH_4^+-N and TN removal were 100% and 87.3%, respectively, when C/N ratio, DO, and HRT were kept at 1, 0.5 mg/L, and 72 h.

Yue et al. (2018) reported that the rise in DO level from 0.3 to 1.8 mg/L would promote the increase in abundance of NOB, and thus lead to the decrease in nitrogen removal rate. However, in this study, when the HRT and C/N ratio were kept constant, the TN removal rate varied directly with DO concentration in the aerobic phase. Take phase 1 and phase 2 for example, when DO decreased from 0.5 in phase 1 to 0.2 mg L^{-1} in phase 2, the TN removal rate decreased from 87.3% to 76.1%. Similar observations were also obtained between phase 3 and 4, phase 9 and 10, phase 12 and 13, and phase 14 and 15. The increase of nitrogen removal rate even at a higher DO may be because of the long anaerobic time (W. Wang et al., 2019; Yang, Trela, Zubrowska-Sudol, &

Plaza, 2015) and low organic loading to the reactor. Previous study (Zhang et al., 2017) showed that NOB would be suppressed by organic carbon to nitrogen ratios lower than 1.0 and the low total solid (0.2 g L^{-1}) in this study. Besides, Xu et al. (2012) reported that hydroxylamine, as an intermediate of partial nitrification, could selectively hinder the growth of NOB. Although the comparison between phase 5 and phase 15 appeared to contradict the observed trend, it could be linked to the bacterial changes in the reactor culture, and more detailed discussion would be presented about this topic later in Section 3.3.

Table 3. Experimental runs using Central Composite Design for partial nitrification anammox process in JMP software.

phase	C/N ratio	DO (mg/L)	HRT (h)	NH_4^+-N removal rate (%)	TN removal rate (%)
1	1	0.5	72	100*	87.3
2	1	0.2	72	100*	76.1
3	1	0.2	24	63.7	61.7
4	1	0.5	24	89.7	65.5
5	2	0.35	48	100*	67.3
6	2	0.35	24	96.3	49.7
7	2	0.35	72	100*	67.0
8	2	0.35	48	100*	62.0
9	3	0.2	24	71.2	45.9
10	3	0.5	24	78.9	53.7
11	3	0.35	48	100*	57.1
12	3	0.2	72	100*	61.4
13	3	0.5	72	100*	63.5
14	2	0.2	48	99.3	59.9
15	2	0.5	48	99.8	64.0
16	1	0.35	48	100*	71.8

Note: 100* indicated that the kits cannot detect NH_4^+-N in the effluent or the concentration of NH_4^+-N exceeded lower limit of the instrument.

When HRT and DO were kept unchanged, the TN removal rate was inversely proportional to C/N ratio, as indicated by the data between phase 5 and 16 in Table 3. Even though the bacterial composition in the liquid changed substantially, the lower C/N ratio still gave rise to a high TN removal efficiency. This result is in good agreement with the result obtained from a study conducted by Mousavi et al. (2018), where inorganic carbon to nitrogen (IC/N) ratio was found to play a critical role in the elimination of total nitrogen. However, Zhang et al. (2016) reported a different optimal IC/N ratio of 2.0 than the one found in this study. In their study, the bioactivity of both AOB and anammox decreased due to the limited amount of IC provided, and the TN removal efficiency would continuously decrease with the decrease of IC/N ratio from 2. That might be caused by the differences in microbial flora present in the two studies because the species, *Nitrosomonas europaea* and *Candidatus Kuenenia stuttgartiensis*, in their study were found to be severe inhibited in IC deficient condition, while those species were not detected in this study.

TN removal efficiency was also proportional to HRT when DO and C/N ratios were set constant, because the anammox community continued to require nitrite and ammonium to maintain their metabolism. A full-scale partial nitrification anammox process to treat anaerobically digested poultry manure wastewater, which required the influent nitrogen range of 20-50 mg/L to protect the reactor, was reported to have the HRT higher than 19 hours (Alejo-Alvarez, Guzmán-Fierro, Fernández, & Roeckel, 2016). Although their study did not show the optimal HRT for the treatment, the trend that higher HRTs would result in better TN removal rates was consistent with the findings obtained from this study. As shown in Table 3, the higher TN removal efficiencies were always associated with longer HRTs, and a substantial increase in TN removal rate was clearly seen for HRT = 72 h as compared to HRT = 24 h. Due to the limited selection of HRTs tested in this study, it might be inferred that higher TN removal rates could still be achieved if longer HRTs than the ones used herein were employed. However, it has to be recognized that longer HRTs can cut short the treatment throughput capacity, which increases the cost of operation. More research is needed to determine the optimal HRTs for the PN/A process without sacrificing the treatment capacity.

Differently, the removal rate of NH_4^+-N, a component of total nitrogen, could reach 99% or above when the HRT was kept at 48 hours in the experimental runs (**Table *3***). Since both nitrifiers (AOB and comammox) and anammox bacteria needed NH_4^+-N for their metabolic activities, high NH_4^+-N removal rates could be achieved with a shorter HRT than that needed for total nitrogen removal. This finding is much better than the previously reported data. One study showed that an HRT of 9 days was needed to achieve 96% NH_4^+-N removal, which was much longer than 48 h used in this study (Lan et al., 2011). Besides, the conclusion - higher ammonium removal rate can be achieved when DO equals 0.5 mg L^{-1} - can be drawn by comparing phase 3 and phase 4, phase 9 and phase 10, and phase 14 and phase 15. Unfortunately, the relationship between C/N ratio and ammonium removal rate could not be established directly using the experimental results from this study, which needs further investigation of the bacterial structure in the reactor.

According to the removal efficiencies of NH_4^+-N and TN and under different C/N ratio, DO and HRT, CCD/RSM was used to establish the regression equation of different responses in the PN/A system. Referring to ANOVA (analysis of variance), the results of the evaluation were presented in **Table 4** and **Table 5**. For the ammonium removal rate model, $F = 4.6423$ and $p = 0.0377$, indicating that the model developed by CCD/RSM for the experiment was significant (< 0.05). Thus, the model can be used to accurately predict the removal of ammonium nitrogen under different operating conditions (C/N ratio, DO, and HRT).

However, DO and C/N ratio were found to be insignificant parameters to impact ammonium removal when compared to HRT. Since DO is an important parameter to support the anammox process, as long as there is AOB and oxygen in the system, ammonium would be consumed by AOB eventually. That said, DO could also be an inhibiting factor to the anammox process as well. One past studied showed that the highest specific nitrogen removal rate was actually observed under non-aerated conditions, resulting in the nitrogen removal efficiency of 81.6% (Yin et al., 2016). Although nitrogen removal was readily inhibited under aerated conditions, an increased anammox activity occurred at the DO concentration of 0.5 mg O_2 L^{-1}. This is in contrast with the directional DO suppression on nitrogen removal in the anammox process, indicating that other nitrogen conversion pathways, such as nitrification and endogenous denitrification, could also be active. These findings were consistent with those from this study. Besides, as the DO was controlled within a certain range and the air pump was controlled according to the DO probe feedback from the reactor, the moderate sensitivity of the DO probe (slightly longer response time) made the measured values lag behind the real values, leading to the measured DO concentrations moving outside the set ranges including overlapping regions among preset ranges. Therefore, the DO measurement and control during the experiments might not be accurate, and the measured DO values might not represent the instant DO concentration in the entire reactor even though the reactor was stirred completely. This could be the reason for the ANOVA analysis that indicated that DO was an insignificant parameter. In addition, as most ammonium removal rates of the experimental runs in this study have reached 100% when the HRT is long enough, e.g., 48 h or 72 h, the effect represented by DO and C/N ratio in the regression model on the nitrogen removal rate may not be accurately reflected. More in-depth research is certainly warranted.

As shown in **Figure 4**, the prediction profiler constructed by JMP visualizes how the response surface changes with the input variables over its range and how the levels of DO, C/N ratio, and HRT affect each other. For example, for the TN removal rate, the DO and HRT have a positive impact in contrast to the C/N ratio, which has a negative impact.

Table 4: ANOVA analysis of NH_4^+-N removal efficiency variance table. (√, significant; ×, not significant.)

Source	d.f.	sum of squares	mean squares	F Ratio	Prob > F	
Model	9	1813.4	201.49	4.6423	0.0377	√
X_1-DO	1	116.96	116.96	2.6948	0.1518	×
X_2-HRT	1	1004	1004	23.132	0.003	√
X_3-C/N ratio	1	1.089	1.089	0.0251	0.8793	×
X_1X_2	1	141.96	141.96	3.2708	0.1205	×
X_1X_3	1	41.861	41.861	0.9645	0.364	×
X_2X_3	1	1.3612	1.361	0.0314	0.8653	×
X_1^2	1	53.141	53.141	1.2244	0.3109	×
X_2^2	1	141.96	141.96	3.2708	0.1205	×
X_3^2	1	41.861	41.861	0.9645	0.364	×
Lack of fit	5	260.42	52.084			×
Pure Error	1	0	0			
Residual	6	260.42				
C. Total	15	2073.8				
R^2		0.874426				

Even though DO and C/N ratio was not significant, the model is significant. Since DO and C/N ratio were independent variables on this model, they still indicated from **Figure 4** that, if the ammonium nitrogen was fully eliminated, the shortest HRT was around 39.88 hours, under which the total nitrogen removal rate was around 62.85%.

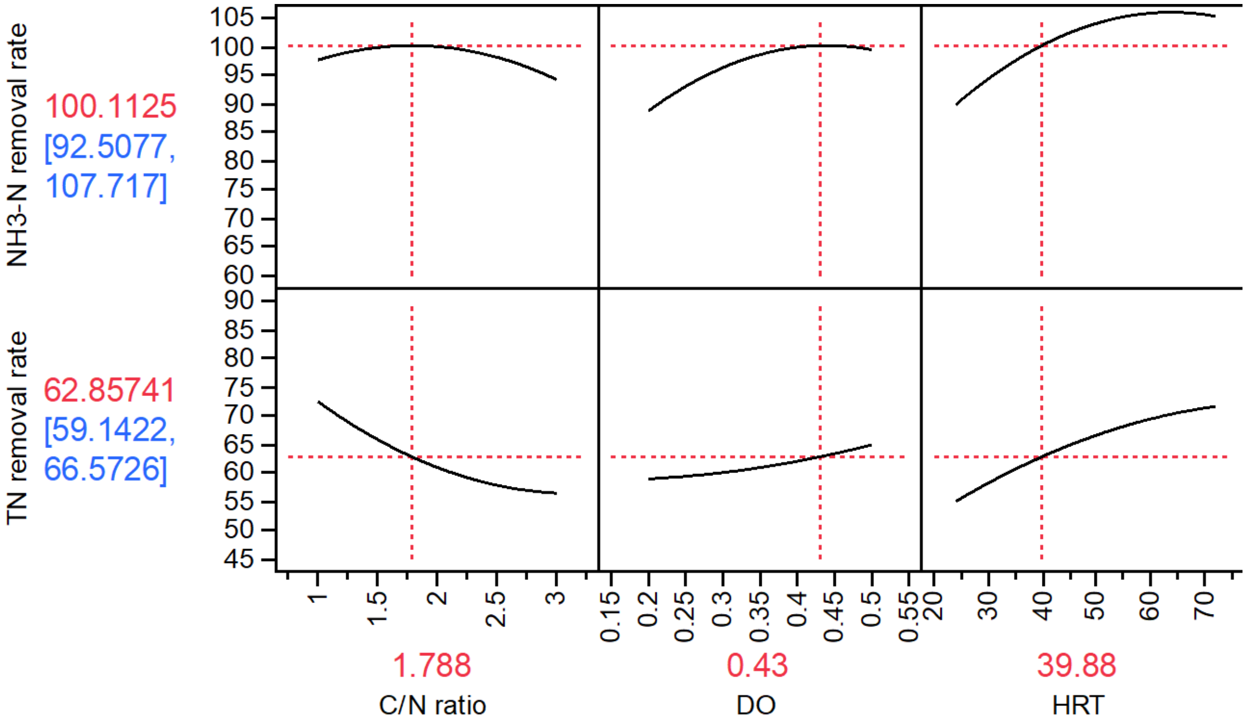

Figure 4. Prediction Profiler when ammonium nitrogen is fully eliminated (shortest HRT)

For the TN removal efficiency model with F = 15.2599 and p = 0.0018, it could be concluded that the model generated by CCD/RSM in the experiment was extremely significant. In this model, a quadratic polynomial equation was acquired by JMP:

$$
\begin{aligned}
TN\ (\%) = {} & 55.8178 + 19.3333 * X_1 + 0.3283 * X_2 - 8.08 * X_3 + (X_1 - 0.35) \\
& * (0.0590 * (X_2 - 48)) + (X_1 - 0.35) * \big((X_3 - 2) * -4.25\big) \\
& + (X_2 - 48) * \big((X_3 - 2) * -0.0568\big) + 44.3678 \\
& * (X_1 - 0.35)^2 - 0.0045 * (X_2 - 48)^2 + 3.4983 * (X_3 - 2)^2
\end{aligned}
\qquad \text{Equation 6}
$$

The corresponding items (X_1, X_2, and X_3) in the model all had $p < 0.05$, indicating that they had an extremely significant influence on the TN removal efficiency. As shown in **Figure 5** (**a**), R^2 was at 0.958, indicating that approximately 95.8% of the observed variation could be explained by the grouping variable and that the actual TN removal rate of the experiment had a good linear relationship with the predicted values. In **Figure 5** (b), (c), (d), the x-axis and y-axis represented HRT, C/N, or DO and the z-axis represented the TN removal efficiency with one variable kept at constant in each plot while the other two varied within the experimental ranges. According to **Figure 4** and **Figure 5**, the total nitrogen removal rate increased with the increase in HRT and DO, but with the decrease of HRT. This observation was the same as the conclusion reached from **Table 3**. Besides, the total nitrogen remove efficiency was strongly affected by HRT and C/ N ratio according to the prevailing direction from data in **Figure 4** and **Figure 5**.

Table 5 Analysis of TN removal efficiency variance table. √, significant; ×, not significant.

Source	d.f.	sum of squares	mean squares	F Ratio	Prob > F	
model	9	1422.6618	158.074	15.2599	0.0018	√
X_1-DO	1	84.1	84.1	8.1187	0.0292	√
X_2-HRT	1	620.944	620.944	59.9439	0.0002	√
X_3-C/N ratio	1	652.864	652.864	63.0253	0.0002	√
X_1X_2	1	0.36125	0.3613	0.0349	0.858	×
X_1X_3	1	3.25125	3.2512	0.3139	0.5956	×
X_2X_3	1	14.85125	14.8512	1.4337	0.2763	×
X_1^2	1	2.62728	2.6273	0.2536	0.6325	×
X_2^2	1	17.84546	17.8455	1.7227	0.2373	×
X_3^2	1	32.26364	32.2636	3.1146	0.128	×
Lack of fit	5	48.10756	9.6215	0.685	0.719	×
Pure Error	1	14.045	14.045			
Residual	6	62.152560				
C. Total	15	1484.8144				
R^2		0.958141				

However, the CCD/RSM results indicated that the limits of the running parameters chosen for the experiments were not able to capture the actual optimal C/N ratio for total nitrogen removal rate. As shown in Figure 5 b, c, and d, no maximal TN removal rates could be determined due to the limitation presented by the upper ranges of the three operating parameters selected because it clearly indicates that the optimal values of these parameters fall outside these ranges. Therefore, continued research is needed to investigate the anammox process to determine the optimal running parameters for the anammox reactor for ammonium removal from the poultry litter.

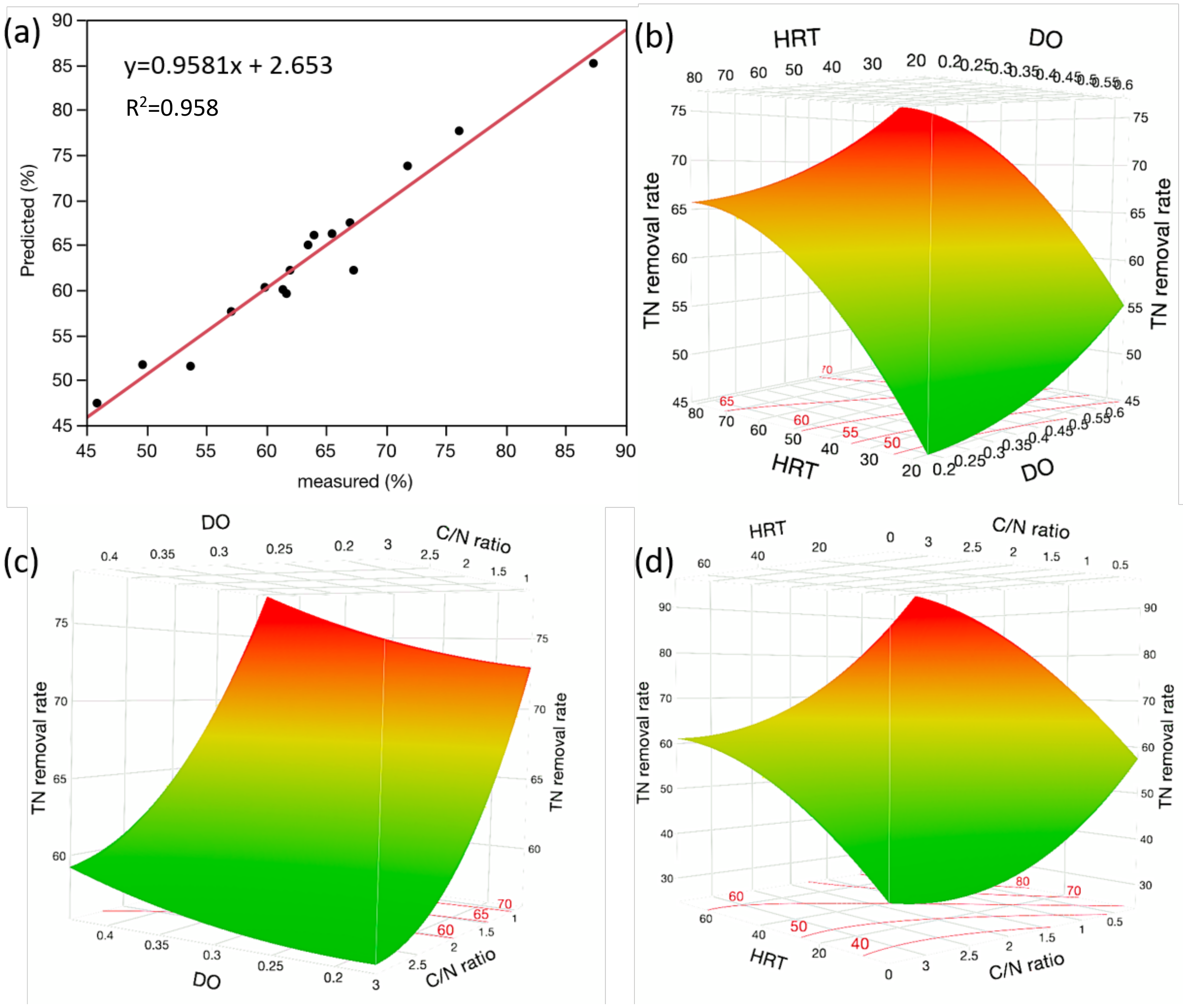

Figure 5 (a) Relationship between the measured and predicted TN removal efficiency; response 3D surface plot for TN removal efficiency: (b) HRT-DO, (c) DO-C/N ratio, (d) HRT- C/N ratio.

3.2 Uncertainty analysis

In order to examine the performance of regression equation in predicting the TN removal rate, the uncertainty analysis was conducted through Linearized Approximation Method. The system uncertainty of this model can be linearly approximated as shown in Equation 7 (Coleman & Steele, 2018).

$$\Delta r \approx \sum_{i=1}^{n} \frac{\partial z}{\partial x_i} \Delta x_i \qquad \text{Equation 7}$$

where Δr is the error in total nitrogen removal rate (%) caused by the experimental errors of three variables in Equation **6**, i.e., C/N ratio, DO, and HRT (n=3).

The experimental errors in this study were assumed to originate from the measurement inaccuracy of the instrument used. The DO meter and the timer had accuracy limits of ± 0.2% and ± 0.1%, respectively, and the analytical balance (VWR, ALW204, USA) used also generated the measurement errors of chemicals (i.e., NH_4Cl_2 and $NaHCO_3$) that were added to the substrate to adjust the C/N ratio, which had a measurement error of ± 0.2%. In order to determine the largest systematic error in this study, the worst-case scenario was assumed, i.e., the measurements of $NaHCO_3$ had a deviation of + 0.2%, and the measurements of NH_4Cl_2 had a deviation of – 0.2%. Thus, the range of systematic error of C/N ratio could be assumed to be within ± 1.2%. Expanding Equation 7 gave the following Equation 8.

$$\begin{aligned}\Delta \mathrm{r} \approx\ & (0.0590 * \mathrm{X}_2 - 4.25 * \mathrm{X}_3 + 88.7356 * \mathrm{X}_1 - 0.6056) * \Delta \mathrm{x}_1 \\ & + (0.8533 + 0.0590 * \mathrm{X}_1 - 0.0568 * \mathrm{X}_3 - 0.0093 * \mathrm{X}_2) * \Delta \mathrm{x}_2 - (4.25 * \mathrm{X}_1 \\ & + 0.0568 * \mathrm{X}_2 - 6.9966 * \mathrm{X}_3 - 17.8593) * \Delta \mathrm{x}_3 \qquad \text{Equation 8}\end{aligned}$$

With Equation 8, Δr could be calculated for each experimental result in **Table 3** to estimate the deviations of TN removal rates. The calculated results were presented in **Table 6**. The systematic error for the model developed using CCD/RSM showed that it was able to predict the total nitrogen removal rate of the SBBR reactor within an error ranged from 0.03% to 2.81% of the modeled value for the three independent variables, i.e., DO (ranging from 0.2 to 0.5 mg/L), HRT (ranging from 24 to 72 h) and C/N ratio (ranging from 1 to 3), which could be regarded as reasonably accurate within the experimented ranges. As the worst-case scenario for C/N ratio was assumed during the calculation, the practical systematic error could be smaller than the range being calculated, which meant that the quadratic model generated by JMP in this study to estimate the TN removal rate was relatively accurate and would not be severely impacted by the instrumental systematic errors.

Table 6: Numerical results for calculating systematic errors for the regression model of TN removal rate.

Run	DO ± ΔDO concentration (mg/L)	HRT ± ΔHRT (h)	C/N ± ΔC/N	TN removal rate (%) model result (w/o biases)	Δr	Δr/TN removal rate
1	0.5 ± 0.001	72 ± 0.072	1 ± 0.012	85.15	0.2787	0.0033
2	0.2 ± 0.0004	72 ± 0.072	1 ± 0.012	77.65	0.2558	0.0033
3	0.2 ± 0.0004	24 ± 0.024	1 ± 0.012	59.59	0.2915	0.0049
4	0.5 ± 0.001	24 ± 0.024	1 ± 0.012	66.24	0.3118	0.0047
5	0.35 ± 0.0007	48 ± 0.048	2 ± 0.024	62.18	0.6957	0.0112
6	0.35 ± 0.0007	24 ± 0.024	2 ± 0.024	51.70	0.7253	0.0140
7	0.35 ± 0.0007	72 ± 0.072	2 ± 0.024	67.46	0.6555	0.0097
8	0.35 ± 0.0007	48 ± 0.048	2 ± 0.024	62.18	0.6957	0.0112
9	0.2 ± 0.0004	24 ± 0.024	3 ± 0.036	47.43	1.3325	0.0281
10	0.5 ± 0.001	24 ± 0.024	3 ± 0.036	51.53	1.3172	0.0256
11	0.35 ± 0.0007	48 ± 0.048	3 ± 0.036	57.60	1.2736	0.0221
12	0.2 ± 0.0004	72 ± 0.072	3 ± 0.036	60.04	1.2260	0.0204
13	0.5 ± 0.001	72 ± 0.072	3 ± 0.036	64.99	1.2132	0.0187
14	0.2 ± 0.0004	48 ± 0.048	2 ± 0.024	60.28	0.6979	0.0116
15	0.5 ± 0.001	48 ± 0.048	2 ± 0.024	66.08	0.7016	0.0106
16	0.35 ± 0.0007	48 ± 0.048	1 ± 0.012	73.76	0.2858	0.0039

3.3 Bacterial consortium analysis result

According to **Figure 6,** the samples showed many notable differences in microbial composition between the original mixed sludge and the sludge in the reactor of day 96. Similar to previous studies, the most dominant phylum of the sludge present in the SBBR was *Proteobacteria* (26.6%), which contained AOB, comammox and denitrifiers (Fan et al., 2020; X. Wang & Gao, 2018; Z. Xu, Zhang, Gao, & Peng, 2020). The dominant bacterial phyla such as *Chloroflexi* and *Bacteroidetes* were also reported in other studies but different in proportion because each reactor had its own characteristic bacterial community composition. Besides, due to the introduction of poultry litter, numerous bacterial groups were introduced into the reactor, thus leading to the changes in proportion of each phylum.

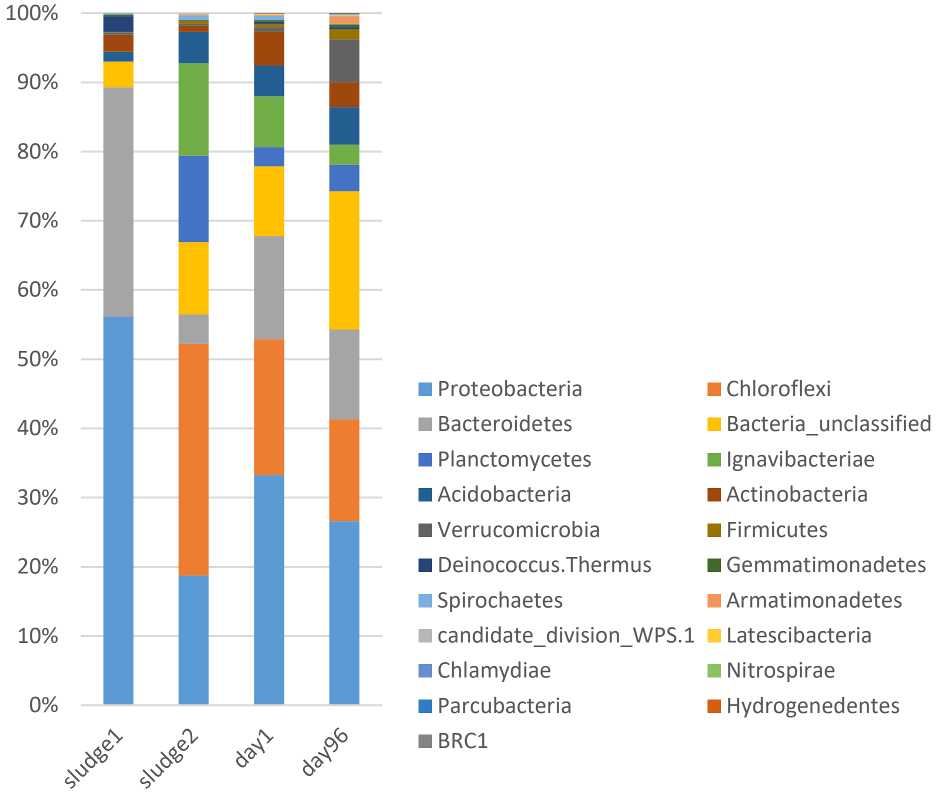

Figure 6. Relative abundance of phylum based on Illumina MiSeq bacterial 16S rRNA genes in ANAMMOX samples

Besides phylum, the bacterial diversity and abundance were also analyzed more specifically at genus level. A total of 919 Operational taxonomic units (OTUs) were observed in the activated sludge of the SBBR based on the 97% identity of 16S rRNA gene sequences. As shown in **Figure 7**, within phylum *Planctomycetes*, a family of AnAOB named *Candidatus Brocadiaceae* with an abundance of 1.18% was detected, which was similar to previous findings. In the single-stage partial nitrification anammox process, Liu et al. (2017) reported an abundance of 1.54% in the suspended flocs of a continuous stirred tank reactor (CSTR). AnAOB abundance of 1.1% was detected in a full-scale moving bed biofilm reactor (MBBR) in the study of Xu et al. (2018) during simultaneous partial nitrification, anammox and denitrification (SNAD) process.

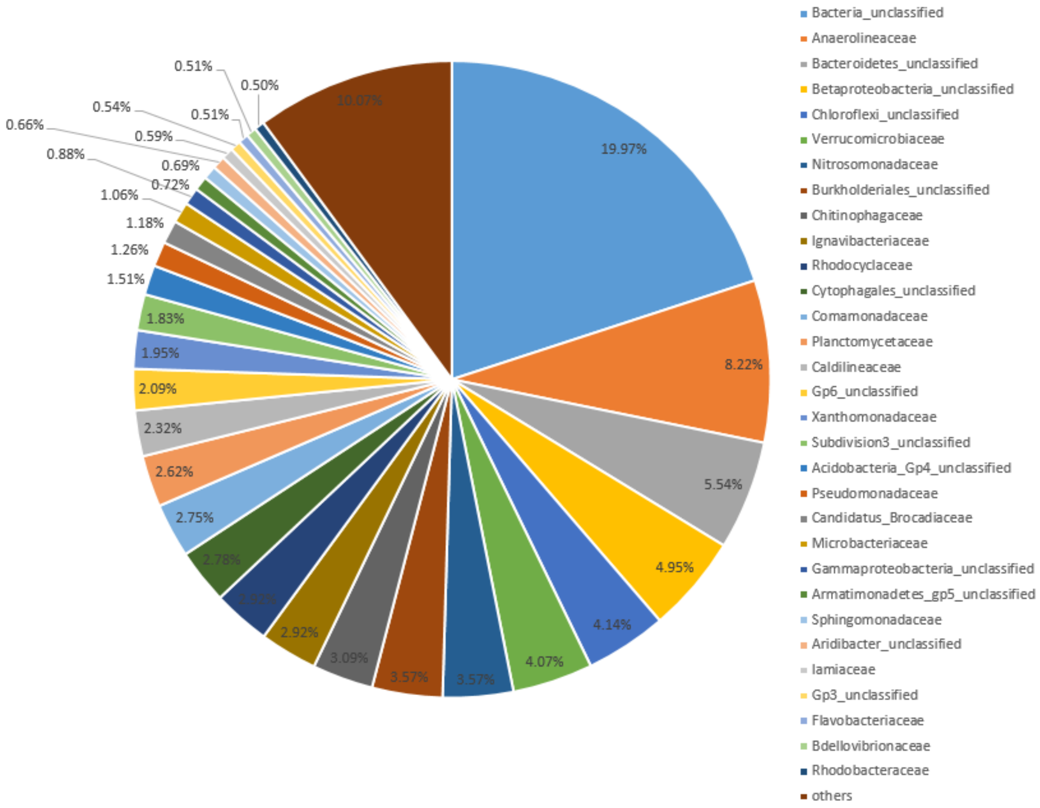

Figure 7. Relative abundance (%) of 16S rRNA gene sequences at the family level

Unclassified bacterial groups such as *Betaproteobacteria* (4.9%), *Nitrosomonadaceae* (3.6%), and Chitinophagaceae (3.1%) were dominating AOB in the bacterial communities (Gomez-Alvarez, Schrantz, Pressman, Speitel Jr, & Wahman, 2013; Roots et al., 2019). The abundance of family *Betaproteobacteria* and *Nitrosomonadaceae* decreased as the experiment proceeded, but the relatively abundance of *Nitrosomonadaceae* increased from 2.75% to 3.57%. The increasing proportion indicated that family *Nitrosomonadacea*, which belonged to *Proteobacteria* (Han, Li, & Liu, 2013), was readily to adapt to the poultry litter wastewater.

In this study, the anoxic period of 46 minutes, which was longer than the suggested anoxic time (at least 20 min) reported by Xu et al.(2020), resulted in the delay of NOB recovery. Besides, the concentration of DO was lower than 1 mg L^{-1}. The combination of these two conditions gave rise to the inhibition of NOB. Thus, no NOB was detected in the reactor except *Nitrospira* (0.06%), which, previously known as NOB, may also belongs to comammox (Daims, Lücker, & Wagner, 2016). **Figure 8** showed the changes of different forms of nitrogen in the reactor over time. The nitrate concentration was kept at very low in the effluent before phase 5 and rose up from 11.1 to 48.7 mg NO_3-N/L on average, which also indicated the presence of comammox activity. *Nitrospira* was only a small portion of the whole microbial community, which was expected because it was not even found in the seed sludge or the mixing sludge at the beginning. These results were consistent with those reported by Roots et al. (2019), who concluded that the cultivation of partial nitrification anammox bioprocesses in the mainstream might inadvertently select for comammox bacteria. In addition, Kessel et al. (2015) also reported that *Nitrospira* could tighten clustering with anammox bacteria under very low oxygen concentration conditions. The cooperation between PN/A and comammox process in the digestion liquid was also investigated (Wu et al., 2019), with the reported abundance of 18.89% for *Chitinophagaceae*, 0.10% for *Candidatus Kuenenia* and 0.2% for *Nitrospira* in a sequencing batch reactor (SBR).

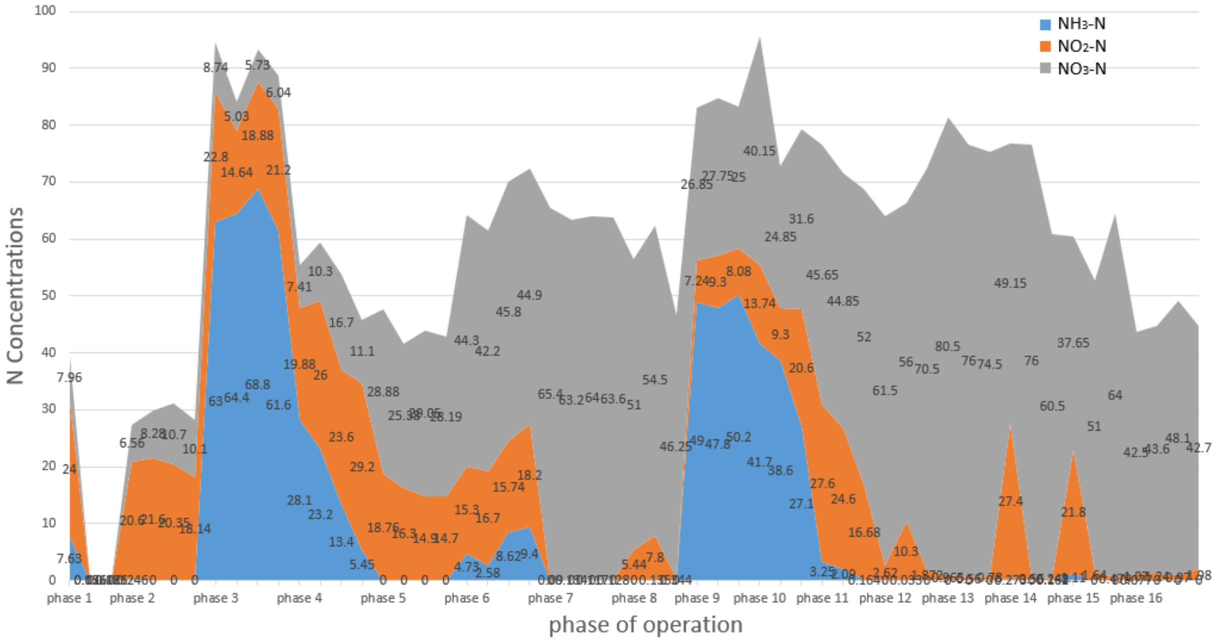

Figure 8. N Concentrations by phase

As no COD was introduced into the system except the organic carbon in the poultry litter, the abundance of heterotrophic bacteria such as *Burkholderiales* remained constant. Denitrifying bacteria *Comamonadaceae* (Hiraishi & Khan, 2003)) and *Thermomonas* deceased from 4.92% and 0.57% to 2.75% and 0.02%, respectively. While the unfalsified *Burkholderiales*, which was known to contain anerobic heterotrophic denitrifiers (Gottshall et al., 2021; Lycus et al., 2017), increased from 1.69% to 3.57% in abundance. No autotrophic denitrifiers such as *Thiobacillus*, *Sulfurimonas*, and *Thioahalobacter* (F. Chen, Li, Gu, Huang, & Yuan, 2018; Shao, Zhang, & Fang, 2010) were detected in this study.

In addition, as the supply of poultry litter to the SBBR continued, more diverse bacterial groups were brought into the system, which resulted in the decrease of existing microbial populations (**Figure 9**). Besides, even though the growth rate of heterotrophic bacteria such as AOB or denitrifiers were much more quickly than that of autotrophic bacteria such as anammox, the abundance of both species reduced in a similar proportion. To be more specific, the successful control of the whole operation system slowed the growth of AOB and other heterotrophic bacteria and incurred a long solid retention time for the SBBR.

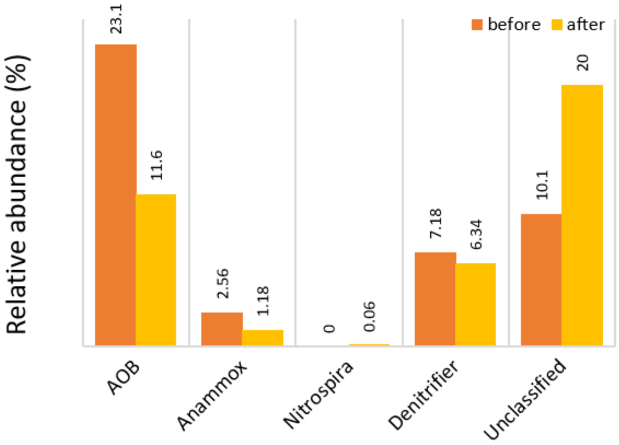

Figure 9. Relative abundance changes of different bacteria

According to **Table 3** and **Figure 8**, accumulation of nitrite and/or ammonium existed before phase 5 even when HRT was 72 h. This observation indicated that the anammox reaction had already reached its treatment limits or the anammox bacteria was overrun by AOB when competing for ammonium. It was assumed in this study that the growth of comammox bacteria was the reason for the TN removal rate decreasing slightly from 67.2% (phase 5) to 62% (phase 8) in the same operating condition. Comammox bacteria, anammox bacteria, and AOB were competing with each other for ammonium and space, and comammox bacteria was also competing for DO and nitrite with AOB and anammox (**Figure 10**)(Gottshall et al., 2021). Thus, although the comammox bacteria might not remove the nitrogen content in the reactor, it instead oxidized the ammonium to nitrate. This competitive relationship with anammox bacteria disrupted the balance between partial nitrification and anammox process, likely leading to a lower total nitrogen removal rate.

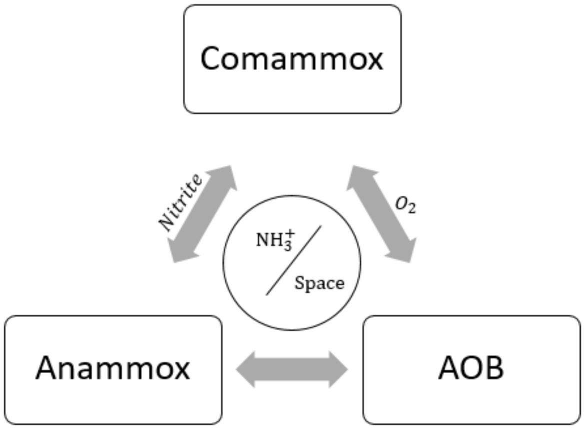

Figure 10. Competitions among Anammox, Comammox and AOB

In recent years, the novel metabolic capacity of complete nitrification by the comammox bacteria (the *Nitrospira* genus) has raised great questions regarding their ecological significance and environmental relevance in different ecosystems (Hu & He, 2017). The evidence that the comammox species exist in many terrestrial and aquatic ecosystems including agricultural soils, freshwater habitats, wastewater treatment plants, and drinking water treatment systems has been reported (Bartelme, McLellan, & Newton, 2017; Palomo et al., 2016; Y. Wang et al., 2017) with the help of metagenomic screening of environmental samples based on the functional gene sequences for ammonia oxidation. The data collected from this study again provided evidence to support the existence of comammox bacteria even in wastewater originating from animal productions. Although these reports revealed the widespread occurrence of commonly agreeable comammox *Nitrospira* in diverse ecosystems, application of this process in removing nitrogen from wastewaters is still in its infancy. In particular, there have been no reports on using the comammox process to treat animal wastewaters such as the poultry litter derived wastewater studied in this thesis. Therefore, given the potential that comammox *Nitrospira* may have a much strong and broader role in dealing with agricultural wastewater treatment to remove nitrogen, it is highly suggested that understanding and exploring the comammox process in agricultural applications should be incorporated into future studies of nitrification in these ecosystems.

Chapter 4 Conclusion

In this study, a one-stage partial nitrification anammox process was successfully operated to treat poultry litter wastewater in an SBBR reactor. This reactor has reached a high total nitrogen removal efficiency of 87.3% during the experimental runs. Central Composite Design (CCD) coupled with Response Surface Methodology (RSM) was used to optimize the facters including DO, HRT and C/N ratio. The model for ammonium removal rate was significant and predicted that the ammonia removal rate could reach 100% at the shortest HRT of 39.88 h. The results also indicated that the quadratic model generated by the CCD/RSM analysis in JMP could satisfactorily predict total nitrogen removal rate with a *p* value of 0.0018. With an error range from 0.03% to 2.81%, the uncertainty analysis for the model in estimating the TN removal rate also supported its accuarcy. According to the model, the optimal total removal rate was found at DO concentration of 0.5 mg L^{-1}, HRT of 72 h, and C/N ratio of 1.0. High-throughput sequencing analysis revealed the successful suppression of NOB and the slow growth of AOB. The bacterial profile also indicated the dominant species that was readily adapted to poultry litter wastewater, i.e., *Nitrosomonadaceae* within AOB and *Candidatus Brocadiaceae* within anammox. However, during operation, the growth of comammox induced the shift of nitrogen metabolism from mainly nitrite to mainly nitrate, thus potentially enhancing denitrification rather than anammox. The competition between AOB, anammox, and comammox also resulted in a slightly reduced TN removal rate.

Chapter 5 Future Works

The limits of running parameters chosen for the experiments were not able to capture the actual optimal C/N ratio for total nitrogen removal rate. More experiments should be conducted to determine the optimal combination of the running parameters. Even through NOB was sucessfully suppressed to maintain stable operation of the PN/A process, the growth of comammox appeared to be inevitable at a low DO concentration condition. If the comammox bacteria become the dominant ammonia oxidizer, the production and accumulation of nitrate may lead to the collapse of the PN/A process. Therefore, although comammox can offer more energy efficient nitrification, it is considered unfavorable in the PN/A process reactors based on the data from this study. Methods to suppress both comammox and NOB need to be developed to maintain the operating stabilty of the PN/A process. Also, technologies and/or strategies need to be investigated to increase the proportion of anammox in the reactor environment, such as reuse of granuled anammox sludge to improve the bacterial population.

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